2011

Production and characterization of value-added biorenewable chemicals

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Production and characterization of value-added biorenewable chemicals

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-Majors: Biorenewable Resources and Technology; Food Science and Technology

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2011

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ABSTRACT

Nisin is the only FDA-approved bacteriocin with a GRAS (Generally Regarded as Safe) status, used as a biopreservative/shelf-life extender in foods and many other products including pharmaceutical, veterinary and health care products. It is produced commercially by fermentation of a milk-based medium, using strains of *Lactococcus lactis* subsp. *lactis.* However, being a dairy-based product requires its labeling since milk is an allergen. Also, since its high production cost is mainly dominated by its medium costs, an emerging trend has been observed with a growing number of studies aimed at finding alternative, non-dairy and preferably non-allergen-based substrates. In order to cut costs, and to also be an environmentally friendly approach, several of these substrates have been sourced from agriculture or aquaculture-based waste streams. The review included in this dissertation highlights the advantages of value-addition to waste and the promising potentials of some of these low-value nutritive sources for the production of high-value nisin. An in-depth study of soy whey (SW) as a non-dairy, nisin fermentation feedstock was performed using *Lactococcus lactis*. Results indicated that SW was able to produce equivalent biomass and nisin yields (2.18g/L and 619mg/L), as compared to the commercial medium, de Man-Rogosa-Sharpe (MRS) broth (2.17g/L and 672mg/L, respectively), without the need for external nutrient supplementation. This indicated the nutritive qualities of this co-product stream in being able to support the growth of a fastidious bacterial culture.

The success of SW as a bacterial growth medium motivated the second study which focused on growing the oleaginous algal strain Chlorella vulgaris under

mixotrophic and heterotrophic culture conditions. Microalgae are cultivated in large scale for several commercially important products among which algal lipids have gained increasing interest due to their use as biodiesel feedstock as well as a source of essential fatty acids with neutraceutical value. Photoautotrophic algal cultivation suffers from low growth rates while heterotrophic/mixotrophic modes have high media costs. In order to boost algal lipid production with lower media costs, two co-product streams, SW and thin stillage (TS) were tested as growth substrates. TS is a high-organic strength co-product generated from the dry-grind corn-ethanol industry. Traditional use of TS involves an energy-intensive concentration process to form “syrup” which finally ends up as animal feed in Distillers’ Dried Grains with Solubles (DDGS). The cost-effectiveness and success of the corn-ethanol industry is highly dependent on the value of its co-products. Therefore, in order to be an economically viable and an environmentally sustainable process, researching new avenues for value-addition to TS is very important. As per the results from the current study, biomass yields (dry basis, db) from TS, SW and a synthetic control medium-MBM (modified basal medium) after 4 days of incubation were 9.8, 6.3 and 8.0 g/L with oil contents at 43, 11, and 27 % (w/w) respectively. Polyunsaturated fatty acids (PUFAs) or essential FAs, were found to be highest in Chlorella-TS_{oil} (56%), followed by 38% in Chlorella-SW_{oil} and 31% in Chlorella-MBM_{oil}. Therefore, mixotrophic cultivation of *C. vulgaris* in TS and SW produced high yields of both algal biomass and lipids at low cost, thus adding value to co-product streams and improving economic viability of algal cultivation.

The high algal oil yields from TS motivated the third study involving the use of oleaginous fungal strain, Mucor circinelloides for its potential in adsorbing/assimilating the oil and nutrients present in TS, for the production of lipid and protein-rich fungal biomass. Fungal batch cultivation for 2 days using a 6-L airlift reactor led to a 92% increase in oil yield from TS, relative to the original oil content, with concomitant reduction in suspended solids and chemical oxygen demand (COD) in TS by 95% and 89% respectively. *M. circinelloides,* when grown on TS gave a biomass yield of 20 g/L (dry basis), with a lipid content of 46 % (dwb). The polyunsaturated fatty acids were 52% of the total lipids. Overall, fungal cultivation on TS produced a high-protein animal feed and high-value fungal oil, thus improving corn-ethanol process economics.

The fourth study focused on employing the “hurdle concept” and developing more efficient antimicrobial systems by combining plant essential oils (EOs) with food-grade organic acids. The seven most active oils among the sixteen tested, were combined with five different organic acids and the antimicrobial interactions (synergistic/additive/antagonistic) were examined against food-related pathogens. Malic and citric acid were the most inhibitory acids which showed synergism with mountain savory, redistilled oregano (RO), and cinnamon oils against *S.aureus* 25923 and with cassia, RO, and cinnamon oils against *E.coli* 25922. The only synergistic EO-acid combination against *C.albicans* 10231 was lemon myrtle-citric acid.

CHAPTER 1 GENERAL INTRODUCTION

1.1 Introduction

The unique metabolic capacities of microorganisms can be used in a variety of practical applications with simultaneous value-addition to low/negative-value industrial by-products and wastewaters. Three primary research problems have been addressed in the course of this dissertation. Firstly, nisin production suffers from the drawbacks of high costs and allergen (milk) labeling and requires alternative, non-dairy, preferably non-allergen-based and less expensive fermentation substrates; secondly, economic viability of the biofuels industry is highly dependent on the value of its co-products which are currently under-utilized and have untapped potentials; and finally, limited application of plant essential oils as antimicrobials in food and feed due to alterations in end-product quality from an organoleptic standpoint. The research objectives of the studies included in this dissertation focus on two main areas – firstly, natural antimicrobials, specifically, improvement in food safety via low-cost production of high-value food preservative, nisin; and enhanced antimicrobial activity of plant essential oils using hurdle technology; and, secondly, biofuels, specifically, value-addition to agro-industrial co-products with simultaneous production and characterization of fungal and algal-based lipids.

Globalization of the food market and rise in processed and ready-to-eat products with longer shelf lives require efficient food chain logistics. This has also increased the time between harvest and consumption. In addition, the rising “green” consumerism demands safe, high-quality, yet minimally processed foods devoid of chemical preservatives (Smid and Gorris, 1999). All these requirements make food safety

management more and more challenging, and are the primary drivers for the expansion of the natural antimicrobials market. Bacteriocins and plant essentials oils (EOs) are two groups of naturally-derived antimicrobial compounds with GRAS (Generally Regarded as Safe) status (Vessoni Penna and Moraes, 2002; Kabara, 1991) and a long history of use in foods and other products. Bacteriocins are a heterogeneous group of proteinaceous anti-bacterial compounds produced by lactic acid bacteria (Arauz, 2009) found as natural microflora in raw milk. Nisin is the only bacteriocin approved by the Food and Drug Administration (FDA) and commercially produced by the fermentation of *Lactococcus lactis* subsp. *lactis* in milk based media (Guerra and Pastrana, 2003; Parente and Ricciardi, 1999; Pongtharangkul and Demirci, 2006). On the other hand, EOs are aromatic liquids obtained from different parts of plants (including bark, leaf, flower, roots) and used in food for flavor, aroma and safety (Burt, 2004). Unlike bacteriocins, EOs are not only anti-bacterial (Mourey and Canillac, 2002), they are also antiviral (Bishop, 1995), antiparasitic (Pessoa et al., 2002), antimycotic (Mari et al., 2003), and insecticidal (Karpouhtsis et al., 1998).

In order to expand nisin usage in food and achieve better food safety, high costs of the rich milk-based fermentation medium need to be addressed (De Vuyst and Vandamme, 1992). The emerging trends in the exploration of food-processing waste streams such as cull potato hydrolysates (Liu et al., 2005), fermented barley extracts (Furuta et al., 2008), and fishery byproducts (Vazquez et al., 2004) as feedstock for nisin production have so far not been able to produce high enough nisin yields for successful commercialization. This inspired us to test and optimize a novel, low-cost,

non-dairy substrate, soy whey (SW) for nisin fermentation. SW is a byproduct from the soybean processing industry generated during the production of soy-protein isolate (Snyder and Kwon, 1987). Although it is generally considered a waste product which poses a burden on the environment if disposed untreated (Smith et al., 1962), we hypothesized that the high nutrient content of soy whey could enable its value-added use as a substrate for lactic acid bacterial fermentation and nisin production (Mitra et al., 2010). In addition, production of nisin using a readily available and non-toxic plant product such as soy whey may ease regulatory approval for food use unlike some of the other animal-waste-derived nisin formulations reported in literature.

Concern over resistance of pathogens to conventional food preservation techniques has also boosted research interests in the antimicrobial “hurdle” concept, where two or more antimicrobial systems are used in concert to achieve higher preservative power and better food safety without altering food quality (Leistner and Gorris, 1995). Among the 3000 known essential oils, 300 are commercially important and used in aromatherapy, and as flavorants and antimicrobials in foods (Van de Braak and Leijten, 1999). However, due to their inherent strong flavor and aroma their use in foods suffers from persistent concerns (Fisher et al., 2007).The concentrations at which the EOs are cidal in their action are too high and above the tolerable taste threshold, and hence restrict their application in foods only till their inhibitory concentrations (Brul and Coote, 1999). If their inhibitory powers could be reinforced with the supplementation of small amounts of another antimicrobial, a balance could be reached between sensory acceptability and antimicrobial efficacy. The wide use of organic acids in foods and their

unique structural properties that provide them with their versatile functionalities promote their inclusion in multi-component antimicrobial compositions (Tamblyn and Connor, 1997). The combined effects of plant essential oils and organic acids have been reported in foods where a select few oils and acids have been tested (Friedly et al., 2009; Dimitrijevic et al., 2007). As per our knowledge, programmed and systematic searches for potential synergies between a broad panel of plant essential oils and organic acids are missing in literature. In this study we have examined a wide variety of essential oils obtained from Van Beek Natural Sciences (Orange City, IA) against a broad panel of food-related pathogens, multi-drug resistant “super bugs” and a veterinary pathogen. The most active oil extracts were combined with food-grade organic acids and tested for synergistic interactions.

The second research focus area involving biofuels aimed at developing novel, low-cost biodiesel feedstocks while simultaneously adding value to corn-ethanol co-products. Depleting resources and increased emissions of green house gases (GHGs) in the environment render the use of fossil fuels as highly unsustainable (Singh et al., 2011). Biofuels such as corn-ethanol and biodiesel serve as attractive, alternative energy sources. Corn-ethanol is produced by the fermentation of corn-starch to ethanol by the yeast, *Saccharomyces cerevisiae* (Kim et al., 2008), while biodiesel or fatty acid methyl esters are made by transesterifying vegetable oils and animal fats in the presence of catalysts and alcohol (Miao et al., 2006). Biofuels offer several advantages such as, alleviation from foreign oil dependence, much lesser greenhouse emission with a carbon neutral process (Carere et al., 2008), and a boost to the local economy

especially to farmers and small-scale industries owing to its scattered production nature. However, despite all the benefits, the economic aspect of biofuel production suffers from the high cost of raw material and the low-value of its co-products, which are the primary hurdles in broader commercialization (Antolin, 2002). There have been many studies which address these challenges (Al-widyan and Al-shyoukh, 2002; Noureddini et al., 2009; Rasmussen et al., in press). Noureddini et al. (2009) investigated the use of whole stillage-extracted corn oil as a raw material for biodiesel production; while Rasmussen et al. (in press) established a profitable way of converting the corn-ethanol co-product, thin stillage to animal feed via fungal processing.

Plant-based oil materials account for almost 85% of total biodiesel production cost and also lead to food vs. fuel controversy (Miao et al., 2006). Microbial lipids, on the other hand, do not require fertile land and have much higher yields (Meng et al., 2009). The cellular content of oleaginous, heterotrophic/mixotrophic microalgae and molds has 30% or more lipids dominated primarily by triglycerides (Sergeeva et al., 2008; Vicente et al., 2009). However, they require costly organic carbon sources for growth which account for almost 80% of the medium costs (Li et al., 2007). In order to improve the economic viability of microalgal/fungal-based biodiesel production, it is imperative to find cheap organic substrates as well as high-yielding fungal/microalgal strains.

In a dry-grind corn-ethanol plant, the removal of ethanol from the fermentate by distillation leaves behind “stillage”, composed of corn fiber, oil, protein, other

unfermented components of the grain and yeast cells (Kim et al., 2008). For every gallon of ethanol produced, 5-6 gallons of stillage is generated (Rasmussen et al., in press) which is centrifuged to produce a liquid (called thin stillage) and a solid fraction (called wet distillers grains). Less than 50% of the thin stillage (TS) is recycled back as backset for liquefaction of ground corn (Sankaran et al., 2010). The rest goes through multiple effect evaporators requiring substantial amounts of energy to make a condensed syrup, that later ends up in DDGS (Kim et al., 2008) and is sold at low margins as animal feed (Moreau et al., 2011). In addition, doubling of corn ethanol production in the United States from 2007 to 2010 (Renewable Fuels Association, 2011) has led to increased production of thin stillage while demands for DDGS have not increased proportionally. A previous study in our lab (Rasmussen et al. in press) indicated the high nutrient content in thin stillage and the feasibility of fungal cultivation to produce a high-protein fungal feed. TS has recently been used as a substrate for biofuel production by Ahn et al. (2011), when they grew a bacterial culture, *Clostridium pasteurianum* for butanol synthesis. Moreau et al. (2011) found that TS contained the highest amount of corn oil on a dry basis, among the various pre-and post-fermentation corn fractions in the corn ethanol process. In recent years, recovery of corn oil from post-fermentation corn fractions has drawn considerable interest for use as a biodiesel feedstock (Noureddini et al., 2009). In addition, the presence of oil in a microbial growth medium is known to increases the lipid accumulation in the microbial cells (Szczesna-Antczak et al., 2006). All the above facts indicated, that stillage could be an excellent medium for growing oleaginous microbes for oil production.

Certain mucoralean fungi like *Mucor circinelloides* are known to grow rapidly on various substrates owing to their excellent enzymatic systems (especially lipase), and have 50-60 % of cellular oil content (Szczesna-Antczak et al., 2006; Sergeeva et al., 2008; Vicente et al., 2009). Fungal biomass has also been documented as excellent biosorbent material (Ozsoy et al., 2008) capable of removing oil from oil - water emulsions (Srinivasan et al., 2010). Based on the above facts, we hypothesized that growing *M.circinelloides* on thin stillage would increase oil yields as the fungal cells would not only adsorb and help to recover the stillage oil but would also metabolize the corn oil and use the end products (fatty acids) for further lipogenesis.

With the same objective of producing low-cost, high oil containing microbial feedstock, the second oleaginous microbe studied was *Chlorella vulgaris.* It is a robust, green, fast-growing microalgal strain capable of metabolizing nutrients from various wastewaters due to their high tolerance to soluble organic compounds (de la Noue and Basseres, 1989). Multiple studies have been done with the goal to reduce algal medium costs by exploring cheaper carbon sources to substitute glucose, such as, corn powder hydrolysate (Xu et al., 2006), sweet sorghum juice (Gao et al., 2010), or domestic/industrial wastewaters (Woertz, et al., 2009) with high bioavailability. The fundamental issue while testing these alternative nutrient sources is to match both lipid and biomass yields at or near the levels obtained from glucose (O'Grady 2011). Therefore, we hypothesized that the balanced nutrient content and low toxicity of the two agro-industrial co-product streams, soy whey and thin stillage could enable mixotrophic, high-density growth of *C. vulgaris* and low-cost lipid production.

The principal test platform in all these research studies (except fungal) was the Bioscreen C automated turbidimeter. This instrument is a self-contained microbial incubation and analysis unit; which allows high-throughput; microplate-based collection of optical density values indicating the growth of the test microbial strains (viz. *L. lactis, C. vulgaris*, and the pathogenic strains used in EO testing). Performing the initial growth optimization studies in the Bioscreen C allowed lower volumes of media and lower waste accumulation, with the generation of detailed and informative growth data sets (Lambert and Pearson, 2000).

1.2 Dissertation Organization

This dissertation is divided into seven chapters and two appendices. The first chapter is a common introduction of the research problems that have been addressed in the dissertation, the objectives that have been targeted and the research hypotheses. Chapters 2, 3, 4, 5 and 6 are written as manuscripts for publication in different peer-reviewed international scientific journals. The second chapter is a review article on *"Nisin production using low-value non-dairy organic substrates: A Review"*, written to be submitted to the Journal of Trends in Food Science and Technology. The third chapter is a research article entitled "*Value-added production of nisin from soy whey"* which has been published in the Journal of Applied Biochemistry and Biotechnology. The fourth and fifth chapters discuss the studies on fungal and algal-based lipid production using soy whey and thin stillage. The fourth chapter is entitled "*Value-added oil and animal feed production from corn-ethanol stillage by oleaginous Mucor circinelloides"* and has

been submitted to the Journal of Bioresource Technology; while the fifth chapter is entitled *"Heterotrophic/mixotrophic cultivation of oleaginous Chlorella vulgaris on industrial co-products"*, and has been submitted to the Journal of Algal Research. The sixth chapter involves the development of efficient multi-component antimicrobial systems including plant essential oils and organic acids in order to control food-related pathogens. It is entitled *"Synergistic action of plant essential oils and organic acids and their group/strain-specific antimicrobial activity against food-related pathogens"* and is written to be submitted to the Journal of Food Protection.

Kindly note that each chapter has its own introduction and conclusion sections, with the figures and tables embedded within the text followed with the cited list of references at the end. Chapter 7, the final concluding chapter, contains the overall summary which highlights the main results from the different studies and the recommendations for future research. Appendix A describes a collaborative study performed with a research group from the Chemistry department on the synthesis and properties of aqueous cationic polyurethane dispersions to be submitted to the Journal of Progress in Organic Coatings. A part of this study which involved the antimicrobial activity testing of the polyurethane dispersions against *Salmonella typhimurium* and *Listeria monocytogenes* has been included in this dissertation as Appendix A. Appendix B contains the curriculum-vitae of the author of this dissertation.

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CHAPTER 2. NISIN PRODUCTION USING LOW-VALUE NON-DAIRY ORGANIC SUBSTRATES: A REVIEW

A paper submitted to the Journal *Trends in Food Science and Technology*

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Keywords: nisin, fermentation, value-addition, non-dairy, agro-industrial co-products

2.1 Abstract: Commercial production of nisin, an FDA approved food preservative, typically uses milk-based media, which substantially adds to production costs. In efforts to reduce the costs of nisin production and to expand its usage, several groups have examined the potential of various non-dairy fermentation substrates for nisin production. We review emerging trends in nisin production using alternative substrates sourced from non-dairy agriculture or aquaculture-based waste streams. The alternative

substrates reviewed are compared in terms of value addition, maximum biomass and nisin yields obtained vis-à-vis milk-based media, and potential motivations and challenges for their commercialization.

2.2 Introduction

Microbial fermentation has been used for ages to preserve food and beverages. Fermentation end products (organic acids or alcohols), production of extracellular enzymes (proteases, cellulases, pectinases), changes in intrinsic parameters (pH, moisture, etc.), and the release of antimicrobial peptides (e.g. bacteriocins), result in the extended shelf life and improved nutrient value of foods. Nisin, a bacterial metabolite, from *Lactococcus lactis* subsp. *lactis* fermentation, is an approved bacteriocin for food use with a wide spectrum of antimicrobial activity. Today the commercial production of this heat stable polypeptide is by *L. lactis* fermentation in a dairy based culture medium followed by peptide recovery. Despite its effectiveness and popularity, nisin production and use in foods faces several challenges. The fermentation medium composition and cost are two key factors in nisin production which have drawn considerable research interest. This review focuses on the production of nisin using alternative, non-dairy, agro-industrial wastes and co-products that may help to address the milk-allergen labeling issue as well as the high media costs associated with the traditional fermentation process.

2.2.1 Lactic acid bacteria (LAB) are one of the most important groups of microbes used in the food processing industry. The food LAB primarily consists of *Lactobacillus**,*** *Lactococcus**,*** *Leuconostoc**,*** *Pediococcus* and *Streptococcus***.** In addition to the production of important flavor compounds and other desirable modifications in foods, the fermentative metabolism of the LAB yields various antimicrobial end products such as lactic acid, diacetyl and bacteriocins that impart valuable preservative effects. LAB are ubiquitous and are present as natural microflora in raw milk, fruits and vegetables and are nutritionally fastidious. They are auxotrophic for amino acids, vitamins and/or fatty acids and are thus unable to grow on minimal media (Heber et al., 2008). Production of bacteriocin is a type I, growth associated, metabolite that is easily observed in complex media such as MRS (de Man, Rogosa and Sharpe, Difco Laboratories), M17 and BHI (Brain Heart Infusion Broth, Difco), among the commonly used formulations to grow LAB (Jozala et al., 2005; Arauz et al., 2008; Rodríguez et al., 2000).

2.2.2 Nisin and its importance

Bacteriocins are wide-spectrum antimicrobial polypeptides produced by bacteria and have bacteriocidal or bacteriostatic activities to select bacteria (Amiali et al., 1998). As a group, the LAB are notable for their production of bacteriocins. While several LAB-produced bacteriocins have been characterized to date, nisin is the only bacteriocin approved for food use by the U.S. Food and Drug Administration (FDA) (Pongtharangkul et al., 2004). Nisin was discovered in the late 1920's, first marketed

in England in 1953, recognized as a food preservative by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969 and awarded generally recognized as safe (GRAS) status in the U.S. in 1988. Nisin is approved for food use in over 50 countries, since the turn of the century (Pongtharangkul et al., 2004; Parente et al., 1999). Nisin is produced commercially by the dairy starter organism *Lactococcus lactis* subsp.*lactis* by fermentation of rich, milk-based media (Guerra et al., 2003; Pongtharangkul et al., 2005). A widely used commercial preparation of nisin is Danisco's Nisaplin™ (Jones et al., 2005; Parente et al., 1999). Nisaplin™ has a potency of one million international units (IU) per gram and contains 2.5% (w/w) nisin, 77.5% sodium chloride, and nonfat dry milk with protein and carbohydrate contents of 12 and 6% (w/w), respectively (Jones et al., 2005; Deegen et al., 2006; Taylor et al., 2007). Consumer demand for safe, high-quality, yet minimally processed foods that do not contain traditional chemical preservatives is a key driver for expanded use of natural antimicrobials, including nisin. Concern over resistance of emerging pathogens to conventional food preservation techniques has also expanded both private and public research interest in antimicrobial "hurdle" approaches, where several antimicrobials are used in concert to achieve greater preservative power and a higher degree of food safety (Leistner and Gorris, 1995). As others have shown, the efficacy of nisin as a preservative may be enhanced when it is combined in a hurdle system along with other antimicrobial substances, such as chelating agents (e.g. citric acid) (Arauz et al., 2009). The GRAS status of nisin and the success of multi-hurdle preservative strategies

incorporating nisin provide incentive for increased and innovative applications for this bacteriocin in the formulation of natural food preservative systems.

2.2.3 Nutritive media for nisin production

Several factors influence nisin production, with media formulation and the nutritive status of the media playing important roles (Lv et al., 2005; Jozala et al., 2007; Arauz et al., 2009). The final bacteriocin titer of the fermentate is influenced by the composition of the media (Yang and Ray, 1994; Vignolo et al., 1995). Therefore for the production of nisin, the use of well-defined, commercially-available media is recommended (Arauz et al., 2009). Semi-synthetic complex media like MRS, All Purpose with Tween (APT), Elliker, BHI, Tryptone Glucose Extract (TGE), Trypticase Soy Broth (TSB) and M17 media support excellent LAB growth and high bacteriocin levels (Cheigh et al., 2002; Guerra et al., 2001). However, the relatively high cost of these media makes them unsuitable for large-scale production of bacteriocins, including nisin. This limitation is driving trends in research towards development of more economical growth media. The literature is replete with reports on development of value-added nisin fermentation media derived from byproducts of the dairy industry, including skimmed milk (Jozala et al., 2007), raw milk whey (Arauz et al., 2008) and cheese whey (Goulhen et al., 1999; Guerra et al., 2001). Arauz et al., (2009) provide a thorough review of dairy-based media for nisin production.

Bacteriocins are used in food products in three ways: 1) *in situ* production by the starter or protective cultures added to the food, 2) as an ingredient (i.e. fermentates generated using bacteriocinogenic strains are added to the food), or 3) as an additive (a semi- or fully-purified form of a bacteriocin is added directly to the food) (Garcia et al., 2010). Commercial nisin (Nisaplin™) is applied in foods via both the second and the third routes. The fermentate containing nisin is typically concentrated, separated, spray-dried, then finely milled and standardized by the addition of sodium chloride (Arauz et al., 2009). Because a major portion of commercial nisin consists of milk solids, use in foods could represent an allergenic and food labeling liability, as milk is one of the eight foods identified by the Food Allergy and Anaphylaxis Network (FAAN) responsible for 90% of all food-allergic reactions (along with egg, peanut, tree nuts, fish, shellfish, soy, and wheat). Therefore, a challenge remains to identify alternative, economical, non-dairy substrates that are suitable for high-titer production of nisin.

2.3 Value-added non-dairy-based substrates as nisin fermentation feedstock

Research on nisin production has mainly been focused on improving fermentation yields in complex media through kinetic modeling and nutrient supplementation. In order to reduce the high costs of nisin production and to promote nutrient recycling and value-addition to industrial wastes, several groups have explored the use of low-value agricultural or aquacultural coproducts and effluents from food-processing industries as potential fermentation feedstocks for nisin fermentation. The

use of these less expensive, alternative substrates may facilitate more cost-effective production of nisin, promote its wider application and allow its use in dairy-free foods. The food-grade nature of many of these substrates may ease regulatory approval and allow the use of the entire fermentate in foods, without the need for purification of nisin prior to use. Currently, these materials are viewed as low-value waste materials. Their high organic strength, which results in high biological and chemical oxygen demands, poses a burden on the environment, unless treated prior to disposal, which is costly (Vazquez et al., 2004). In some cases (soy whey), these wastes may be concentrated via heat treatment, and then disposed of in landfills – an energy-intensive, no-profit process (Mitra et al., 2010). Other wastes, like condensed corn solubles are dried and used as animal feed, which requires energy-intensive processing and generates only minimal revenue (Wolf-Hall et al., 2009; Van Leeuwen et al., 2012). Use of these low-value substrates to produce a high-value product like nisin could be an exciting approach for value-addition to these byproducts or wastes, and a desirable alternative to their current applications. The benefits of such a proposition can be widely felt. Industries generating these co-products would be able to reduce costs of waste disposal and generate more revenue, and lower costs of nisin might foster wider use of this preservative, enhancing the microbial safety of foods. In contrast to dairy-based substrates, studies on non-dairy based media for nisin production are fewer and relatively more recent. To our knowledge, a review of approaches for value-added production of nisin from alternative, non-dairy substrates has not yet been published.

The goal of this review is to give an overview of the various *non-dairy* agricultural waste streams that have been explored for LAB cultivation and subsequent nisin production. Nisin production from each substrate is discussed, and a standard set of variables are considered. These include –

1) importance of value-addition to the particular co-product/waste stream,

2) availability of the rawmaterial,

3) modifications/pretreatment required prior to use for LAB cultivation,

4) fermentation conditions used,

5) analytical methods used for cell growth and determination of nisin yield,

6) reported biomass and nisin yields, and

7) advantages and limitations of each substrate

The non-dairy substrates for nisin production reviewed here fall into two main categories – plant-derived and animal-derived. Plant-based substrates include sugar molasses (Egorov et al., 1980), cull potato hydrolysate (Liu, et al., 2005), organic waste from the date production industry (Al-Zahrani et al., 2006), barley extract (Furuta et al., 2008), sago starch (Zarrabal et al., 2009), condensed distillers solubles (Liu et al., 2007; Wolf-Hall et al., 2009), and soy whey (Mitra et al., 2010). Animal-based substrates from the aquaculture sector include mussel waste (Guerra et al., 2002), octopus protein waste (Vásquez et al., 2004) and fish protein waste (Vazquez et al., 2004).

2.4 Plant-Derived Substrates

2.4.1 Molasses

Egorov et al. (1980) reported use of fermentation media based on sugar molasses for growth of the nisin producing strain *Streptococcus (Lactococcus) lactis* strain MGU. These authors recommended the use of 1-2% (w/w) of molasses, 10-20% (w/w) of yeast autolysate and 1-2% (w/w) of KH_2PO_4 in the nisin fermentation medium. Although an abstract is available in PubMed, the unavailability of the entire research article in English prevents a complete review of this research study.

2.4.2 Cull potato hydrolysate (CPH)

Cull potatoes are potato tubers that fall below acceptable standards of size, shape or quality, or that are damaged due to disease or physical injury during transportation. Cull potatoes constitute 8% of the total potato production (Liu et al., 2005). With a total of 43.1 billion pounds of potatoes produced in the US (National Potato Council, 2009 data; www.nationalpotatocouncil.org), this represents approximately *3.5 billion pounds* of cull potatoes. Cull potatoes are an undervalued agricultural waste, currently used as an animal feed supplement. Additional uses of cull potatoes have been investigated, including its use as a feedstock for ethanol (Thiele et al., 2005) or lactic acid production (Oda et al., 2002). Liu et al**.** (2005) studied the feasibility of using CPH as a substrate for simultaneous production of nisin and lactic acid. The CPH in this study was prepared by cutting and homogenizing cull potatoes using 10% (w/w) NaOH, followed by enzymatic hydrolysis using α-amylase and α-

glycosidase. Solids were removed by filtration, and the resulting hydrolysate was used as the base material for the nisin production medium. Nisin fermentations were performed in 250 mL Erlenmeyer flasks with *Lactococcus lactis* subsp. *lactis* 11454 as the nisin-producing strain. The control medium contained (in 1 L): 50 g milk whey, 12 g yeast extract, 5 g polypeptone, 0.6 g KH_2PO_4, 06 g $MgSO_4$, 1 g Tween 80, and 30 g $CaCO_3$. The *L. lactis* biomass was quantitatively analyzed by absorbance at 600 nm, and nisin concentration was measured as described by Shimizu et al. (1999), using the indicator strain *Micrococcus luteus* 9341.

Results of this study indicated that growth in CPH was poor, with an Abs_{600} reaching only 0.28 within 24h. The authors sought to stimulate growth and nisin production through supplementation of the basal CPH medium with additional nutrients, including yeast extract (YE), peptone from soy (PS), peptone from meat (PM), distillers dried grains with solubles (DDGS) and corn steep solid (CSS). The combination of CSS and PS showed the highest improvement in nisin production with yields equal to the control medium (as defined earlier) (89.4 mg/L). Though potatoes are rich in starch, minerals and proteins, they apparently lack vital micronutrients that are essential for the growth of *L. lactis*. When seeking to utilize any agricultural or industrial waste product, ready availability of the raw material is an important aspect to consider. Industry news sources have reported the closure of potato-based ethanol plants in Idaho (the leading potato production state in the U.S.) due to the lack of feedstock needed to operate profitably. A further limitation is the need to use 10% (w/w) NaOH and the disposal problems this could create.

2.4.3 Organic waste from the date production industry

Date palm cultivation and date production in Saudi Arabia contributes 12% of the world's date supply (USAID, 2008). Al-Zahrani *et al.* (2006) investigated use of waste material from the date processing industry to support the growth of 4 strains of *Lactococcus lactis* subsp. *lactis* (strains G1, G2, G11 and G14) previously isolated from goat and camel milk. Two types of dates grown in Saudi Arabia, Alkhlas and Alsuccharia, were used in this study. The date medium used was obtained by mixing date waste material with distilled water, followed by filtration to remove solids. Results from the date medium were compared to 1) MRS medium, and 2) whey supplemented with yeast extract (2.5 g/L) and glucose (20 g/L). Batch fermentations were carried out in 250 mL Erlenmeyer flasks. Nisin concentration was determined by agar well diffusion assay using *Staphylococcus aureus* as the test strain. Biomass yield was estimated from absorbance at 660 nm.

These authors reported better growth for all four strains in MRS than in either the date or whey media. For strains G1 and G2, the highest nisin concentrations were found in MRS, however, strain G11 produced the highest nisin yield in the medium derived from Alkhlas dates (Al-Zahrani et al., 2006). An ideal value-added fermentation feedstock should be of consistent quality in order to minimize variation in product yield from batch to batch. Therefore, the variability observed for results obtained from the two varieties of dates should be taken into consideration, especially if mixed-source feedstocks are to be used. Interestingly, these authors found that, high bacteriocin production did not correlate with high biomass concentration, unlike results reported for

some of the other substrates reviewed here. The complex relationships between bacteriocin production and cell growth are still not fully understood (Guerra and Pastrana, 2002a). Difficulties in making direct correlations between cell growth and nisin production may stem from cryptic phenomena such as adsorption of the bacteriocins onto cell surfaces and/or the post translational processing of the pre-peptides to active forms (Biswas et al, 1991; Yang and Ray, 1992). Several studies report that nisin is a primary metabolite, with a positive relationship existing between cell concentration and nisin yield (De Vuyst and Vandamme, 1992; Guerra et al., 2001; Pongtharangkul et al., 2004). However, results from other studies suggest the opposite (Biswas et al., 1991; Matsusaki et al., 1996; Vazquez et al., 2006; Furuta et al., 2008; Mitra et al., 2010). Complex signals triggering nisin production and affecting nisin titers could vary according to the medium used, with factors such as the presence of specific peptides (De Vuyst et al., 1995; Liu et al., 2005) or the rate of acid production in the medium (Guerra et al., 2001) playing important roles.

2.4.4 Fermented barley extracts

Shochu is a traditional Japanese alcoholic beverage prepared through fermentation of plant starch sources including barley, rice, sweet potatoes or buckwheat. Shochu kasu is a byproduct stream generated during the shochu distillation process. The common practice of dumping this nutrient-rich byproduct as waste has now been partially replaced by its utilization as animal feed or fertilizer. However, a

large fraction of this waste stream still remains underutilized. Value-added uses of this resource could help solve current disposal problems. Similar to cull potatoes, shochu kasu has also been investigated as a substrate for production of other products, including enzymes and chitosan (Yokoi et al., 1998). Furuta *et al.* (2008) explored the use of shochu kasu as a fermentation base for nisin production using *Lactococcus lactis* subsp. *lactis* ATCC 11454. Starting with shochu kasu, fermented barley extract (FBE) was obtained by centrifugation, followed by filtration. The concentration of FBE was measured as Brix values with a handheld refractometer. The control culture medium contained (in 1 L): 5 g yeast extract, 5 g polypeptone, 5 g sodium chloride, and 10 g glucose. FBE concentration in the main fermentation medium was adjusted from 1 Brix [(solid content - 0.92% (w/v)] to 4 Brix [solid content - 3.68% (w/v)] and supplemented with 45 g/L of glucose when required. The main bacterial cultivation was performed in a 2 L jar fermentor and cell density was monitored by absorbance at 560 nm, then converted to cell dry weight (CDW) using a standard curve. Nisin A was quantified by High Performance Liquid Chromatography (HPLC).

Although cell growth and nisin yields improved with increasing concentrations of FBE, biomass and nisin production were very low in unsupplemented FBE medium. The authors attributed the poor performance of FBE to an insufficient supply of fermentable sugars. The authors surmised that sugars from the barley were likely consumed by yeasts for ethanol production in the primary shochu fermentation. Supplementation of FBE with glucose greatly improved both biomass and nisin yields. The maximum biomass yields were seen in 3 and 4 Brix FBE, while 2 and 3 Brix FBE supported the

highest nisin production (31.5 mg/L/d). Interestingly, this nisin yield was higher than that obtained in the control medium (as defined earlier). Results from this study show that conditions needed for optimal growth of *L. lactis* and for production of nisin were not the same.

2.4.5 Sago starch

Zarrabal et al. (2009) examined the production of nisin in a medium based on sago starch, obtained from the pith of sago palm stems. Glucose syrup obtained from enzymatic hydrolysis of sago starch was used as the sole carbon source for the growth of *Lactococcus lactis* IO-1 (JCM 7638). The starch was liquefied and saccharified using two commercial amylase preparations, Kleistase T10 (Daiwa Kasei, KK. Ltd., Osaka, Japan) and Glucozyme (Amano Pharmaceutical Co., Ltd., Nagoya, Japan). Cell density was measured by absorbance at 562 nm and was converted to cell dry weight (CDW) using a standard curve. Nisin activity was determined using a modified spot-on-lawn method (Ennahar et al., 2001) using the indicator strain *Bacillus circulans* JCM 2504. The fermentation medium contained: 40 g/L glucose (from hydrolyzed sago starch), 10 g/L yeast extract, and 2 g/L polypeptone. Batch cultures were performed in 5-L jar fermentors. The highest nisin yield obtained was 8 AU/mL. Based on their batch-mode fermentation study, where the highest nisin concentration was obtained in the log phase, the authors designed a continuous culture system where a constant high nisin titer was achieved by maintaining a high cell density with the maximum of the population

in the log phase. In this study, although the authors targeted the use of a non-dairy, low-cost nutrition source for the nisin producing strain, it should be noted that the entire fermentation medium was not a low-cost substrate. Glucose was substituted by a less expensive carbon source, i.e. hydrolyzed sago starch while the rest of the media comprised of yeast extract and polypeptone.

2.4.6 Condensed Distillers Solubles and Condensed Corn solubles

After distillation of ethanol from fermented corn mash, the residual fluid, called whole stillage is centrifuged into two fractions, distillers wet grains and thin stillage. Thin stillage is generally heated to concentrate it to a total solid content of 30–50% (w/v) which is then called as Condensed Distillers Solubles (CDS) or Condensed Corn Solubles (CCS). Dried distillers grains with solubles (DDGS) is formed when CDS/CCS is combined with distillers' wet grains and dried. These processes are energy-intensive. Therefore, value-addition to CDS/CCS by using it as a nutrient source for secondary fermentation is a desirable alternative to inclusion in DGGS. Currently, CDS is used as an animal feed, but growing ethanol production rates are expected to result in a surplus that exceeds demand for its use in feeds. Therefore, development of additional value-added applications is desirable. Liu et al. (2007) studied the use of CDS as a nutrient supplement to sweet whey for simultaneous production of nisin and lactic acid by *Lactococcus lactis* subsp. *lactis* ATCC 11454. The main fermentations were performed in a 5-L Bioflo 110 fermentor (New Brunswick Scientific, Edison, NJ) using sweet whey

supplemented with 5% (v/v) CDS. In order to quantify the performance of CDS, the optimal medium for nisin production obtained from an earlier study (Liu et al., 2004) was used as a positive control. This medium contained (in 1 L): 0.6 g KH_2PO_4, 0.6 g $MgSO_4$, 12 g YE, and 20 g sweet whey. Unsupplemented whey was used as a negative control medium. Viable *L. lactis* cell concentration was measured as colony forming units (cfu) on agar plates, while nisin concentration was measured using the bioassay procedure reported by Shimizu et al., 1999 (indicator strain - *Micrococcus luteus* ATCC9341).

Results indicated that whey alone (without CDS supplementation) produced low yields of both biomass and nisin. A two-fold increase in nisin yields was seen with 5% CDS supplementation and found to decrease with higher amounts of CDS. These authors reported their results in the form of relative nisin production i.e. whether CDS supplementation produced higher or lower nisin yields as compared to the control whey medium (without CDS). Hence exact figures for the yields were not available. Toxicity of CDS at higher concentrations could be due to the presence of some byproducts from the ethanol process like acetic or lactic acids in CDS, which could be inhibitory to nisin biosynthesis. CDS typically contains 2.4 and 0.2 % (w/w) lactic and acetic acids, respectively (Liu et al., 2007). Since lactic acid is a primary metabolic product of the nisin producing bacterial strain, additional lactic acid in the medium could lead to feedback inhibition and restrict the bacterial growth and simultaneously nisin production. The inhibitory effect of lactic acid could also be due to the antimicrobial nature of its undissociated form which is capable of crossing the lipid bi-layer of bacterial cell walls, dissociating into protons and anions upon internalization and stressing the cellular

metabolism (Ricke, 2003; Guerra et al., 2007). However, CDS supplementation did not provide all the nutrition required by the nisin producer and auxiliary supplementation with YE was required. The media containing (in 1 L): 37 g CDS and 7 g YE yielded 85 mg/L nisin. Thus, it was concluded that although CDS supplementation did increase nisin production, higher concentrations of CDS were inhibitory; additionally, CDS did not supply all the nutrients needed for growth and nisin production.

In another study, Wolf-Hall et al. (2009) explored the possible use of diluted Condensed Corn Solubles (CCS) as a nisin production medium instead of as a media supplement. Lauryl-Tryptose broth was used as the control medium. CCS was obtained from a local ethanol facility and supplemented with 10 g/L of glucose. Cell growth was measured by plating, with subsequent conversion to cell mass yields. Nisin was assayed using Tramer and Fowler's (1964) agar diffusion technique, modified by Wolf and Gibbons (1996). Shake flask trials were conducted using both the cheese whey and CCS-based fermentation media. Results from this study indicate that 10% CCS + glucose could support excellent cell growth (1 mg/L) and nisin yields (6.8 mg/L/d). Higher concentrations of CCS (>10%) resulted in lowered nisin yields. A similar outcome for higher concentrations of CDS was reported by Liu et al. (2007). The possible mechanisms underlying this kind of substrate behavior could be feedback inhibition by lactic acid found in CCS/CDS, or it could be higher proportions of CDS/CCS in the growth media resulting in higher salt/osmolyte concentrations not conducive to the bacterial growth. One of the drawbacks of using CCS/CDS as a nisin

fermentation substrate was the need for external sugar supplementation, which naturally increased the media formulation cost.

2.4.7 Soy whey

Soy protein isolate (SPI) is one of the high-value products made from the wet-processing of soybeans. This process leads to the generation of large volumes of concentrated liquid waste material, known as soy whey (SW). This waste stream is nutritionally rich and typically contains 3.6– 4.4% (w/v) nitrogen (50% of which is protein nitrogen) and 25–35% (w/v) soluble sugars, significant amounts of phosphorus, minerals, and numerous trace elements essential for fastidious LAB. Being too dilute for economical nutrient recovery, this high-organic strength stream causes significant disposal problems (Smith et al., 1962). Mitra *et al.* (2010) hypothesized that the high-nutrient content of SW could enable its value-added use as a LAB fermentation feedstock for the production of nisin. The nisin producing strain used was *L. lactis* subsp. *lactis* ATCC 7962 and the indicator strain was *Micrococcus luteus* ATCC 10240. To establish proof of concept and ensure consistency in quality, SW was prepared in the laboratory using defatted soybean flakes. Iso-electric precipitation (pH 4.5) and extraction of soy protein from defatted soy flakes leads to the generation of a liquid by-product called SW. Bacterial batch fermentations were carried out in 250 mL Erlenmeyer flasks containing 100 mL SW. Cell density was measured by absorbance at

600 nm and converted to dry cell weight (DCW). Nisin activity was determined by the agar well diffusion bioassay (Tramer and Fowler, 1964).

Results from this study indicated that SW was able to support *L. lactis* growth and nisin production without the need for external nutrient supplementation. Addition of supplements (e.g. sucrose, NaCl, KH_2PO_4, yeast extract, $MgSO_4$) did not lead to any significant increase in nisin yields. The combined treatment of filtration and heat sterilization of SW, gave the highest biomass yields (2.18 g/L) as compared to the control medium (MRS). While heat-sterilized SW proved most successful for nisin production (619 mg/L). Therefore, similar to the observations made by Vazquez et al., 2006; Al-Zahrani et al., 2006; Furuta et al., 2008; on fish peptone, date and barley-based substrates, respectively; Mitra *et al.* also noted a growth-independent behavior for nisin production on SW. The advantages of using SW as a nisin fermentation substrate include its ready availability as a byproduct of the soybean processing industry along with its ability to meet the nutritional demands of the fastidious *L. lactis* bacteria without supplementation. The food-sourced nature of SW could also help ease barriers in regulatory approval for food applications of SW-fermented nisin. As noted, the model SW used in this study was prepared in the laboratory. Further research using SW recovered as an effluent from a soybean processing facility should be tested as the next step towards commercial scale-up and practical utilization of this processing waste. A potential drawback of using SW could stem from the fact that like cow's milk, soy is also a food allergen.

2.5 Animal-Derived Substrates

2.5.1 Mussel-processing waste

Mussel-processing wastes (MPW) with an average chemical oxygen demand of 25 g/L and glycogen as the main carbon source (5–10 g/L) are an important eutrophication factor along the coast of northwest Spain. Guerra and Pastrana (2002b) used MPW to prepare a culture medium for *Lactococcus lactis* subsp. *lactis* CECT 539. MPW have also been used by researchers for the fermentative production of other economically valuable products such as amylases, gibberellic, gluconic and citric acid (Murado et al., 1994; Pintado et al., 1997). The MPW obtained from a local processing plant was adjusted to pH 4.5, centrifuged, and glycogen in the resulting supernatant was hydrolyzed to glucose by α-amylase. The final MPW medium contained (in 1 L): 5.33 g glucose, 0.65 g total nitrogen, 0.14 g total phosphorus, 1.82 g proteins. The saccharified MPW was adjusted to pH 6.3 and sterilized by autoclaving. Batch fermentations were carried out in 250 mL Erlenmeyer flasks. Cell growth was monitored by absorbance at 700 nm and converted to cell dry weight (CDW). Nisin concentrations were measured by a photometric bioassay method (Cabo et al. 1999) using *Carnobacterium piscicola* as the indicator organism. Results indicated that nisin synthesis in the MPW medium ceased when the pH dropped to less than 4 and it was highest (8 AU/mL) when the final pH was 5.8. Similar to the observations by Cabo et al. (1999), an increase in the pH fall gradient or the rate of acidification in the medium during the *L. lactis* growth increased the nisin concentrations achieved. The nisin titer was increased almost 4 times to 33 AU/mL when the MPW medium was buffered with

0.1 M potassium hydrogen phthalate/NaOH (final pH=4.8). The buffer at this concentration allowed the optimum rate of acidification in the medium which resulted in a final pH level suitable for nisin production. Supplementation of MPW media with glucose, glycine and KH_2PO_4 did not enhance nisin production. As future studies, the authors suggested testing other nitrogen sources such as cotton-seed meal, soya-bean meal and fish meal to increase nisin production.

In a follow-up study, Guerra *et al.* (2002c) sought to formulate a nisin fermentation medium from mussel-processing waste (MPW) that would enable maximal nisin production, at the minimal possible cost. The authors tested the effect of increased glucose concentrations and five different nitrogen sources (NH_4Cl, glycine, glutamic acid, yeast extract and Bacto casitone) on the growth of and nisin production from *Lactococcus lactis* subsp. *lactis* CECT 539 in hydrolyzed and buffered MPW medium. The indicator strain used was *Carnobacterium piscicola* CECT 4020. Among the nitrogen sources, yeast extract and Bacto casitone used at a concentration of 3.28 g/L, had strong positive effects increasing the nisin concentration from 32 to 100 AU/mL. The positive effects of yeast extract and Bacto casitone could be related to their high content in minerals, vitamins and amino acids such as serine, cysteine and threonine, which are known to have a precursor role during nisin biosynthesis (De Vuyst, 1995). However, these nitrogen sources would be too expensive for a large scale process.

The authors found that increasing the glucose concentration did not improve either biomass or nisin production. The authors suggested substrate inhibition as the

probable mechanism underlying this inhibitory action of glucose and referred to the study by De Vuyst and Vandamme (1992). In this particular study, the authors had examined and modeled the kinetics of nisin production in a sucrose-based complex medium. They reported that sucrose, although a disaccharide was actively metabolized by *L. lactis* up to 40 g/L concentration, above which the nisin titer was seen to fall. They explained that the genetic linkage previously established (De Vuyst, 1990) between nisin biosynthesis and sucrose fermentation capacity of *L. lactis,* and/or their common metabolic control system, may be responsible for the inhibitory effect of sucrose on nisin production at higher concentrations. Based on this, it is more likely that since the observations made by Guerra *et al.* (2002c) involved nisin inhibition by glucose overdose and not sucrose it would probably be due to a metabolic control system rather than a genetic regulatory system.

Guerra *et al.* (2002c) concluded that the C/N ratio of the growth medium is a key factor and should be between 1.66 and 1.99 for optimal nisin production. Therefore, close attention should be paid to this parameter while investigating different industrial effluents as possible nisin-production substrates. However, a potential problem in using MPW for nisin production could be due to its inherent nature of being an animal waste which could contain potentially toxic components. Therefore, nisin derived from such a source would require extensive purification, and could not be applied directly to food as a concentrated fermentate.

The same group of researchers constructed a dynamic mathematical model to describe the growth and nisin production by *L. lactis* subsp. *lactis* CECT 539 in a fed-

batch and re-alkalized fed-batch cultures using MPW (Guerra et al., 2007). This novel, pseudo-mechanistic model helped to better describe the biological phenomena influencing the growth of *L. lactis* during fermentation in MPW media. Based on their observations the authors concluded that glutamic acid is an absolute requirement for *L. lactis* growth and increasing its concentration in MPW stimulated nitrogen consumption and growth.

2.5.2 Octopus protein waste

LAB growth and nisin production on an industrial scale requires peptidic sources in the nutrient media like peptone, tryptone, meat extract or yeast extract, all of which contribute substantially to the cost of the fermentation media. Vásquez et al. (2004a) explored other inexpensive protein sources like soluble proteins present in the waste-water from octopus processing facilities for cultivating *Lactococcus lactis* subsp. *lactis* HD1-IIM. The octopus wastewater was provided by an industry partner and was composed of 23 g/L of protein (Lowry) and 0.22 g/L of reducing sugars. Supplements added to the OP medium were (in 1 L): 20 g glucose, 2 g yeast extract, 3 g sodium acetate, 2 g ammonium citrate, 2 g di-potassium hydrogen phosphate, 0.2 g magnesium sulfate, and 0.05 g manganese sulfate. The OP medium was compared to medium B, containing Bacto peptone and MRS. The nisin producing strain was grown in 300 mL Erlenmeyer flasks and cell density was monitored by absorbance at 700nm and converted to cell dry weight (DCW). Nisin was quantified as described by Cabo et al.

(1999) and Murado et al. (2002), using the indicator organism *Leuconostoc mesenteroides* WYO Ly.

Increased biomass and nisin production was observed with OP medium (0.963 g/L and 55.38 AU/mL respectively), compared to either MRS (0.598 g/L and 36.54 AU/mL) or B medium (0.671 g/L and 37.59 AU/mL), suggesting a potential advantage for the use of octopus-derived peptones in nisin production. This study indicated slight secondary metabolic characteristics of this metabolite. However, as noted above for other fishery wastes, a concern about the production of nisin from this substrate is the potential presence of toxic compounds. Another drawback to this study was that OP was only a minor component of the medium, the balance of which was comprised of expensive ingredients typical of existing synthetic media formulations.

2.5.3 Fish visceral and muscle protein waste

As established above, one of the most prominent features in the fastidious nutritional requirements of LAB for bacteriocin production is the need for an adequate source of peptides in the growth medium. Peptide sources serve not only as a source of organic nitrogen for the LAB, but are also a source of the essential amino acids or specific peptides which serve as precursors or stimulants for bacteriocin synthesis. Vazquez et al. (2004b) studied the potential of fish viscera and fish muscle protein wastes in providing the required peptides to the LAB growth medium. Peptones from fish have been successfully used for many other microbial metabolites including the

production of proteases by *Bacillus subtilis* (Ellouz et al., 2001), and glycerol by *Saccharomyces cerevisiae* (Kurbanoglu and Kurbanoglu, 2004). An abundant supply of fish waste available for use makes this nutrient source a potentially attractive option from a value-added standpoint. The FP (fish peptone) medium was prepared in this study by grounding the fish viscera of different fish species with ~10% (v/w) distilled water. Homogenates were distributed into aliquots and by adjusting the pH to different levels; they were autohydrolysed to varying degrees. Two other media used for comparison were BP (Bacto peptone) and MRS. Nisin fermentation was carried out using *Lactococcus lactis* CECT 539 in 300 mL Erlenmeyer flasks. Cell growth was monitored by absorbance at 700 nm and converted to cell dry weight (CDW). Nisin concentrations were measured by a photometric bioassay (Cabo et al. 1999) using *Carnobacterium piscicola* CECT 4020 as the indicator organism. These authors observed that peptones from different fish species resulted in different biomass and nisin yields. Trout viscera hydrolysate gave the highest biomass yield (1.6 g/L), while the highest nisin yield was given by yellowfin tuna viscera hydrolysate (8.6 AU/mL). In comparison, biomass yields from BP and MRS were 0.68 and 0.82 g/L, respectively, while the nisin yields were 7.01 and 10.13 AU/mL, respectively. Therefore, in general, *L. lactis* growth in FP medium was higher than both BP and MRS, while nisin yields were lower than MRS but higher than BP.

In a later study by the same group (Vazquez et al., 2006) viscera from rainbow trout and squid and muscle wastes from swordfish were used as the peptide sources in the FP medium. Medium D used in this study was equivalent to Medium B (Vazquez et

al., 2004b). Results from this study indicated that fish peptones were able to meet the complex peptidic requirements of the fastidious *L. lactis.* FP medium containing autohydrolyzed trout viscera had the highest biomass and nisin yields (0.994 g/L and 6.06 AU/mL) as compared to 0.752 g/L, 3.83 AU/mL and 0.665 g/L, 6.76 AU/mL found in Medium D and MRS, respectively.

Based on the Luedeking and Piret (1959) model on bacteriocin synthesis and the Logistic model on biomass, a metabolite can be classified as primary by nature if its production rate is dependent on biomass production rate, secondary, if its production rate depends on biomass present, and mixed, when its dependant simultaneously on both biomass production rate and biomass present (Vazquez et al., 2004b; 2006). An interesting observation in these two studies was the mixed metabolite characteristics of nisin produced on the FP medium from the first study (Vazquez et al., 2004b) and slightly secondary metabolite characteristics of nisin in the second study (Vazquez et al., 2006). Also important to note is that being an animal waste, FP medium not only suffers from the drawback of being toxic which is similar to MPW and OP media, additionally, nisin production using an allergen-containing medium, such as fish, could be problematic from a labeling (and possibly from an organoleptic) standpoint.

2.6 Comparative analysis of the different substrates

The chemical composition of the various non-dairy nisin fermentation media covered in this review has been summarized in Table 1. Overall, reports on the use of plant-based substrates (potato, date, barley, sago, corn, and soy) demonstrate their

better efficiency in supporting *L. lactis* growth and nisin production as compared to the animal-based substrates (mussel, octopus and fish). The highest reported biomass yield among all the substrates was from sago starch (2.76 ± 0.55 g/L) and shochu kasu (barley) (2.7 ± 0.1 g/L), followed closely by soy whey (2.18 g/L). In the case of animal-based substrates, FP medium gave the best results (1.6 g/L from trout). In case of nisin production, the highest concentration was found in soy whey (619 mg/L) (plant-based) and OP medium (27.7 mg/L) (animal-based). Both the growth and nisin data obtained from the various media covered in this review have been illustrated in Table 2 with their maximum yields. Results from two dairy-based media are also included for comparative purposes. Since the various authors have used different measuring units for expressing biomass and nisin yield data in their studies, necessary conversions were done to a common unit to facilitate a true comparison.

It is interesting to note the two distinct trends which have been followed by the research groups. In one set of studies, the nisin fermentation substrate was composed primarily of a waste/byproduct material. For example in the case of fermented barley extract and soy whey, the main goal of the researchers (Furuta et al., 2008 and Mitra et al., 2010, respectively) was to promote the recycling of shochu kasu/soy whey by converting it into a value-added food product with preservative functions with simultaneous value-addition to the nisin fermentation process. Other similar studies were those involving mussel waste, potato waste and date processing wastes. While in the other set of studies, the primary intent of the researchers was to use processing wastes as supplements to standard synthetic media (e.g. CDS, octopus waste, fish

protein waste and sago starch). Therefore, one approach clearly adds value to agricultural or aquacultural waste streams or byproducts, and promotes recycling of nutrients, while the other approach utilizes these substrates as supplements.

Another interesting observation emerging from the literature is that with certain substrates, such as soy whey, date processing wastes, barley extract, and fish peptone waste, the fermentation conditions needed for high LAB growth did not correlate with those needed for high nisin production, thus indicating secondary metabolite characteristics of nisin. By contrast, in media like sago starch, cull potatoes, mussel processing wastes and octopus protein, nisin behaved as a primary metabolite, with its production being proportional to cell growth. The fact that nisin does not always behave as a primary metabolite suggests that the nutrient profiles of candidate substrates may impact nisin biosynthesis in different ways, perhaps through provision of signal molecules that may affect metabolism and gene regulation. A key objective of this review was to provide a comparative analysis of the various non-dairy substrates that have been tested for nisin production. This task is complicated by variations in experimental procedures and conditions used by the different research groups, including different nisin producer strains, different indicator strains, different methods for measurement of nisin and bacterial biomass, etc. Despite these differences, certain general trends in the comparative utility of the different substrates examined to date can be seen.

Table 1. Comparison of chemical composition of non-dairy-based nisin fermentation media

Value-added industrial wastes/by-products as the base fermentation media	**Non-dairy substrate**	**Nutrient supplements [a]**	**Reference**
	Soy whey (SW)	Tested alone and supplemented with sucrose, sodium chloride, potassium hydrogen phosphate, yeast extract (YE), magnesium sulfate High nisin yields when tested alone	Mitra *et al.* (2010)
	Cull potato hydrolysate (CPH)	Tested alone and supplemented with YE, peptone from meat, peptone from soy, corn steep solid (CSS), and distillers dried grains with solubles (DDGS) Low yields when tested alone.	Liu *et al.* (2005)
	Date waste	Tested alone and in combination with cheese whey	Al-Zahrani *et al.* (2006)
	Fermented barley extract (FBE)	Tested alone and supplemented with Glucose Low yields when tested alone	Furuta *et al.* (2008)
	Mussel processing waste (MPW)	Tested alone and supplemented with glucose, ammonium chloride, glycine, glutamic acid, yeast extract, and Bacto casitone Low yields when tested alone	Guerra *et al.* (2002b&c)
	Condensed corn soluble (CCS)	Tested alone and supplemented with glucose	Wolf-Hall *et al.* (2009)
Industrial wastes/by-products used as supplements in fermentation media	**Non-dairy supplement**	**Base components of fermentation media [a]**	**Reference**
	Sago starch	Glucose (from hydrolyzed sago starch), YE, polypeptone	Zarrabal *et al.* (2009)
	Condensed distillers solubles (CDS)[a]	Sweet whey, YE	Liu *et al.* (2007)
	Octopus muscle waste protein (OP)	Glucose, YE, sodium acetate, ammonium citrate, di-potassium hydrogen phosphate, magnesium sulfate, and manganese sulfate	Vazquez *et al.* (2004a)

Table 1. Comparison of chemical composition of non-dairy-based nisin fermentation media (contd.)			
	Fish visceral waste protein (FP)	Glucose, YE, sodium acetate, ammonium citrate, di-potassium hydrogen phosphate, magnesium sulfate, and manganese sulfate	Vazquez *et al.* (2004b)
a – Supplements used in the media for pH control are not included			

Table 2. Maximum biomass and nisin production from batch fermentation of *Lactococcus lactis* strains in different non-dairy substrates as compared to a few dairy-based media

	Fermentation substrate composition [a]	Nisin producer strain	Biomass yield (g/L, db) / growth rate (h^{-1})	Indicator strain	Nisin yield
Plant-based organic wastes	Cull potato hydrolysate + supplements	*L. lactis* subsp. *lactis* ATCC 11454	2.6 mg/L [b]	*M. luteus* ATCC 9341	89.4 mg/L
	Date waste	*L. lactis* subsp.*lactis*	11.8 mg/L	*S. aureus*	20 mm (ZOI)[f]
	Fermented barley extract + supplements	*L. lactis* subsp. *lactis* ATCC 11454	2.7 g/L	NA [c]	1260 IU/mL or 31.5 mg/L [d]
	Sago starch + other media components	*L. lactis* IO-1 (JCM 7638)	2.76 g/L	*B. circulans* JCM 2504	8 AU/mL or ~0.2 mg/L [d]
	Condensed corn soluble + supplements	*L. lactis* subsp. *lactis* ATCC 11454	1 mg/L [b]	*M. luteus* ATCC10240	273 IU/mL or 6.8 mg/L [d]
	Condensed distillers solubles +yeast extract + sweet whey	*L. lactis* subsp. *lactis* ATCC 11454	0.63 mg/L [b]	*M. luteus* ATCC 9341	85 mg/L
	Soy whey	*L. lactis* subsp. *lactis* ATCC7962	2.2 g/L	*M. luteus* ATCC10240	619.2 mg/L
Animal/aquaculture-based organic wastes	Mussel processing waste + supplement	*L. lactis* subsp. *lactis* CECT 539	0.31 h^{-1} [e]	*C. piscicola* CECT 4020	100 AU/mL or ~3.2 mg/L [d]
	Octopus muscle waste + other media components	*L. lactis* subsp. *lactis* HD1-IIM	0.96 g/L	*L. mesenteroides* WYO Ly	1107.6 IU/mL or 27.7 mg/L [d]
	Fish viscera + other media components	*L. lactis* CECT 539	1.6 g/L (trout)	*C. piscicola* CECT 4020	8.6 AU/mL or ~0.2 mg/L [d] (yellowfin tuna)

Table 2. Maximum biomass and nisin production from batch fermentation of *Lactococcus lactis* strains in different non-dairy substrates as compared to a few dairy-based media (contd.)

Dairy wastes	Cheese whey	*L. lactis* subsp. *lactis* CECT 539	0.41 g/L	*C. piscicola* CECT 4020	~ 23 AU/mL = or ~0.5 mg/L [d]
	Skimmed milk	*L. lactis* subsp. *lactis* ATCC 11454	1.91 g/L	*L. sakei* ATCC 15521	502 mg/L
	Milk whey	*L. lactis* subsp. *lactis* ATCC 11454	NA [g]	*L. sakei* ATCC 15521	11,120 mg/L

IU = International units; AU = Arbitrary units.
a - External nutrient supplements and fermentation conditions vary among the different substrates and not included in table.
b - Biomass yields have been converted from CFU/mL (as reported in original research article) to mg/L for comparison, based on experimental calculations (2×10^9 CFU/mL = 1 mg/L)
NA [c] – Disc diffusion assay not performed. Nisin was quantified by High Performance Liquid Chromatography (HPLC).
d - Nisin yield units have been converted from IU/mL or AU/mL to mg/L based on a relation reported by Arauz et al., 2008.
e - Growth rate expressed as generations per hour
f – ZOI: Zone of Inhibition
NA [g] – Growth data not given
Source: Adapted from Liu et al. 2005; Al-Zahrani et al., 2006; Furuta et al., 2008; Zarrabal et al., 2009; Wolf-Hall et al., 2009; Liu et al., 2007; Mitra et al., 2010; Guerra et al., 2002(b&c,2007); Vazquez et al., 2004(a); Vazquez et al., 2004(b); Guerra et al., 2003; Jozala et al., 2007and Arauz et al., 2008.

2.7 Concluding remarks

Global food additives market has grown at an average annual rate of 2.4% between 2001 and 2004 and bacteriocin-based preservatives which play a major part of this market, is expected to continue growing at a steady to strong pace due to the rising Ready-To-Eat market (RTE) (Jones et al., 2005). According to this report by the Texas State Department of Agriculture, several U.S-based as well as multinational food companies are seeking additional ways to incorporate nisin in their operations. The popularity of nisin is owed to its relatively long history of safe use in food, pharmaceutical and health care products and its documented effectiveness against important foodborne pathogens and spoilage agents. But the high production cost of nisin makes it an expensive product and restricts its cost-effective application in bulk fermentation processes (Ogden et al., 1988). As mentioned earlier, medium composition plays an important role in triggering nisin biosynthesis and final yields (Biswas et al., 1991; Parente and Hill, 1992; Daba et al., 1993; Parente and Ricciardi, 1994; Yang and Ray, 1994; Vignolo et al., 1995). Therefore, it is clear that in order to decrease production costs of nisin, it is important to identify suitable substrates that are both inexpensive and nutritionally rich**.** As a result, research towards finding suitable alternative media which are non-dairy (and if possible, non-allergen based) and which are able to support fastidious LAB growth should be encouraged.

In the quest to identify new and inexpensive substrates on which to produce nisin, the primary concern is to identify the reliability of supply and quality of such

feedstocks. The fermentation substrates mentioned in this review paper are all co-products from different food-processing operations. There are certain limitations in utilizing such byproducts as raw materials for a fermentation process. Number one is the inconsistencies in quality leading to variability in nisin yields; secondly, inconsistent raw material price. Another important aspect of a suitable nisin fermentation medium is its availability. Seasonal variations in availability and/or quality depending on the time of the year could be a concern regarding these substrates. Logistics management would also require a close look. Nutrient sources like octopus, mussel and fish protein waste will be hard to access in bulk in non-coastal locations. Cultivation of cereals like barley, soybeans, corn and sago is also dependent on proper soil and/or weather conditions for growth.

An additional factor that can weigh against the use of very crude byproducts is the ultimate market for the nisin produced. If high-purity nisin or food-grade nisin is required, some byproducts may result in high downstream purification costs. When nisin is applied in foods as an ingredient to increase its microbial safety and/or shelf life, the entire fermentate is concentrated, dried and added to the food product. In doing so, it is important to verify that the nisin fermentation medium does not contribute or change the original texture, taste or aroma of that food product. For example, soy whey-fermented nisin could be a suitable additive in soy-based foods, while it may give undesirable “green” or “beany” flavors when added to products such as meat. Similarly, nisin derived from fermentation of fisheries wastes may be

most appropriate for seafood applications, and the absence of potentially toxic compounds would need to be verified.

In summary, a desirable nisin production medium should be of low cost, abundant, able to achieve high productivity, and at the same time should not be an allergen or negatively affect the taste or texture of food. It is interesting to note that some of the non-dairy media are able to support the growth of the fastidious *Lactococcus lactis* strains and consequent nisin production without the need for external nutrient supplementation. This indicates the high nutrient content of these waste materials and their potential for further utilization by recycle and reuse. Harder economic times and stricter environmental norms have compelled the food processing companies to find new ways to add value to their byproducts and effluents before disposal. Using them as substrates for the growth of nisin producing lactococcal strains definitely holds promise and is worth the initial required investment. Further research to explore the economical and practical feasibility of these ideas and the commercialization potential of these non-dairy based substrates is awaited.

2.8 Future trends

There is a keen interest from industries in finding non-traditional substrates for nisin production, which would enable them to lower the production costs, and also improve the versatility of nisin applications (IFT, 2010). Nisin production formulation using low-cost ingredients may have potential in the U.S. market since

nisin is currently imported from European countries. Fuel ethanol production in the U.S. is booming with 13.23 billion gallons produced in 2010, a considerable rise from 2007 (6.5) and 2008 (9.0) (RFA, 2011). The major challenge with a rapidly growing ethanol industry is the utilization of the co-products like CDS. Use of this low-value co-product in producing a high-value product like nisin definitely holds potential for the future. Another high-potential co-product of the ethanol industry is 'thin stillage'. Each gallon of ethanol produces 5-6 gal of stillage (Sankaran et al, 2010) which can be diluted and used as a nisin fermentation feedstock. Using a corn-based nisin fermentate will allow safe and bulk use in food products without regulatory hassles, and is worth further investigation. Fermented barley extract (FBE), another successful nisin production medium is obtained from a by-product (shochu kasu) of the barley-based alcohol (shochu) industry. About 15 t of shochu kasu is generated from the production of 10 kiloliters of shochu and presently the volume of this wastewater has exceeded 800,000 t per year in Japan (Furuta et al., 2008). This substrate could present a promising alternative to the high-cost dairy-based nisin production medium. Similar to corn, sago and barley are also non-allergen cereal crops consumed by humans and their regulatory approval for food-based applications should be easier.

Genetic engineering of *L. lactis* strains or synthetic biology approaches could also be interesting avenues worth pursuing in the future. These strains could be modified such that they are able to synthesize enzymes that can metabolize complex carbohydrates and proteins found in waste/byproduct streams. This could

enable more economical yields of nisin although consumer acceptability and regulatory status of nisin produced in this way are issues that would need to be resolved before moving ahead with this approach.

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CHAPTER 3: VALUE-ADDED PRODUCTION OF NISIN FROM SOY WHEY

A paper published in

***Journal of Applied Biochemistry and Biotechnology* 162 (7): 1819-33 (2010)**

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3.1 Abstract

The objective of this study was to evaluate the potential of low/negative value soy whey (SW) as an alternative, inexpensive fermentation substrate to culture *Lactococcus lactis* subsp. *lactis* for nisin production. Initially, a microtiter plate assay using a Bioscreen C Microbiology Plate Reader was used for rapid optimization of

culture conditions. Various treatments were examined in efforts to optimize nisin production from SW, including different methods for SW sterilization, ultrasonication of soy flake slurries for possible nutrient release, comparison of diluted and undiluted SW and supplementation of SW with nutrients. In subsequent flask-based experiments, dry bacterial biomass and nisin yields obtained from SW were 2.18g/L and 619mg/L, respectively, as compared to 2.17g/L and 672mg/L from a complex medium, de Man-Rogosa-Sharpe (MRS) broth. Ultrasonication of soybean flake slurries (10% solid content) in water prior to production of SW resulted in ~2% increase in biomass yields and ~1% decrease in nisin yields. Nutrient supplementation to SW resulted in ~3 and ~7% increase in cell and nisin yields, respectively. This proof-of-concept study demonstrates the potential for use of a low/negative value liquid waste stream from soybean processing for production of a high-value fermentation end product.

Keywords: soy whey, nisin, value-added production, *Lactococcus lactis*, Bioscreen, fermentation

3.2 Introduction

An increasing demand for natural food preservatives has created a niche market for bacteriocins, which are natural antimicrobial peptides produced by lactic acid bacteria (LAB). Nisin is the only bacteriocin approved by the Food and Drug Administration (FDA) and commercially produced by the fermentation of the lactic

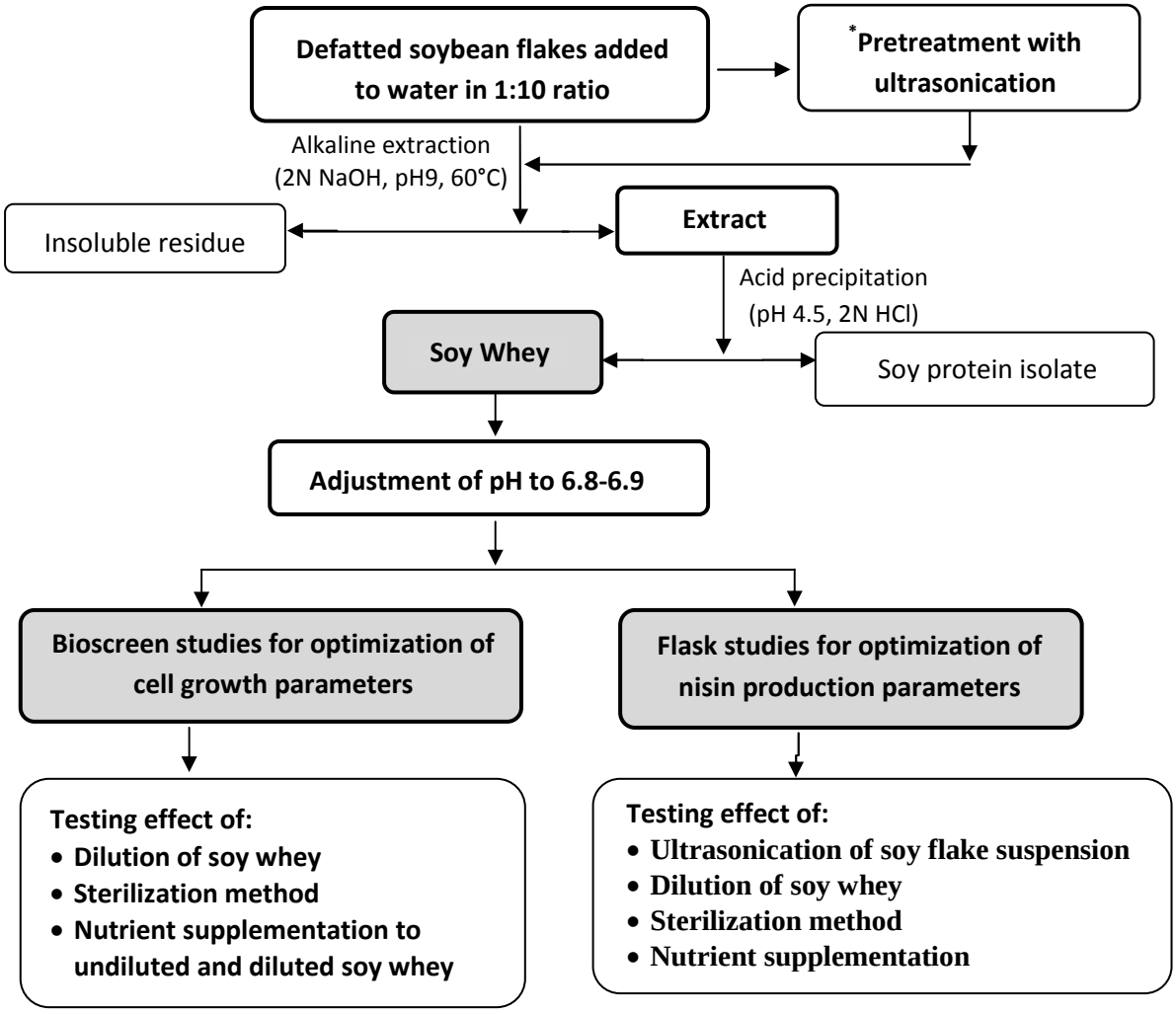
acid bacterium, *Lactococcus lactis* subsp. *lactis* in milk based media under well-controlled culture conditions *(1- 3)*. Nisin is a 34-amino acid peptide with a molecular mass of 3.5 kDa. Nisin occurs naturally in two different forms, nisin A and nisin Z. Nisin Z differs from Nisin A by one amino acid residue. It contains asparagine at position 27 instead of histidine. The two nisin variants have identical minimum inhibitory concentrations against most microbes with nisin Z showing better diffusion ability in agar. Nisin demonstrates wide-spectrum activity against almost all Gram positive bacteria including the food-borne pathogens *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus cereus (4).*

The limitations of nisin production are low yields, low product concentrations and high medium costs among others *(5)***.** In view of the growing consumer demand for natural food additives, there is a great need for more cost-effective means to produce this product. Thus, there has been a rising research trend in exploring the use of food processing waste streams as low-cost, value-added growth media for nisin production. In the quest for suitable materials, dairy byproducts have been widely studied *(1, 6, 7)*. Recent work has also focused on the use of non-dairy substrates. For example, agricultural wastes such as cull potato hydrolysate and fermented barley extracts as well as fishery byproducts, including octopus and mussel processing wastes, have been examined as possible feedstocks for nisin fermentation (*8-11*). In this study, we have evaluated soy whey, a byproduct stream from the soybean processing industry for nisin production as substrate for cultivating the strain *Lactococcus lactis* subsp. *lactis* ATCC 7962*.* Production of nisin using a readily available and non-toxic plant product such as soy whey may help minimize

processing requirements and ease barriers in regulatory approval for food use of SW-derived nisin *(12)***.** To our knowledge, this is the first report on potential usage of soy whey to support *L. lactis* growth and nisin production.

Wet processing of soybeans is commonly used for the production of high-value products such as soy protein isolates (SPI). This process, however, leads to the generation of large volumes of concentrated liquid waste material, known as “soy whey”. As an economical recovery method is yet to be established, whey generation leads to a serious waste disposal problem *(13)*. Figure 1 shows a schematic of lab preparation of soy whey *(modified from [12, 14, 15]).* Soy whey accounts for nearly one-third of the defatted desolventized soybean flakes used in the production of soy protein isolate (SPI) and contain about 11% (w/v) of the total nitrogen in soybean meal**.** In other words, every kilogram of wet-processed soybean generates almost 0.33 kilogram of soy whey *(14, 16)*. Because soy whey contains only 1-3% (w/v) solids, it is generally considered too dilute for economical recovery of the whey solids *(14, 17)*. Smith et al. *(18)* showed that soy whey solids content and composition depend on origin of the soybean meal and method of whey preparation. On a dry basis, soy whey typically contains 3.6-4.4% nitrogen (50% of which is protein nitrogen) and 25-35% soluble sugars like sucrose, raffinose, and stachyose, various glycosides, galactans and hemicelluloses *(14)*. Substantial amounts of phosphorus and minerals, largely as phytate salts and numerous trace elements essential for microbial fermentation of fastidious LAB are also present in soy whey *(14, 18)*. Because of its inherent nitrogen content, soluble sugar levels and high

Chemical Oxygen Demand (COD) content (25,000 mg/L); soy whey poses a burden on the environment if disposed of untreated.



* This step was performed only in some experiments (potential effects of ultrasonic pretreatment on growth and nisin production).

Figure 1. Lab preparation of soy whey *(modified from 12, 17, 23)* and experimental outline for its use as a growth medium for *Lactococcus lactis* subsp. *lactis* ATCC 7962

Although it is generally considered a waste product, we hypothesized that the high nutrient content of soy whey could enable its value-added use as a substrate for microbial fermentation. Specifically, we sought to investigate the use of soy whey as a fermentation feedstock for the production of the high-value food preservative nisin. The goals of this study were (a) evaluation of soy whey as a substrate for LAB growth and subsequent nisin production, (b) optimization of soy whey medium and fermentation parameters and (c) comparison of the biomass and nisin yields obtained from soy whey to MRS broth (Difco Laboratories), a nutritionally rich complex growth medium.

3.3 Materials and methods

3.3.1 Microorganisms and media

Lactococcus lactis subsp. *lactis* ATCC 7962 (nisin producer) and *Micrococcus luteus* ATCC 10240 (nisin indicator strain) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained as frozen stocks at -75℃ in MRS or nutrien t broth (Difco Laboratories, Lawrence, KS, USA) media, respectively, containing 20% (v/v) glycerol. Working cultures of *L. lactis* and *M. luteus* were maintained as slants on MRS and nutrient agar at 4℃, respectively.

3.3.2 Soy whey preparation and partial characterization

Commercially available soybean flakes (Cargill, Inc., Cedar Rapids, IA) were obtained from the Center for Crops Utilization Research (CCUR) at Iowa State

University, Ames, IA. Soy flakes were packed in air-tight plastic bags and stored at 4℃ until further use. According to analyses provided by the manufacturer, these flakes contained 57.3 % (dry basis) crude protein and had a protein dispersibility index (PDI) of 93.8. A method for lab-scale production of soy whey from hexane-defatted, desolventized dry soybean flakes was developed, yielding a final product with properties similar to soy whey generated through commercial-scale wet milling of soy beans. Briefly, ground and defatted soybean flakes (50 g) were mixed in a 1:10 ratio (w/v) with tap water *(19)*. The pH was raised to 8.5 with 2N NaOH and the resulting slurry was stirred for 30 min at 60℃. After centrifugation at 10,000 *g* (20 min, 4℃), the supernatant was collected. The water soluble proteins in the supernatant were precipitated by lowering the pH to 4.5 with addition of 2N HCl. The slurry was kept at 4 ℃ for ~ 4 h and was again centrifuged at 10,000 *g* for 20 min at 4℃. The supernatant (soy whey) was collected; the pH was adjusted to 6.8-6.9 and after sterilization it was ready to be used as a growth medium. Use of this lab-scale method for production of soy whey yielded a reproducible substrate for our proof-of-concept studies.

The soy whey produced in this manner was characterized for its total nitrogen and protein content, as well as its sugar composition. Total nitrogen content was estimated using the Dumas nitrogen combustion method *(1)* with an Elementar Vario MAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) and was found to be 1,210 mg/L and after multiplying by conversion factor 6.25, the crude protein content was calculated to be 7,575 mg/L. Whey sugars were analyzed using

a Varian High Performance Liquid Chromatograph (HPLC) with a Bio-Rad HPX-87P column for carbohydrates, a guard column, and equipped with a Varian's refractive index (RI) detector. The sample volume was 20 µl, eluant used was filtered (Millipore nylon membrane filter, pore size 0.2 µm) and degassed HPLC grade water at a flow rate of 0.6 ml/min. The column temperature was 85℃ a nd run time was 22 min. Standards used were glucose, galactose, fructose, sucrose, stachyose and raffinose prepared at a concentration of 5 mg/ml in mili-Q water. The soy whey was found to contain 7.14 mg/ml sucrose, 6.56 mg/ml stachyose, 0.84 mg/ml galactose and 0.74 mg/ml glucose.

3.3.3 Inoculum preparation

In preparation for its use as a microbial growth substrate, the pH of the whey was adjusted from 4.4-4.7 to 6.8-7.0 and the whey was sterilized via heat (121℃ for 15 min) or filtration (0.2 µm nylon filter). Inocula for batch fermentation were prepared by adding 1 ml of culture stock (10^9 CFU [colony forming units] /ml) to 10 ml of sterilized soy whey and incubating at 28℃ for 10 h under stationary conditions. Additional dilution was performed as needed to obtain a final cell concentration of 10^9 CFU /ml, equivalent to an absorbance of 0.45 at 600 nm (Abs_{600}).

3.3.4 Batch fermentation

Batch fermentations were carried out in 250 ml Erlenmeyer flasks containing a final volume of 100 ml of soy whey. A 10% (v/v) inoculum was added to the flasks

to obtain a cell density of 10^8 CFU/ml and flasks were incubated at 28℃ for 48 h. All soy whey batch fermentations were carried out under static conditions. At regular time intervals during the fermentation, samples were taken and pH, cell density (Abs_{600}), total sugars, total soluble proteins, lactic acid, acetic acid and bacteriocin concentration were measured. For comparison, parallel batch fermentations were carried out in MRS broth using growth conditions identical to those used for soy whey. All batch fermentations and analytical determinations were performed in triplicate. Between 1-3 ml of sample was removed for analysis at each sampling time, depending on which analytical tests were performed.

3.3.5 Microtiter plate rapid bioassay

A Bioscreen C Microbiology Reader (Growth Curves, Inc., Piscataway, NJ) was used to rapidly determine the effect of various culture parameters on bacterial growth, assuming a positive correlation between cell growth and nisin production. The Bioscreen instrument is a combined incubator and microplate reader that enables collection of high-resolution optical density data for up to 200 individual wells (well capacity 400 µl). Plates can be incubated at temperatures ranging from ambient to 60°C, and automated optical density readings of individual wells can be taken at discrete intervals to yield detailed growth curves useful in process optimization. Growth medium (200 µl) and inoculum (50 µl), were added to individual wells to yield a final cell density in each well of 10^8 CFU/ml or Abs_{600}=0.17. Inoculated plates were placed in the Bioscreen and incubated for up to 24 h at 28°C. Abs_{600} was recorded at 30 min intervals with automatic shaking for 5 s before each

reading to ensure proper mixing of well contents. All treatments were evaluated in replicates of five (n=5).

3.3.6 Analytical procedures

Biomass yields

A standard curve was derived by plotting cell dry weight (CDW) measurements (g/L) against corresponding spectrophotometric Abs_{600} readings (Spectronic 20 Genesys, Thermo Electron, Cambridge, UK). CDW was measured by harvesting the cells of known Abs_{600} by centrifugation (12,000*g* for 15 min at 4℃), washing twice with sterile saline (0.85% [w/v] NaCl) and drying to a constant weight at 80℃. A regression equation was obtained for CDW as a function of Abs_{600}. A correlation coefficient of 0.9955 was derived for the best fit regression line for the CDW - Abs_{600} curve. During fermentation, absorbance readings of samples were converted into equivalent CDW values (g/L) using this standard curve and plotted against time (h) to obtain growth-time curves.

3.3.6 Determination of sugars, proteins and fermentation end products

After removal of the bacterial cells from the culture broth by centrifugation (12,000*g*, 15 min, 4℃), the culture supernatants were tested fo r total sugars by the phenol–sulfuric acid method of Strickland et al., (1968) *(20)*, with glucose as the standard. The total soluble proteins were measured by Lowry's method *(21),* with bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard. The sugar and protein contents were determined spectrophotometrically at 490 and 750 nm, respectively. Lactic acid and acetic acid concentrations were measured using a

Waters HPLC (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 RI detector, column heater, auto-sampler and computer controller. A Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) was used with 0.012N sulfuric acid as the mobile phase with a flow rate of 0.8 ml/min, injection volume of 20 µl and a column temperature of 65℃. All measurements were performed in triplicate (n=3).

3.3.7 Nisin quantification

The nisin titer in the fermentation broth samples was determined by the method of Tramer and Fowler *(22)*, with the modifications of Wolf et al. *(15)*. The volume of the fermentate present in individual Bioscreen wells was too low to allow accurate manipulation of the sample needed to determine nisin titer. Therefore, nisin titer was determined only for samples prepared using Erlenmeyer flasks. Briefly, fermentation broth samples from flasks were adjusted to pH 2.0 with dropwise addition of 2N HCl. This was done to release nisin bound to producer cell surfaces to facilitate measurement of cell-associated nisin. Samples were then heated at 90℃ for 5 min to inactivate any interfering proteases in the medium without affecting nisin, which is thermostable at acidic pH. After centrifugation at 4,000*g* for 10 min at 4℃, the clear, nisin-containing supernatant (nisin extract) was neutralized in order to eliminate erroneous inhibition zones resulting from low pH. Nisin concentration was determined by agar well diffusion bioassay *(22, 23)* using *Micrococcus luteus* as the indicator strain. Briefly, Nutrient Agar (NA) overlays containing a concentration of 10^8 CFU/ml *M. luteus* were poured over NA base plates, allowed to harden and wells

were bored in the agar aseptically using a sterile stainless steel borer (5 mm inner diameter). One hundred microliter aliquots of nisin extract were added to individual agar wells and the plates were incubated overnight at 37ºC. The diameter of resulting zones of inhibition around each well were measured horizontally and vertically to the nearest 0.01 mm using a digital caliper (Digimatic caliper, Mitutoyo, Kanagawa, Japan) and the average value recorded. These values were used to determine the nisin concentration from the standard curve (below). Each zone of inhibition determination represented the average of three replicate wells.

Standard curve: The commercial nisin used here (Nisaplin, Sigma Chemical Co., St. Louis, MO), was reported to contain $1x10^6$ IU per gram. A stock solution of this nisin preparation at 1,000 IU/ml was made using sterile 0.02N HCl. This stock solution was diluted further as needed in sterile 0.02N HCl, neutralized and a standard curve relating zone size to nisin concentration was prepared by plotting inhibition zones versus known units of activity (IU). The regression equation obtained for inhibition zone diameter as an exponential function of nisin concentration: Y (log nisin conc.) = 0.1515 * (zone diameter) - 3.9467, resulted in a correlation coefficient of 0.989. Nisin concentrations (in mg/L) in test samples were estimated from inhibition zone measurements using this standard curve.

3.3.8 Optimization of fermentation parameters

Several different pre-fermentation treatments were examined for their impact on cell growth and nisin production in an effort to optimize our results. Factors

examined included different methods of soy whey sterilization, physical treatment (sonication) of defatted soy flakes for possible enhanced nutrient release, the use of diluted vs. undiluted soy whey and micronutrient supplementation of diluted or full strength soy whey. These treatments are described below.

3.3.8.1 Soy whey sterilization

Four methods for soy whey sterilization were evaluated to determine the procedure with the minimal impact on nutrient bioavailability as determined by microbial growth and nisin production. The first method tested was membrane filtration using a Millipore nylon membrane filter of pore size 0.2 µm (Millipore Corp. Billerica, MA, USA). The second method was heat sterilization at 121°C, for 15 min. In the third method, heat sterilization at 121°C for 15 min was followed by centrifugation at 5,000*g* for 15 min at 20°C to remove precipitated materials. In the fourth method, soy whey was filter-sterilized followed by heat sterilization and centrifugation as described above. Filtered and autoclaved soy whey mixed in different proportions was also examined. Heat-sterilized MRS was used as control. Cell growth and nisin production were monitored and measured at regular time intervals in the Bioscreen as well as flask level. Table 1 gives the nomenclature used in this paper to describe these different preparations of the soy whey media.

3.3.8.2 Pre-treatment of soybean flake suspension by ultrasonication

The impact of high-power ultrasound on sugar and protein release from soybean flakes *(24, 25)* and subsequent biomass and nisin yields from soy whey

made with sonicated flakes was investigated. Defatted soy flakes (10 g) were dispersed into 100 ml of tap water in a glass beaker. The batch experiment was conducted in a Branson 2000 series bench-scale ultrasonic unit (Branson Ultrasonics, Danbury, CT) with a maximum output of 2.2 kW. A standard 20-kHz half wavelength titanium horn with a gain of 1:8 and a booster with a gain of 1:1.5 were used. Samples were sonicated at amplitudes 60 and 192 μm_{pp} (peak to peak amplitude in μm) for 15 and 120 s. Soy whey was prepared from the sonicated soybean flakes suspension by the aqueous alkaline method summarized in Figure 1. Whey prepared from non-sonicated defatted soy flakes was used as a control. All whey samples were filter sterilized and characterized for protein and sugar contents, and tested for their ability to support cell growth and bacteriocin production in batch fermentation studies.

3.3.8.3 Soy whey dilution

Three different dilutions of 10, 50 and 80% (v/v) of Medium FW (Table 1) were prepared with sterile distilled water in 250 ml Erlenmeyer flasks. Undiluted Medium FW was used as a positive control. All flasks were inoculated as described earlier and samples taken aseptically were tested for cell growth and nisin concentration and the yields were compared.

3.3.8.4 Supplementation of diluted soy whey

The effect of carbon source supplementation to diluted soy whey (Medium DFW-ss) was compared to micro-nutrients supplementation (Medium DFW-ns) and

cell yields were calculated from Bioscreen absorbance data. It was found from previous experiments that 4-fold dilution of Medium FW (Medium DFW) could support growth and hence in this experiment, supplementation was done in Medium DFW. Unsupplemented Medium DFW and Medium FW were used as controls.

Supplementation of soy whey

In order to evaluate the potential of enhancing nisin activity by external nutrient supplementation, different micronutrients (sucrose, NaCl, KH_2PO_4, Yeast Extract, $MgSO_4$) were added to Medium FW in defined amounts *(25)* and their individual as well as combined effects were studied in the Bioscreen. Unsupplemented Medium FW was used as the control.

Table 1. Nomenclature of soy whey media used

Soy whey media	Composition
Medium FW	Filter sterilized (0.2 µm nylon membrane) soy whey
Medium AW	Autoclaved (121°C for 15 min) soy whey
Medium ACW	Autoclaved and centrifuged (5000 *g* for 15 min at 20°C)soy whey
Medium FACW	Filtered, autoclaved & centrifuged soy whey
Medium 1:1	1:1 Filtered: Autoclaved &centrifuged soy whey
Medium 3:7	3:7 Filtered: Autoclaved &centrifuged soy whey
Medium SFW	Soy whey with nutrient supplementation (FW + 2% (w/v) sucrose + 0.5% (w/v) KH_2PO_4 + 0.01% (w/v) $MgSO_4 \cdot 7H_2O$ + 0.1% (w/v) NaCl + 0.01% (w/v) yeast extract [Difco])
Medium DFW	4-fold diluted Medium FW
Medium DFW-ss	Diluted soy whey with sucrose supplementation

	(4-fold diluted FW + 2% (w/v) sucrose)
Medium DFW-ns	Diluted soy whey with nutrient supplementation (4-fold diluted FW + 2% (w/v) sucrose + 0.5% (w/v) KH_2PO_4 + 0.01% (w/v) $MgSO_4 \cdot 7H_2O$ + 0.1% (w/v) NaCl + 0.01% (w/v) yeast extract)

3.3.9 Comparative study with Lactobacillus casei

To examine whether nisin activity was the sole cause of the inhibition zones observed for *L. lactis* grown on soy whey, a comparative study using a modified agar overlay method *(26)* was done using *Lactobacillus casei* ATCC 7469, a homofermentative LAB that produces lactic acid but not nisin. Briefly, a colony of each organism was grown on MRS agar, a Nutrient Agar overlay containing 10^8 CFU/ml of the *Micrococcus luteus* indicator strain was poured over the colonies. These plates were incubated overnight at 37ºC, zones of inhibition in the overlay resulting from the production of inhibitory substances by the *L. lactis* or *L. casei* colonies were measured and additional testing was performed as needed to characterize the nature of any inhibition phenomena observed (see Results and Discussion).

3.3.10 Statistical Analysis

The significant difference between results obtained from different treatments was evaluated and analyzed by the Method of Derived Variables *(27)*. A logistic growth model was fitted to the time profile of each well, using non-linear least squares. Using this fit, the entire time profile for each well was reduced to one

number denoted by the letter, ‘a’, which can be scientifically interpreted as the limit of bacterial growth over time. All pair-wise treatment comparisons were done using the Wilcoxon Rank Sum Test. The *p*-value was derived after comparison of the mean ‘a’ values for each experimental sample. The *p*-value was calculated for every experimental sample in comparison with the others. A p-value<0.01 indicated statistically significant different mean values of ‘a’ for the two samples. Samples having higher ‘a’ values were then judged to represent the optimal condition/treatment under investigation. The performance of different media was statistically analyzed and the ones having statistically significant differences in their performance were further selected to identify the best culture growth condition.

3.4 Results and Discussion

3.4.1 Sterilization method

The sterilization method of soy whey played an important role on the growth rate of *L. lactis* in soy whey. The combined treatment of filtration, followed by autoclaving and centrifugation (Medium FACW) resulted in a significantly higher (p<0.01) growth of *L. lactis* while the autoclaved soy whey medium AW resulted in a significantly lower growth (p<0.01) of *L. lactis* as compared to MRS. This is summarized in Table 2 and Figures 2a and 3.

It is possible that heat sterilization led to a loss of proteins from the whey via denaturation and precipitation. If so, then Medium ACW would likely be nitrogen deficient, which negatively impacted cell yields (Table 2). However, Medium ACW

also supported the highest nisin production of all the soy media examined. Soy is known to contain protease inhibitors *(28, 29)*. Post-translational modification of pre-nisin to nisin requires the action of proteases. It is therefore possible that heat denaturation of endogenous soy protease inhibitors could have played a role in the higher nisin activity observed in Medium ACW, as proteases responsible for activating nisin would have remained active. This observation was further supported by the fact that equal proportions of filtered soy whey and autoclaved soy whey in Medium 1:1 gave a nisin yield which was almost midway between that obtained separately from filtered soy whey and autoclaved soy whey. Medium 3:7 which had an increased percentage of autoclaved soy whey, showed an increased nisin yield but lower cell density than Medium 1:1 (Table 2).

We found that growth of *L. lactis* in soy whey differed from that previously reported for cheese whey *(30)*. Specifically, we found the nisin yield from soy whey was growth-independent. Among the soy whey media tested, Medium FACW led to the highest growth rate (253.86 mg/L/h) and biomass yield (2.18g/L), while Medium ACW yielded the highest nisin activity (24,767IU/ml, corresponding to a nisin concentration of 619.2mg/L). Overall, MRS gave the highest nisin concentration (672.8mg/L). However, Medium ACW resulted in a higher production rate for nisin, after an initial lag from 0 – 12 h (Table 2).

A similar decoupling of nisin and cell biomass production has been reported by other groups. For example**,** Vásquez et al. *(10)* found higher nisin yields under conditions conducive to lower cell growth. There is some controversy as to whether

nisin should be considered a primary or a secondary metabolite *(5, 30-33)*. Difficulties in making direct correlations between cell growth rate and nisin production may stem from cryptic phenomena such as adsorption of the bacteriocins onto cell surfaces and/or the post-translational processing of the prepeptides to active forms *(9)*. Additional media-specific variables (pH, nutrient availability, etc.) may also play a role in bacteriocin synthesis and further confound efforts at direct correlation between growth and nisin production *(2, 34-36)*.

A clear link between the nutrient content of media in which *L. lactis* is grown and final nisin concentration has been established *(37,38)*. With this in mind, it was interesting to note that the average biomass yield from the soy whey media tested (average for all treatments was 2.15 g/L) was comparable to MRS (2.17 g/L), while the growth rate in soy whey (Medium FACW, 253.86 mg/L/h) was higher than MRS (217.48 mg/L/h) (Table 2). This indicates a high nutrient content for soy whey, underscoring the latent potential of this agricultural waste stream and its potential use as a value-added substrate for supporting microbial growth.

Table 2. Biomass and nisin yields from soy whey sterilized by different methods in static culture flask studies (n=3)

	Biomass Yield			Nisin Yield		
Soy whey media	Maximum absorbance at 600 nm	Maximum biomass yield (mg /L)	Maximum growth rate[b] (mg/L/h)	Maximum nisin activity (IU/mL)	Maximum nisin concentration (mg/L)	Maximum nisin production rate[c] (mg/L/h)
FW	1.03	2151.3	218.13	23,230	580.75	53.44
AW	0.71	2112.4	176.03	1,192	29.79	1.98
ACW	1.12	2141.5	308.79	24,767	619.16[a]	72.58[a]
FACW	1.32	2183.2[a]	253.86[a]	23,740	593.49	63.26
1:1-FW:ACW	1.01	2144.0	252.24	23,988	599.7	61.24
3:7-FW:ACW	0.81	2122.2	282.96	24,114	602.85	67.84
MRS	1.2	2174.8	217.48	26,912	672.81[a]	57.75[a]
25%,15s	1.23	2175.95	217.59	23,886	597.15	54.29
25%, 120s	1.24	2177.37	217.74	24,279.2	606.98	55.18
100%, 15s	1.17	2168.10	216.81	24,248	606.20	55.11
100%, 120s	1.22	2174.01	217.40	24,339.6	608.49	55.32

[a]Signifies highest yields/rates of biomass/nisin production in the group.

[b]Maximum growth rate was determined by calculating the growth rates over constant time intervals during log phase of growth curve and selecting the highest.

[c]Maximum nisin production rate was determined by calculating the nisin production rates over constant time intervals during entire growth curve and selecting the highest.

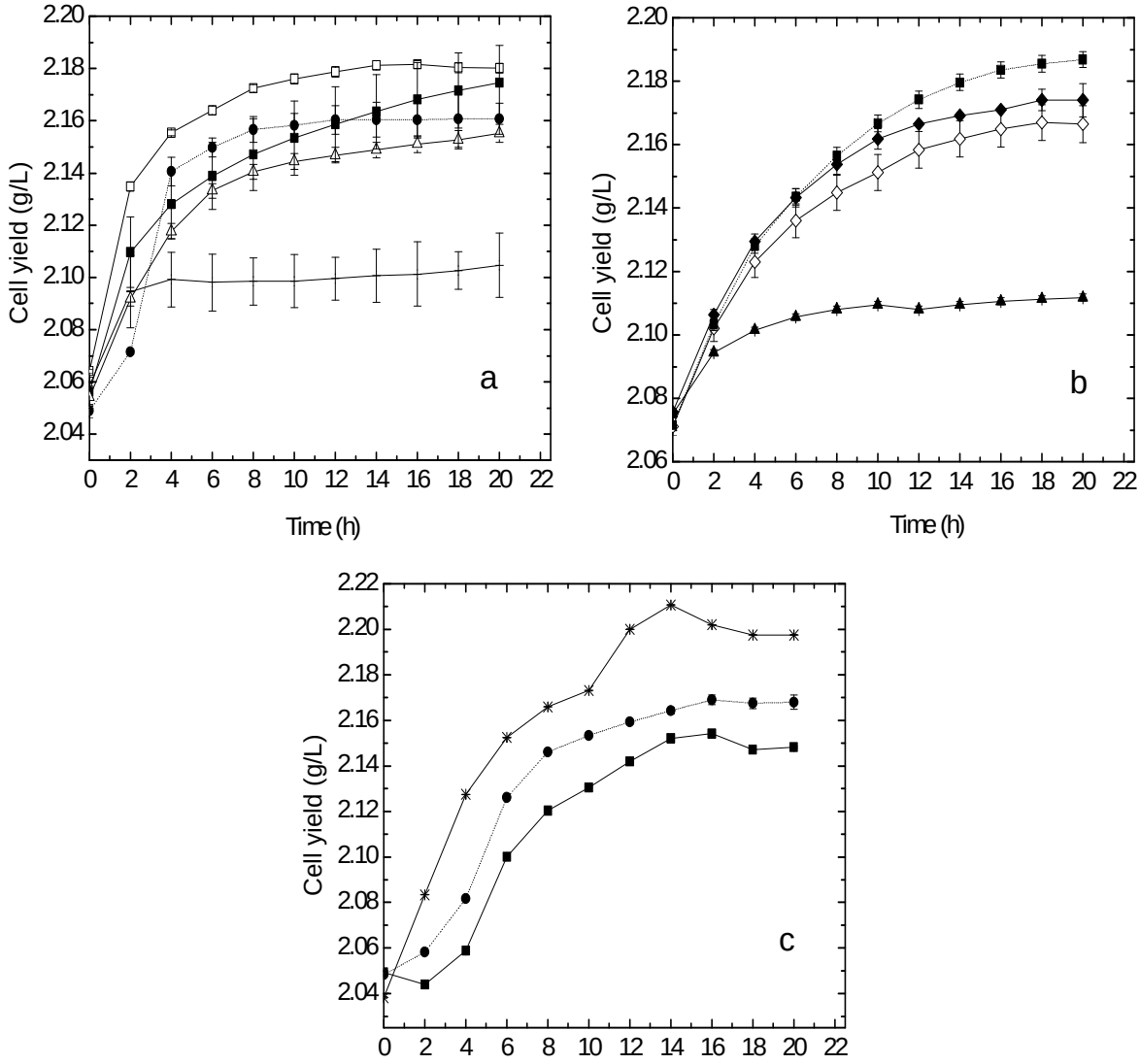


Figure 2. Time course of growth of L. lactis subsp. lactis ATCC 7962 in soy whey media under different conditions using the Bioscreen C Microbiological Reader. a Soy whey sterilized by different methods as compared to standard MRS medium: filled circles MRS, filled squares Medium FW, unfilled triangles Medium ACW, solid line Medium AW, unfilled squares Medium FACW; (n=5). b Medium FW diluted to different levels as compared to original Medium FW: filled triangles 10% or 10-fold diluted Medium FW, unfilled diamonds 50% or 2-fold diluted Medium FW, filled diamonds 80% or 1.25-fold diluted Medium FW, filled squares undiluted Medium FW; (n=5). c Soy whey supplemented with

nutrients as compared to unsupplemented soy whey and MRS: asterisks Medium SFW, filled squares Medium FW, (filled circles) MRS; (n=5)

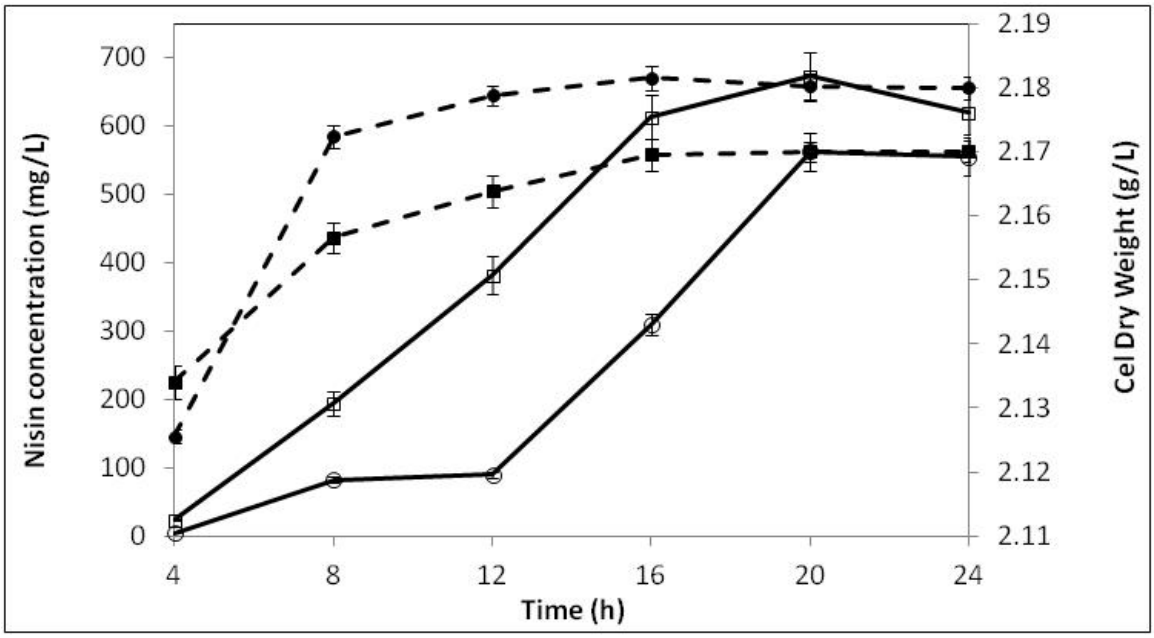


Figure 3. Growth and nisin production rates in soy whey as compared to MRS in flask culture studies: filled circles Cell dry weight-MRS, filled squares Cell dry weight-Medium FACW, (unfilled circles) Nisin concentration-MRS, unfilled squares Nisin concentration-Medium FACW; (n=3)

3.4.2 Ultrasonication of soybean flake slurry

Pre-treatment of the soy flake slurry by ultrasonication prior to lab scale production of soy whey did not affect the protein and sugar concentration in the soy whey (data not shown). Soy whey made from sonicated soy flake slurry resulted in slight increase in cell yields and slight decrease in nisin yields (Figure 4). These results suggest that sonication did not result in the release of nutrients essential for nisin production.

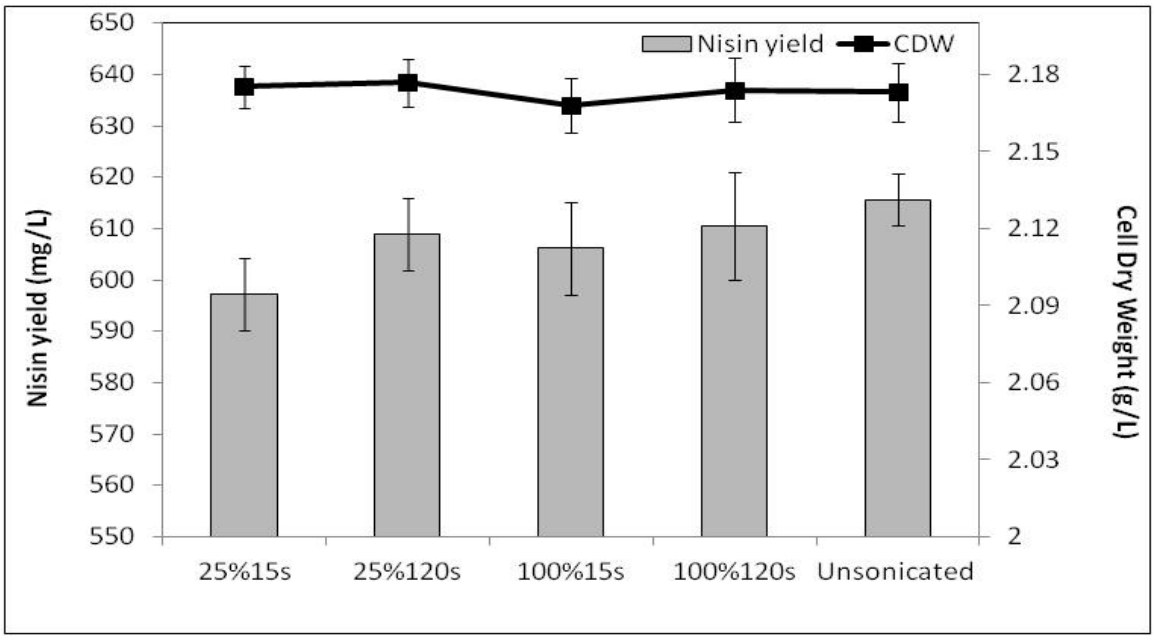


Figure 4. Flask culture study of *Lactococcus lactis* subsp. *lactis* ATCC 7962 growth in Medium FW prepared from ultrasonicated soy flake suspension and consequent nisin yields after 48 h of incubation at 28℃, as compared to Medium FW prepared from unsonicated soy flakes. The 25 and 100% amplitude of ultrasonic waves corresponding to 21 and 84 μm_{pp} (peak to peak amplitude in μm) were used for 15 and 120 s; (n=3)

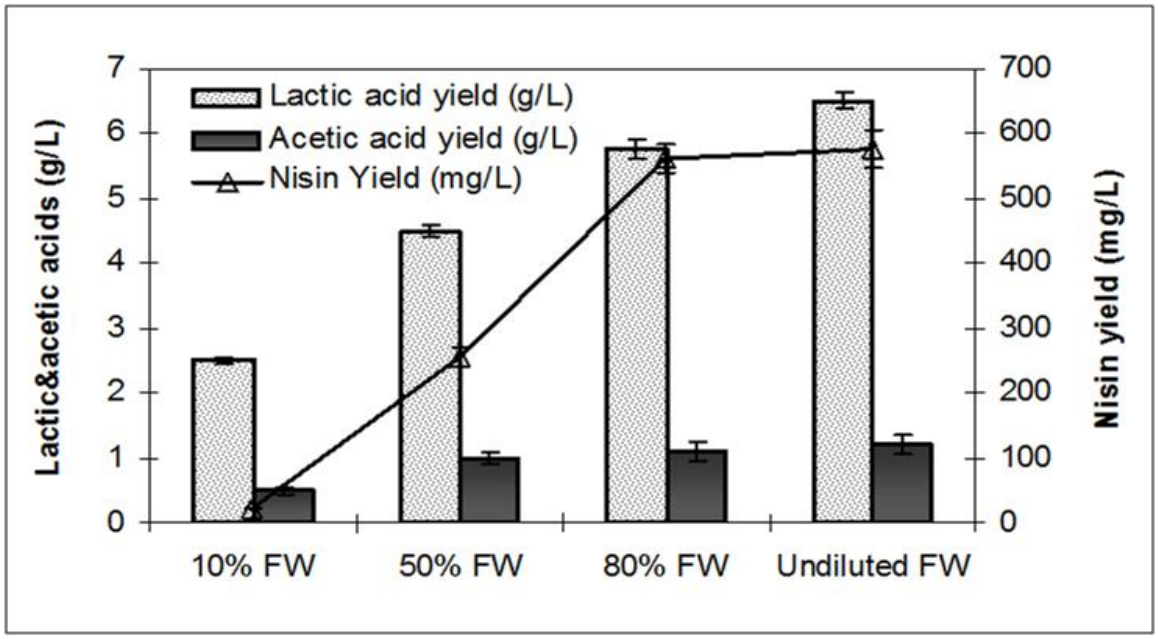


Figure 5. Flask culture study of lactic acid, acetic acid and nisin yields after 24 h of growth of *Lactococcus lactis* subsp. *lactis* ATCC 7962 in Medium FW at different dilution levels; (n=3)

3.4.3 Dilution of soy whey

Both lactic acid (R^2= 0.9571) and nisin yields (R^2= 0.7867) were directly related to the whey concentration (Figure 5). However, ten-fold diluted Medium FW was still able to support *L. lactis* growth and nisin production (Figure 2b). Hence, the nutrient level in soy whey was found to be quite high and dilution could be done without significant decrease in biomass yield.

3.4.4 Nutrient supplementation to diluted soy whey

From the results of the dilution studies done above, it was evident that the growth of *L. lactis* in 4-fold diluted soy whey was significantly lower than undiluted whey (p<0.01). Hence 4-fold diluted whey was used in this test to examine the effect of nutrient supplementation on *L. lactis* growth and nisin yields. Supplementation of diluted soy whey with sucrose as a carbon source (Medium DFW-ss) did not lead to a significant increase in biomass yield vis-à-vis unsupplemented, diluted soy whey (p>0.01), whereas supplementation with nitrogen and phosphorus sources (Medium DFW-ns) led to a significant increase in growth (p<0.01) (data not shown). Interestingly, Medium DFW-ns was almost as efficient as undiluted soy whey in its ability to support bacterial growth. This suggests that the growth-limiting nutrient in soy whey is not the carbon source.

3.4.5 Nutrient supplementation to undiluted soy whey

When sucrose, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl and yeast extract were individually supplemented to Medium FW, significant increase (p<0.01) in biomass yield was observed in almost every case (data not shown). Among the nutrients, KH_2PO_4 proved to be the most effective growth promoter/supplement. Our hypothesis is that KH_2PO_4 acts as a growth promoter in soy whey by supplying phosphorus, as supported by Liu et al. *(30)*. Soybeans do contain phosphorus, but the primary source of phosphorus in soy is phytate which is in a biologically unavailable form *(39)*.

Biomass yield from Medium SFW was significantly higher than Medium FW and MRS both (p<0.01) (Figure 2c, Table 3). Thus, this indicated that addition of small amounts of mainly a phosphorus source could greatly increase *L. lactis* growth in soy whey.

As determined via HPLC, lactic acid production in soy whey during *L. lactis* growth was seen to rise slightly with nutrient supplementation, paralleling our observation of increased biomass yield in supplemented whey treatments. Growth of *L. lactis* was seen to reach a stationary phase before the complete depletion of sugars in the whey (Figure 6). There could be two possible explanations to this observation; either the growth-limiting nutrient in soy whey was not the carbon source, or *L. lactis* grew and exhausted the simple sugars in the whey and were not able to breakdown the remaining disaccharides and complex sugars for carbon uptake. This latter possibility is supported by the fact that *L. lactis* does not produce α-galactosidase, the enzyme needed to degrade stachyose and related sugars *(40)*.

Table 3. Cell yields of *Lactococcus lactis* subsp. *lactis* ATCC 7962 and nisin production when grown on MRS, supplemented soy whey (Medium SFW) or un-supplemented soy whey (Medium FW); (n=3)

Media	Maximum nisin activity (IU/ml)	Maximum nisin concentration (mg/L)	Maximum absorbance at 600 nm	Maximum biomass yield (mg /L)
Medium FW	23,230	580.80	1.03	2151.3
Medium SFW	24,858	621.46	1.5	2211.2
MRS	26,912	672.81	1.2	2174.8

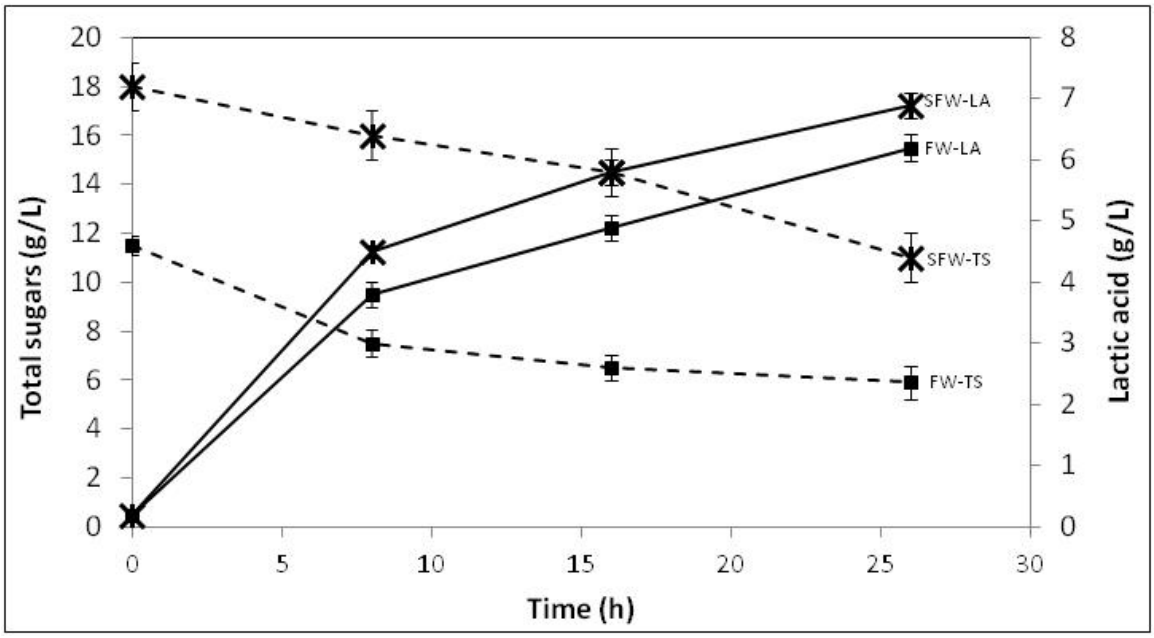


Figure 6. Total sugar depletion and lactic acid production during flask culture study of L. lactis subsp. lactis ATCC 7962 in Medium SFW and Medium FW. Filled squares in broken lines Total sugars in Medium FW, ex symbols in broken lines Total sugars in Medium SFW, filled squares in solid lines Lactic acid in Medium FW, ex symbols in broken lines Lactic acid in Medium SFW; (n=3)

3.4.6 Antimicrobial activity of Lactococcus lactis and Lactobacillus casei against M. luteus

The modified agar overlay method *(26)* used resulted in inhibition zones around both *L. lactis* and *L. casei* colonies (data not shown). We surmised that the unexpected zones observed for the nisin-negative *L. casei* could have arisen from the production of lactic acid, which has weak antimicrobial effects against the indicator strain. To investigate this, plates were incubated for an additional 2-3 days. After additional incubation, growth of *M. luteus* was observed within the previously clear inhibition zones on plates containing *L. casei* colonies. Parallel tests performed with the nisin-producing *Lactococcus lactis* resulted in persistent and clear zones of inhibition around *L. lactis* colonies. These results suggest that the antimicrobial effects initially seen with *L. casei* colonies were transitory effects due to metabolites such as lactic acid, which could be differentiated from the unambiguous inhibitory effects of nisin produced by colonies of *L. lactis*. These results, along with the fact that nisin extracts from soy whey fermentates were pH-adjusted, confirm that the clear and persistent zones of inhibition produced by these extracts were indeed due to nisin and not caused by confounding factors such as the presence of other inhibitory metabolites such as lactic acid.

3.5 Conclusions

This work provides proof-of-concept data for the use of soy whey as a suitable and inexpensive medium to grow the nisin-producing strain, *Lactococcus lactis*. Because soy whey is a byproduct of food processing operations, the nisin

produced from this feedstock is not expected to face steep regulatory hurdles for its ultimate use in foods. A clear advantage of the present work is that nisin yields obtained from soy whey were substantially higher than those previously reported from other non-dairy based media *(8-11, 35, 41)***.** In fact, soy whey, without any additional nutrient supplementation resulted in a nisin yield of 619.2 mg/L (or 24,767 IU/ml), as compared to the 92.9 mg/L reported by Liu et al. *(30)* from cheese whey. Other researchers have reported 88.7 mg/L of nisin from cull potatoes *(8)*, 1,233 IU/ml of nisin from fermented barley extract *(9)*, and 16,000 IU/ml of nisin from hydrolyzed sago starch *(41),* to name a few.

The ability to efficiently produce nisin using value-added agricultural waste materials such as soy whey may facilitate wider use of this once cost-restrictive antimicrobial. In the simplest example, the entire fermentate containing the soy whey, *L. lactis* and nisin could be concentrated and dried and used as a shelf life extender in foods or also as a value-added animal feed additive. Depending on the processes used, this product could potentially have both probiotic and antimicrobial properties due to the presence of viable *L. lactis* and nisin, respectively.

In summary, we have demonstrated the successful use of soy whey, a soy-processing waste material, for fermentation of *L. lactis* and subsequent high-yield production of nisin. Our ability to use this readily available and inexpensive agricultural byproduct as a base for fermentative production of nisin suggests the possibility for more cost-effective, sustainable production of nisin, which is currently produced using higher cost milk-based materials. Based on the strengths of this

study, future work should be performed, focusing on the use of commercial waste soy whey and on scaled-up production of soy-fermented nisin to industrially relevant levels. Additional monitoring of *L. lactis* gene expression (*nisP*) during growth on soy whey could also enable us to more fully characterize this substrate as a medium for nisin production.

3.6 Acknowledgement

The authors wish to thank the Iowa Biotechnology Byproducts Consortium and Iowa State University, Institute for Food Safety and Security (IFSS) for financial support. We also extend our thanks to the Center for Crop Utilization (CCUR) at Iowa State University, Adam Pintar for help with statistical analysis, Stephanie Jung, Devin Maurer, Steve Fox, Ilankovan Paraman, Supriyo Das and Carol Ziel for experimental and analytical assistance. The defatted soy flakes were kindly provided by Cargill through CCUR.

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CHAPTER 4: VALUE-ADDED OIL AND ANIMAL FEED PRODUCTION FROM CORN-ETHANOL STILLAGE BY OLEAGINOUS *MUCOR CIRCINELLOIDES*

A paper submitted to *Journal of Bioresource Technology*

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Note: Some of the research results from this study resulted in an R&D 100 Award from R&D Magazine in 2009 (http://www.rdmag.com/Community/Blogs/RDBlog/Helping-The-Third-World/).

4.1 Abstract: This study highlights the potential of the oleaginous fungus, *Mucor circinelloides* in adsorbing/assimilating oil and nutrients in thin stillage (TS), and producing lipid and protein-rich fungal biomass. A 92% increase in oil from 4.8 g/L

(originally present in TS), to 9.2 g/L yield was obtained after fungal batch cultivation for 2 days using a 6-L airlift reactor; along with a reduction in suspended solids and chemical oxygen demand (COD) in TS by 95 and 89%, respectively. *M. circinelloides,* when grown on TS gave a biomass yield of 20 g/L (dry basis), with a lipid content of 46 % (dwb). The polyunsaturated fatty acids were 52% (w/w) of the total lipids. Supplementing TS with crude glycerol, a biodiesel byproduct (10%, v/v) during the fungal cultivation under nitrogen-depleted conditions, led to a further 41% increase in oil content in the cells (from 46 to 65%). Overall, fungal cultivation on TS produced a high-protein animal feed and high-value fungal oil, thus improving corn-ethanol process economics.

Keywords: Thin stillage, *Mucor circinelloides*, oleaginous, value-addition, fungal lipids

4.2 Introduction

Corn ethanol production in the United States has doubled from 6,500 in 2007 to 13,230 MGY in 2010 (Renewable Fuels Association, 2011). For every gallon of ethanol produced, 5-6 gallons of stillage is generated (Rasmussen et al., in press) which is composed of corn fiber, oil, protein, other unfermented components of the grain and yeast cells (Kim et al., 2008). This whole stillage is then centrifuged to produce a liquid (called thin stillage) and a solid fraction (called wet distillers grains). Less than 50% of the thin stillage (TS) is recycled back as backset for liquefaction of ground corn (Sankaran et al., 2010). The rest goes through multiple effect

evaporators requiring substantial amounts of energy to make a condensed syrup, that later ends up in DDGS (Kim et al., 2008) and is sold at low margins as animal feed (Moreau et al., 2011). In a study by Moreau et al. (2011), it was found that TS contained the highest amount of corn oil on a dry basis, among the various pre- and post-fermentation corn fractions in the dry-grind corn ethanol process. In recent years, recovery of corn oil from post fermentation corn fractions has drawn considerable interest for use as a biodiesel feedstock (Noureddini et al., 2009).

The presence of oil in a microbial growth medium increases the lipid accumulation in the microbial cells, and the composition of the accumulated intracellular lipid reflects the length of the carbon chain and structure of the oil source (Szczesna-Antczak et al., 2006). Certain mucoralean fungal strains like *Mucor circinelloides* are known to be efficient producers of intracellular lipases, which display very high hydrolytic and synthetic activities (Szczesna-Antczak et al., 2006). Fungal biomass has also been documented as excellent biosorbent material (Ozsoy et al., 2008) capable of removing oil from oil - water emulsions especially vegetable oil based emulsions (Srinivasan et al., 2010). Similar oil adsorption behavior was observed by Ahmad et al. (2005) using chitosan, the cationic amino-polysaccharide found in mycelia of mucoralean fungi, having adsorption and flocculation abilities (Srinivasan et al., 2010). Based on the above facts, we hypothesized that growing *M. circinelloides* on TS would help in both oil recovery and production as the fungal cells would not only adsorb and remove the oil from TS but would also be able to metabolize the corn oil and use the end products (fatty

acids) for further lipogenesis. Being grown on TS, the fungal oil quality would resemble corn oil. Since, triglycerides are the main constituent of both corn and fungal lipids, (Vicente et al., 2009) they could serve as feedstock for biodiesel production, which could then be used in-house by the corn-ethanol plants or sold. Our hypothesis is further supported by the fact that *M. circinelloides* is oleaginous in nature and accumulates high levels of lipids in its mycelia (Vicente et al., 2009). More significantly, its oil has been found to be rich in high-value γ--linolenic acid (Ratledge, 2004).

Despite the numerous favorable impacts of biodiesel production, its economic aspect suffers from the high cost of raw material (Antolin et al., 2002). Plant-based oil feedstocks account for almost 85% of total biodiesel production cost and also lead to food vs. fuel controversy (Miao et al., 2006). Microbial lipids, in contrast, do not require fertile land and hence do not compete with food production, have much higher yields and their production is better controlled without dependency on climate and geographical location (Meng et al., 2009). Filamentous oleaginous fungi hold promise in this respect. They can grow rapidly and accumulate notable amounts of lipids intracellularly, dominated by triglycerides (Sergeeva et al., 2008). Especially mucoralean fungi have been found to contain almost 40-50% of oil (Sergeeva et al., 2008; Vicente et al., 2009). These are also excellent enzyme producers and can be grown on various substrates. For example, *Rhizopus microsporus* (var. *oligosporus*) has been successfully grown on TS from corn ethanol plants (Rasmussen et al., in press); and on vinasse from sugar-based ethanol plants (Nitayavardhana and Khanal, 2010) for use as an animal feed. To our

knowledge, this is the first study of TS as a growth medium for the production of *M. circinelloides* biomass and fungal oil. This is also a first- time report on the change in fatty acid composition of oil extracted from *M. circinelloides* when grown on an oil-free synthetic medium, namely, Yeast Mold (YM) broth (Difco Laboratories, Sparks, MD, USA) as compared to an oil-containing TS medium.

Microbial cell disruption in order to release intracellular lipids is a fairly new technique and has been employed in a few studies. Ultrasonication assisted oil extraction was used by Cravotto et al., (2008) to extract intracellular oil from a marine microalga and higher yields were obtained with ultrasonication compared with microwaves or Soxhlet extraction. In this study, we have applied ultrasonication to disrupt the cell wall of *M. circinelloides* and have used a novel solvent system (toluene and methanol, 1:1) for lipid extraction. The Folch, Lees and Stanley method (Christi and Han, 2010) is one of the most popular oil extraction methods and involves the use of chloroform, which is very toxic and hazardous to human health with increasing restrictions on its use. In this study we have investigated the efficacy of the toluene/methanol solvent pair for oil extraction instead of chloroform/methanol, and compared the yields from both methods.

Some of the research results from this study resulted in an R&D 100 Award from R&D Magazine in 2009 (http://www.rdmag.com/Community/Blogs/RDBlog/Helping-The-Third-World/).

4.3 Materials and Methods

4.3.1 Thin stillage (TS)

Thin stillage was obtained from Lincolnway Energy (Nevada, IA, USA), a local dry-grind corn ethanol plant and stored in sterile 10-L carboys at 4 ℃ prior to use. Two batches of stillage were used for the entire study. Both batches were chemically characterized and were noted to have similar composition. The solids in the TS were seen to settle upon storage for 3-4 days, leaving a clearer liquid on top. The top layer was decanted and centrifuged at 5000 × *g* for 15 min. Both regular thin stillage (TS) without settling (%TS=6.1) and centrifuged thin stillage (CTS, %TS=3.8) were used as fungal growth media following heat sterilization at 121℃ for 15 min.

4.3.2 Fungal strain, media and inoculum preparation

Lyophilized culture of *Mucor circinelloides* f. *lusitanicus* ATCC1216B (CBS 277.49) was purchased from CBS-KNAW Fungal Biodiversity Centre, The Netherlands. The frozen culture was soaked in sterile deionized water and revived by spread plating onto Bacto PDA (Potato Dextrose Agar, Difco Laboratories, Sparks, MD, USA) plates and incubating for 5-7 days at 37 °C until white sporulation was observed. Fungal spores and mycelia were lightly scraped off from the plates aseptically using a sterile solution of 0.1% (w/v) Bacto peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). The spore and mycelial suspension was then passed through a 20 mL syringe containing glass wool which removed the mycelia and only allowed the passage of spores. The filtrate or spore suspension was mixed with Bacto potato dextrose broth (PDB) in a 1:1 ratio and

glycerin was added to a final concentration of 20% (v/v). The glycerin-spore stock was distributed into sterile 2 mL cryovials and preserved in a -75 °C freezer. The spore count of the stock was determined by a haemocytometer and found to be ~ 8 x 10^4 spores/mL.

The fungal inoculum for the shake flask studies was prepared by adding 0.2 % (v/v) of spore suspension to 50 mL of heat sterilized (121°C, 15 min) YM broth (Difco Laboratories, Sparks, MD, USA) in 200 mL Erlenmeyer flasks with foiled covered mouth. The inoculum for the 6-L bioreactor was prepared by adding 0.2 % (v/v) of spore suspension to 500 mL YM broth contained in a 2-L flask with cotton plugged mouth. Centrifuged thin stillage (CTS) was also tested for inoculum preparation in an attempt to acclimate the fungal cells to stillage nutrients, for enabling higher biomass yields from the main cultivation process in TS. Inoculum flasks were incubated on a shaker at 150 rpm, 37°C for 24 h. The fungal cells that grew as small compact mycelial pellets were then filtered using Whatman No. 1 filter paper, rinsed with sterile saline to remove media components and used as the inoculum. Quality control tests were performed to confirm uniformity in inoculum cell weight added to each cultivation batch. In a separate batch experiment, the fungal mycelial inoculum was shredded using a homogenizer to test the ability of smaller mycelial fragments in producing higher fungal growth as compared to compact pellets.

4.3.3 Fungal cultivation

Growth conditions of *Mucor circinelloides* were optimized in a series of shake flask experiments in 2 L Erlenmeyer flasks containing 500 mL of TS. The TS was heat sterilized (121°C, 15 min) unless otherwise mentioned. A 10 % (v/v) mycelial inoculum was added and the flasks were incubated on a shaker at 150 rpm agitation speed and 37°C for 2 days or longer when required. The optimized culture conditions were scaled up in the 6-L airlift draft-tube bioreactor with a 5 L working volume. An aeration rate of 7 SLPM (Standard Liters per Minute; 1.4 vvm) was used. The draft tube facilitated proper air circulation and mixing of the reactor contents.

4.3.4 Optimization of growth conditions in flasks

The growth of the fungus was tested in whole thin stillage (TS, total solids=6.1%) and centrifuged thin stillage (CTS, total solids=3.8%). Since significantly higher ($p<0.05$) fungal biomass yield (g/L) was obtained from TS as compared to CTS (as discussed in the Results and Discussion section), TS was chosen to be used in the rest of the optimization studies. The effect of sterilization on fungal growth was checked by autoclaving TS in flasks at 121°C for 15 min. Since sterile TS gave consistently higher fungal cell yields than non-sterile TS under the same culture conditions (as discussed in the Results and Discussion section), further growth optimizations were done using autoclaved TS. The effect of pH on fungal growth was tested by adjusting the initial pH of TS to 4, 5, 6 and 7, before sterilization using 1 N HCl/ 1N NaOH. The flasks with TS adjusted to different pH levels were then inoculated with mycelia and incubated under similar conditions. A range of incubation temperatures viz. 25, 30, 37 and 45°C were examined and

optimized conditions favoring highest growth were selected. In another optimization study, the length of the incubation period required for the highest biomass and oil yield was determined. Flasks containing TS inoculated with fungal mycelia were incubated for 1, 2, 4, 6, 8, and 10 days. At each time period, two flasks (duplicates) were randomly removed from the shaker and screened to determine the fungal biomass and oil yield. The effect of the physiological state of the fungal inoculum (i.e. spores vs. mycelia on the fungal growth rate and final biomass yield) was also evaluated. One set of flasks with TS was inoculated with 0.2% (v/v) spore stock, while the other set was inoculated with 10% (v/v) mycelia and the biomass yields were compared.

4.3.5 Optimization of growth and oil production conditions in 6-L airlift reactor

The optimized growth conditions determined by the shake flask studies were scaled up to the 6 L draft-tube airlift bioreactor. The fungal culture was grown for 72 h on sterile TS with a working volume of 5-L adjusted to pH 6, at 37 ℃ and 7 SLPM aeration rate. Samples were periodically removed from the broth during the cultivation process, filtered aseptically using a screen (pore-size of 1mm×1mm) and the fungal cells were returned to the reactor. The effluent/filtrate was analyzed for total solids, COD, soluble sugars, acids, glycerol and nitrogen content. At the end of each run, the entire culture broth was filtered and the biomass yield and oil content were measured.

In another batch of fungal cultivation on TS, a supplemental carbon source in the form of crude glycerol (10%, v/v) was fed to the fungal culture and its effect on

fungal oil production was tested. The crude glycerol was obtained as a by-product from a local soy-biodiesel plant. The glycerol was added to the fungal culture broth at the stage where the glycerol and lactic/acetic acid concentration in TS had just started falling. This phase indicated the exhaustion of available sugars and the shift of the fungal metabolism towards consumption of alternative carbon sources in the stillage. After glycerol addition, the fungi were allowed to grow for another 2 days. As a control, the fungal culture was grown in un-supplemented TS for the same time period. Fungal biomass and lipid yields from both cultivation batches were then measured and compared.

Analytical methods

4.3.6 Fungal biomass yield

The fungal biomass with attached stillage solids was filtered out from the culture broth using a stainless steel screen of pore size of 1 mm. The biomass was then oven dried at 80 ℃ for 24 h. The dried solids were measured gravimetrically and the biomass yield was reported in terms of 'grams of dried biomass per liter of stillage'.

4.3.7 Fungal biomass characterization

Proximate analysis of dried fungal cells was done to investigate its suitability as an animal feed. Total crude protein was measured by the AOAC official combustion method (AOAC 990.03) and an Elementar Vario Max Carbon Nitrogen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Total

carbohydrate content in the biomass was calculated by adding the starch, fiber and soluble sugar. Starch was measured by the amyloglucosidase/ α-amylase method (Knudsen, 1997); while the amylase neutral detergent fiber method (Van Soest et al., 1991) and phenol-sulfuric acid colorimetric assay (Dubois et al., 1956) were used to measure the fiber and soluble carbohydrate contents, respectively. Total fat was measured using the SoxCap 2047 with Soxtec extraction systems (Foss Analytical AB Soxtec System Application Note AN3906 (2007), followed by the ether extraction method for crude fat estimation (AOAC Official Method 920.39). The ash content was determined as the residue after ignition of the biomass at 600℃ for 3 h. The percent moisture content was measured by heating the samples to 105℃ and calculating the loss in moisture gravimetrically as described in the Standard Methods for the Examination of Water and Wastewater (2005). The total carbohydrate and fat measurements were performed by Dairyland Laboratories Inc., WI.

4.3.8 Chemical analyses of culture broth

The effluent TS after removal of fungal cells was analyzed for nitrogen, soluble sugars, acids, glycerol, and soluble chemical oxygen demand (SCOD). The soluble sugars, lactic and acetic acids, and glycerol were measured using a Waters Model 401 HPLC system with an RI detector, equipped with a column heater, auto-sampler, and computer controller. A Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad Chemical Division, Richmond, CA, USA) was used with 0.012 N sulfuric acid as the mobile phase with a flow rate of 0.8 ml/min, injection volume of

20 µl, and a column temperature of 65℃. Total crude prot ein was measured as described earlier. SCOD was calculated using the Hach COD kit (COD Vials 200-15,000 mg/L, High Range Plus, Hach company, Loveland, CO, USA). Total and suspended solids in TS were measured as described in the Standard Methods for the Examination of Water and Wastewater (2005).

4.3.9 Intracellular fungal-oil extraction using high-power ultrasonication and organic solvents

The oven-dried fungal cells were subjected to ultrasonic waves in the presence of organic solvents in order to mechanically disrupt the cell wall and enable extraction of the intracellular oil by the solvents. A Branson 450 Series bench scale ultrasonics unit (Branson Ultrasonics, Danbury, CT) with a maximum power output of 400 W and frequency of 20 kHz was used. The ultrasonic cell-disruptor horn used was a tapered microtip titanium horn with a flat 1/8" (3mm) diameter face. The solvent system comprised of methanol and toluene (1:1). Toluene was tested as a substitute for chloroform, which is the standard solvent used in the standard Folch, Lees and Stanley method (Christi and Han, 2010). As a control, the oil content (wt %) in the fungal biomass was also measured by the standard method (Christi and Han, 2010) and compared.Other solvent systems that were investigated were – toluene, methanol, hexane, and methanol: toluene (1:5), methanol: hexane (1:1). One gram of dried fungal biomass was mixed with 20 mL of the solvent system and sonicated at 70% amplitude (494 μm_{pp}) for 3 min. The optimum time of sonication was found to be 3 min after testing 0.5, 1, 2, 3, 4 and 5 min (data not shown). The

glass container with the fungal cell samples was placed in an ice bath during the sonication process to prevent temperature rise. After sonication, anhydrous magnesium sulfate was added to remove any water present and the mixture was filtered through Whatman1 filter paper. The filter paper was rinsed with toluene to wash away any residual oil sticking to the paper. The solvents were removed from the filtrate using a rotary evaporator at 65℃ (Buchi rotavapor R124) and the weight of the oil was determined gravimetrically.

The lipid concentration was calculated as percent oil yield (g of extracted oil per 100 g of dry biomass). The lipid productivity (g L^{-1} day^{-1}) obtained from each culture medium was determined as the product of biomass productivity (g L^{-1} day^{-1}) and lipid content (g of oil per g of dry biomass) as follows: $P_L = C_L \times \Delta CDW/ t$; where P_L is the lipid productivity, ΔCDW is the accumulated dry cell weight from inoculation to harvest time, t; and C_L is the cellular lipid content. The efficiency of the sonication method of cell disruption was compared to other mechanical shearing techniques such as magnetic stirring (with glass beads) and rotary mixing, using the same solvent system and the same batch of fungal biomass. The oil content in TS itself was determined by freeze-drying 1 liter of TS and extracting the corn oil from the dried stillage solids using the sonication-solvent method. All the lipid samples were analyzed for their fatty acid composition by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

4.3.10 Fatty acid analysis of lipids

The oil samples extracted from fungal biomass grown on TS and YM as well as the stillage oil samples were subjected to fatty acid profiling using GC. Methyl heptadecanoate (C17:0) was added as an internal sample to 30 mg of oil sample. The total lipids were esterified to fatty acid methyl esters (FAMEs) by treatment with 3% (v/v) sulfuric acid in methanol for 2 days at 60°C. The FAMEs were extracted by hexane and washed with water and then analyzed with a Hewlett-Packard 5890 series II GC equipped with a flame ionization detector and a SPB-2330 fused silica column (15 m x 0.25 mm id and 0.20 μm film thickness) (Supelco, Bellefonte, PA, USA). The initial oven temperature was 100°C, the oven temperature program was ramped up from 100- 200°C at a rate of 5°C /min and the injector and detector temperatures were 230°C. The sample injection volume was 1μL. The carrier gas (helium) flow rate was 3.5 mL/min. The lipid classes in the oil samples were characterized by thin layer chromatography (TLC). About 50-60 mg of oil sample was dissolved in hexane (1 mL) and acetone (2 drops) and applied to a preparative TLC plate (20 x 20 cm, 500 μm thickness). The plate was developed using hexane/diethyl ether/acetic acid (70: 30: 1, v/v/v), then sprayed with 2, 7-dichlorofluorescein and viewed under UV light. The TAG and FFA bands were scraped off and extracted twice with 10 mL of diethyl ether. The DAG and polar lipids bands were extracted twice with 10 mL of chloroform / methanol (2:1, v/v). The solvents were removed by using a flow of nitrogen. The fatty acids were esterified into FAMEs following the procedure given above. All analyses were done as duplicates.

4.3.11 Statistical analysis

The Analysis of Variance (ANOVA) was performed using JMP 8.0.2 (Cary, NC, USA), to determine significant differences among the various treatments. Least Significant Means Differences (LSD) were calculated at $p = 0.05$ by Student's t test. All treatments were carried out in triplicates and the results are shown as the means of three replicates ± standard deviation (SD).

4.4 Results and Discussion

Optimization of M. circinelloides growth in flasks

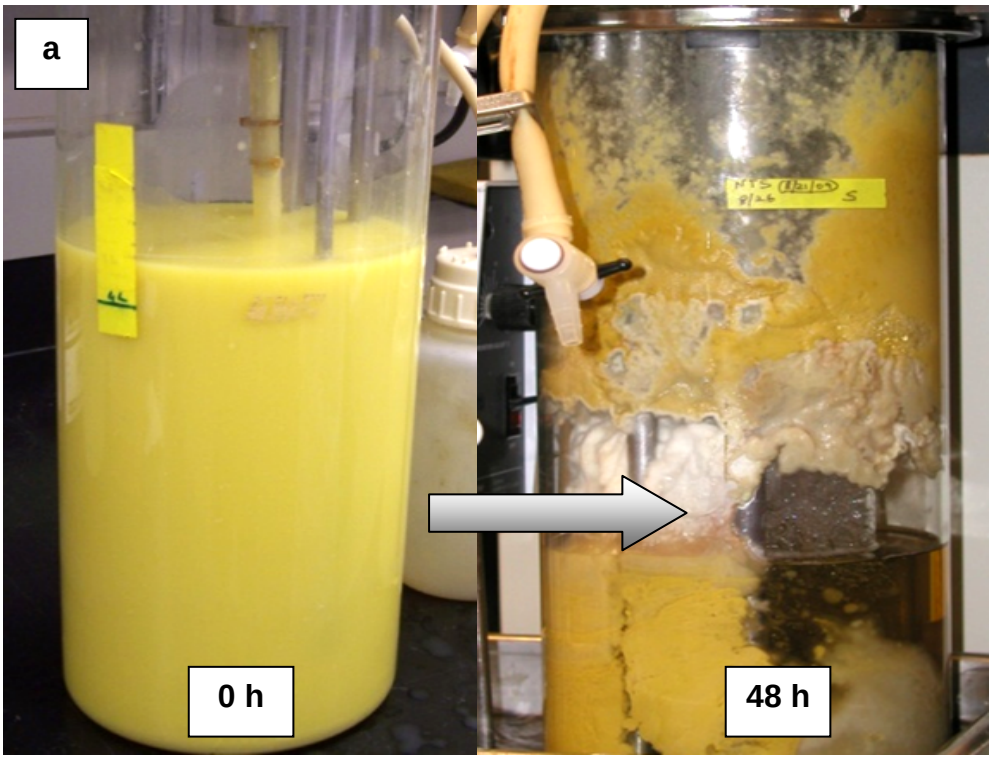
4.4.1 Effect of corn solids on fungal morphology and biomass yields

The dry fungal biomass yield from CTS averaged about 8 g/L while un-settled (original) TS gave an average biomass yield of 20 g/L when cultivated in a 6-L airlift bioreactor for 2 days. When un-settled TS (without fungal cells) was passed through the same screen (pore size, 1 mm × 1 mm), 2 g/L (dry) of corn solids were retained. Several factors contributed to the lower yields obtained from CTS, among which lower nitrogen and insoluble sugar contents due to the removal of suspended solids, were most influential. The suspended solids consisted of both corn solids left-over after ethanol separation from the corn mash, as well as some residual yeast cells and some possible bacterial contaminants too. Due to the higher biomass yields, TS was used in all further experiments instead of CTS. *M. circinelloides* is known to produce cellulolytic and other extracellular enzymes (Botha et al., 1997) which could have aided in degrading and utilizing the insoluble di- and polysaccharides present in TS. The total dissolved solids were quite similar in CTS (40 g/L) and TS (43 g/L),

while the total nitrogen content was much lower in CTS (1.5 g/L) compared to TS (5.5 g/L).

Filamentous fungi are known to grow as loose hyphal aggregates called "mycelial clumps", or as denser, often spherical aggregates called "pellets" (Paul and Thomas, 1998). Visual observations showed distinct morphological differences in the growth of *M. circinelloides* in TS as compared to CTS. In TS it grew as branched mycelia, while in CTS it grew as compact pellets. Similar observations were made by Rasmussen et al. (in press) in the case of *Rhizopus oligosporus,* where they found a distinct change in fungal morphology from compact pellets to filaments when the fungus was grown on settled thin stillage supernatant and TS respectively. The presence of corn solids in TS might serve as supports for attachment by the fungal mycelia. We found that the fungal mycelial biomass adsorbed and separated most of the corn solid matter from the TS leaving behind a relatively clearer effluent as shown in Fig 1. Since the mycelial form is known to contain higher chitosan than the yeast-like form (discussed later), the mycelial biomass of *M. circinelloides* facilitated the agglomeration of corn solids within and around the fungal filaments resulting in the efficient removal of corn solids from the thin stillage. Solids content in stillage between 3% and 6% supported good fungal growth, but 8% or more was found unfavorable for fungal cultivation and dilution was required. Concentrated thin stillage also known as "syrup" or "condensed distillers' solubles (CDS)" has a high solid content (~25% total solids). When it was tested as a growth medium for *M.*

circinelloides cultivation, no screenable (pore size, 1 mm) fungal growth could be seen unless the syrup was diluted.



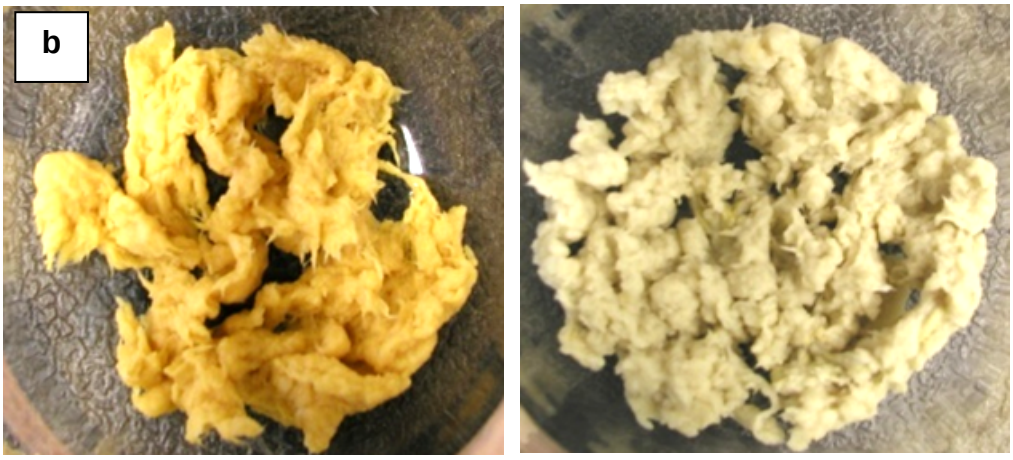


Figure 1 (a). Growth of *Mucor circinelloides* in TS cultivated in a 6-L airlift draft-tube bioreactor with 5 L working volume, after 48 h of incubation at 37 ℃ and 7SLPM aeration rate;

1b). Screened fungal mycelia grown on TS and containing entrapped corn solids imparting yellow color (left), screened fungal mycelia grown on CTS in its natural creamy-white color (right).

Dimorphic growth is a typical feature of representatives from the genus *Mucor* (Bartinicki-Garcia, 1968; Orlowski, 1991; McIntyre et al., 2002). Sporangiospores are specialized fungal cells which germinate under favorable conditions to produce mycelia, while the yeast-like budding cells are the pre-dominant forms present under stressed conditions such as low pH, high glucose, low nitrogen or anaerobic atmosphere (Funtikova & Mysyakina, 2003). Extensive research has been done on the correlation between fungal morphology and intracellular lipid composition. The lipids in these yeast-like cells differed from the mycelial cells in that they were found to contain lower concentrations of unsaturated fatty acids, higher concentrations of polar lipids and less glycolipids, which makes it unsuitable as a biodiesel feedstock (Mysyakina & Funtikova, 2008). The protein levels were also lower in the yeast-like form (Mysyakina & Funtikova, 2008). Therefore, careful study of the fungal morphological adaptations in response to the characteristics of the TS, (e.g. solid content, nitrogen and glucose levels) is vital for lipid production. Microscopic observations of *M. circinelloides* when grown on CTS and CDS indicated more yeast-like cells than mycelia. The possible explanation could be the low nitrogen content in CTS and high solid content in CDS creating unfavorable culture conditions for the fungus and promoting its growth in yeast-like form. As a consequence, the fungal growth in CTS and CDS could not be harvested via screening (pore size, 1

mm × 1 mm) and required high-speed centrifugation. On the other hand, mycelia were easier to harvest and gave higher yields than the yeast-like form. Thus, our findings indicate that *M. circinelloides* should be grown in its mycelial form for ease of operation and for better quality biomass.

4.4.2 Effect of TS sterilization on biomass production

Autoclaved TS was found to produce consistently high fungal biomass yields (~21 g/L). However, unsterilized TS used after prolonged storage (> 15 days) in carboys at 4 ℃, showed lower fungal yields. On plating a 20-day old TS sample on nutrient agar (Difco Laboratories, Sparks, MD, USA), bacterial growth was observed confirming contamination of TS during storage probably by psychrophilic bacteria. Bacterial growth lowered the nutrient content of TS especially sugars and prevented fungal growth. Therefore, heat sterilization was recommended as a pretreatment step for stillage before *M. circinelloides* cultivation, especially when it is stored for long periods. However, on a commercial scale, a continuous system enabling the transfer of TS directly from the corn-ethanol plant into the fungal reactor could omit the storage period and heat-sterilization steps.

4.4.3 Effect of pH, temperature, inoculum preparation and incubation period on biomass production

M. circinelloides was found to give the highest biomass yields when grown in TS within a pH range of 5.5 ± 1.5 and a temperature of 37℃. Previous studies on the fungal members of the Mucorales order report a pH range of 5 to 7 for their

cultivation (McIntyre et al., 2002; Funtikova & Mysyakina, 2003; Mysyakina & Funtikova, 2008). A temperature range of 25℃ to 30° C is the commonly reported incubation temperature for growing *M. circinelloides* (McIntyre et al., 2002; Mysyakina & Funtikova, 2008; Roux-Van der Merwe et al., 2005). Our findings indicate a higher growth rate of 15 g/L/day at 37℃ as compared to 10 g/L/day at 25℃ in TS. The highest biomass yields were 18.5 ± 0.5 g/L after 8 days at 25℃, 19.2 ± 0.4 g/L after 5 days at 30℃ and 20.0 ± 0.3 g /L after 2 days at 37℃. Thus, on Day 2 a significantly higher ($p<0.05$) biomass yield was observed at 37℃ compared to 25℃ and 30℃. A temperature of 45℃ was found t o be too high and resulted in negligible fungal growth.

When spore stock was used as the inoculum in the main fungal cultivation process, a long lag phase of 2 days was followed by the log phase from day 2 to day 4 (Fig 2). A mycelial inoculum prepared in both YM and CTS produced a shorter lag phase (18-24 h) with the growth rate being highest from day 1 to day 2 of incubation. The final biomass yields on Day 4 produced by the different inoculum preparations were however not significantly different ($p>0.05$). Homogenization of the fungal inoculum did not lead to any significant increase in yields ($p>0.05$).

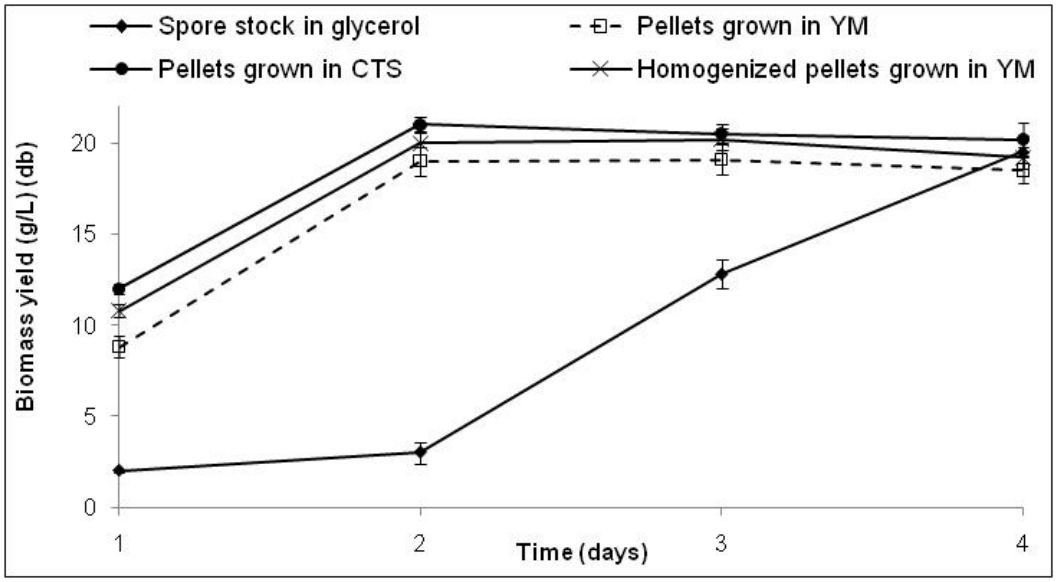


Figure 2. Effect of inoculum morphology on the growth characteristics of *Mucor circinelloides* in thin stillage contained in 2-L Erlenmeyer shake flasks and incubated for 4 days at pH 6, 37 ℃ and 150 rpm agitation. Data are means ± SD (n=3)

Growth and oil production by M. circinelloides in airlift bioreactor

4.4.4 Change in TS characteristics during fungal growth

During the fungal cultivation, periodic sampling of the fungal culture broth was done and the TS 'effluent' after removal of fungal biomass was analyzed for compositional changes. Sugar analysis by HPLC showed a sharp fall in the concentration of the monosaccharides present in TS (glucose and fructose) in the first 18 h of growth. Samples collected after 18 h showed a gradual fall in the concentration of the more complex sugars, maltose and DP4+ sugars (sugars with 4 or more monomeric units) as shown in Fig. 3a. *M. circinelloides* is known to produce α-D-glucosidase enzyme needed for breaking down and assimilating maltose sugar.

It also produces other essential enzymes for sugar assimilation like β-glucosidase and amylase (Botha et al., 197) required for breakdown of cellobiose and starch respectively. Although our HPLC was not standardized to measure the polysaccharides in TS, other research suggests that *M. circinelloides* is capable of metabolizing xylose, arabinose, cellulose, and starch, known to be present in TS (Kim et al., 2008).

Original TS contained glycerol and organic acids (lactic and acetic) carried over from the yeast fermentation. An initial rise (between 0 and 24 h) was recorded in their concentrations in the TS effluent during *M. circinelloides* cultivation. However, culture broth samples collected after 24 h showed a gradual decrease in these concentrations, as illustrated in Fig 3b. A probable explanation could be the exhaustion of simple sugars during fungal growth on TS, triggering a shift in fungal metabolism, where the formation of glycerol and acids was replaced by their utilization as carbon sources. A similar observation was also made by Rasmussen et al. (In press) for *Rhizopus oligosporus* grown on TS. After 48 h of *M. circinelloides* cultivation in the airlift bioreactor, yellow-colored fungal mycelial clumps were observed in the bioreactor leaving the rest of the culture broth with substantially lower amounts of corn solids (Fig.1). The SCOD in TS was reduced from 45±4 to 5±0.5 g/L; while the total solid content was decreased from 66±5 to 6.6±0.3 g/L after 48 h of fungal growth. Initial total nitrogen load of 5.5 g/L in TS was reduced to ~0.5 g/L, a part of which could be from the soluble fraction of dead fungal cells (Table 1). The filamentous nature of the fungal growth is known to increase the viscosity of the cultivation broth unlike pelleted fungal suspensions (Metz et al., 1979). In our study

with *M. circinelloides* cultivation in TS, we made similar observations. The high viscosity caused by the fungal filaments led to some mixing problems with a few stagnant and oxygen-limited zones in the airlift bioreactor. A fungal biomass yield of 20.0 ± 1.2 g/L was obtained from the fungal cultivation in the bioreactor after 48 h of cultivation.

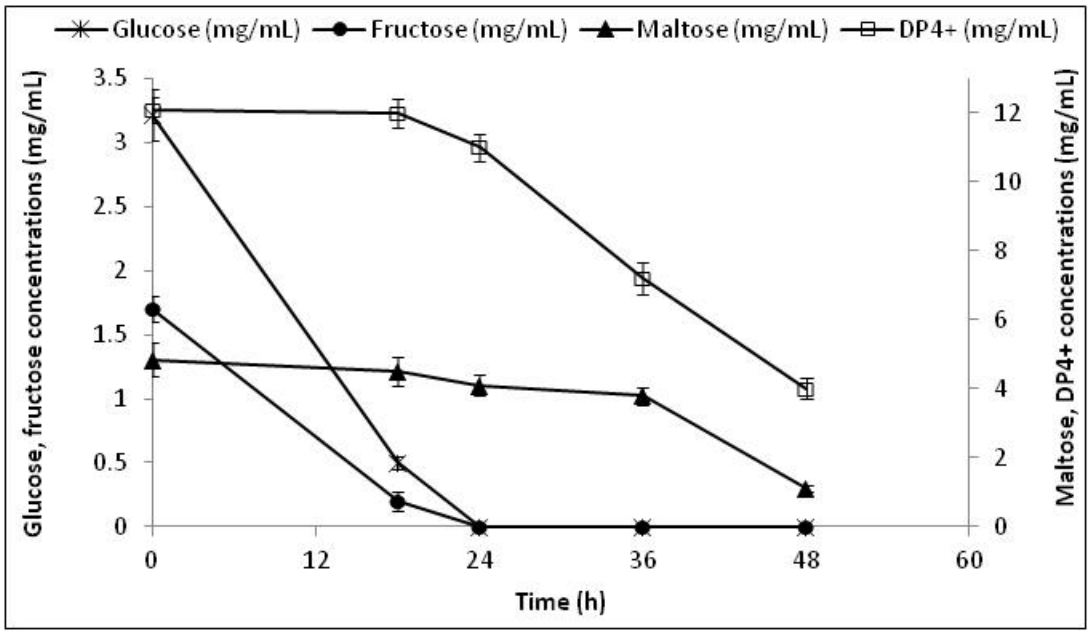


Figure 3a. Change in glucose, fructose, maltose and DP4+ (sugars containing 4 or more monomers) concentrations in thin stillage during the growth of *Mucor circinelloides* for 48 h at pH 6, 37 ℃ and 7 SLPM aeration rate i n a 6-L airlift bioreactor. Data are means ± SD (n=3)

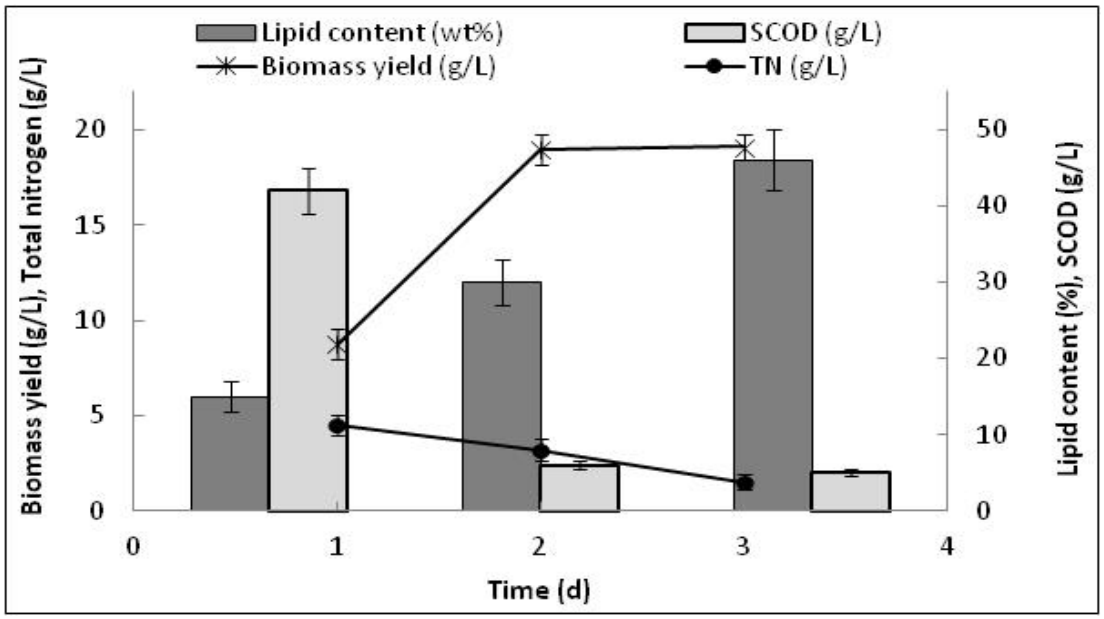


Figure 3b. Change in fungal biomass yield, lipid content in biomass, soluble chemical oxygen demand (SCOD), and total nitrogen (TN) in thin stillage during the growth of *Mucor circinelloides* for 72 h at pH 6, 37 ℃ and 7 SLPM aeration rate in a 6-L airlift bioreactor. Data are means ± SD (n=3)

Table 1. Change in thin stillage (TS) characteristics before and after fungal processing (without pH control). *Mucor circinelloides* was grown for 48 h on sterile TS in a 6-L airlift reactor with a working volume of 5-L, at pH 6, 37 ℃ and 7 SLPM aeration rate. Data are means ± SD (n=3)

TS characteristics	Before fungal processing	After fungal processing
pH	4.2 ± 0.3	5.4 ± 0.3
Total solids (g/L)	66.0 ± 5.0	6.6 ± 0.3
Total nitrogen (g/L)	5.5 ± 0.3	0.5 ± 0.04
Total suspended solids (g/L)	19.0 ± 2.0	1.0 ± 0.1
SCOD (mg/L)	45.0 ± 4.0	5.0 ± 0.5

Proximate analyses of *M. circinelloides* cells grown on TS indicated higher fat and crude fiber content as compared to DDGS (Belyea et al., 2004). The protein contents of the *M. circinelloides* biomass and DDGS were quite similar (Table 2). Thus, high fiber and protein content in the fungal biomass indicated its potential application as an animal feed. It should be noted that *M. circinelloides* also has GRAS (Generally Regarded As Safe) status from the Food and Drug Administration.

Table 2. Proximate analysis showing the chemical characteristics of *Mucor circinelloides* biomass grown on thin stillage (TS) for 48 h in a 6-L airlift bioreactor, harvested by screening and oven-dried at 80 °C for 24 h, as compared to the properties of DDGS. Data are means ± SD (n=3)

Chemical component (%) (db)	*M. circinelloides* biomass	DDGS[a]
Moisture	2.1 ± 0.3	NA
Ash	4.0 ± 0.5	4.6
Total carbohydrates	23.1 ± 1.3	44
Starch	0.8 ± 0.1	5.1
Crude fiber	19.6 ± 1.5	10.2
Soluble sugars	2.2 ± 0.2	NA
Total fat	39.4 ± 2.1	11.9
Total protein	30.4 ± 2.5	31.3

a: Belyea et al., 2004

NA: Data not provided in referred article, Belyea et al., 2004

4.4.5 Optimization of oil extraction

Among the different solvent systems tested, toluene: methanol (1:1) solvent system was quite comparable ($p>0.05$) to the methanol: hexane (1:1) and chloroform: methanol (2:1) solvent pairs. Due to toluene being a relatively safer solvent than either hexane or chloroform, the toluene: methanol solvent pair was selected for rest of the oil extractions in this study. A significant difference ($p<0.05$) in oil yields was observed when methanol, hexane and toluene were used as single solvents and when they were used as pairs. The yields from the different solvent systems have been included and compared in Table 3. Among the three cell-disruption methods, both magnetic and rotary mixing took almost 12 h to produce the same oil yields (9.2 g of oil/L stillage) as achieved by the sonication method in 3 min. Figure 4 shows the success of sonication in breaking open the fungal cell wall to release the stored oil. Sonication was also faster than the standard Folch, Lees and Stanley method (Christi and Han, 2010) which required a homogenization step before solvent addition and ~4 h of mixing/contact times. The oil yield using this standard method involving chloroform: methanol (2:1) was found to be 43 % (g of oil/ 100 g dry fungal cells), which was comparable to the yields from the ultrasonication-solvent method (Table 3).

Table 3.Oil yields from *Mucor circinelloides* biomass grown on thin stillage for 48 h, using various solvent systems. Data are means ± SD (n=3)

Solvent system	Oil yield (g oil/100 g biomass)
Toluene	21.0 ± 1.8
Methanol	16.8 ± 2.3
Hexane	23.5 ± 1.5
Toluene: Methanol (1:1)	46.2 ± 3.1
Toluene: Methanol (3:1)	46.7 ± 2.2
Methanol: Hexane (1:1)	45.5 ± 3.5
Chloroform: Methanol (2:1)	43.6 ± 3.3

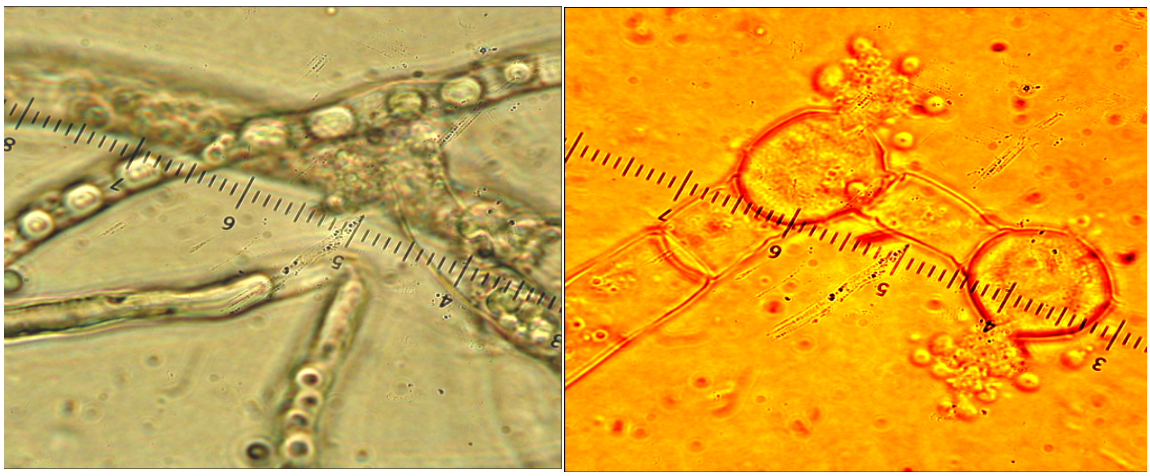


Figure 4. Micrographs (100×10 x magnification) of *Mucor circinelloides* filaments depicting intracellular oil bodies (a), which were released after cell disruption using ultrasonication (b) at 70% amplitude or 494 μm_{pp} (peak to peak) for 3 min

4.4.6 Fungal oil production

Freeze dried TS solids were found to contain 8.0 ± 2.0% oil (g of oil/100 g stillage solids). Hence, TS with 6% total solids contained 4.8 ± 1.2 g of oil per liter.

After fungal cultivation on TS, the oil concentration in the effluent was negligible. The corn oil was almost completely removed from TS during the growth of *M. circinelloides.* The oil could have been utilized as a carbon source for intracellular lipid production. The fatty acids from the corn oil breakdown may have been used as precursors or as a backbone to synthesize the fungal storage lipids. Or they could have been degraded into basic skeletons (such as acetyl CoA) serving biomass synthesis (Ratledge, 1989). The corn oil could also have been removed from TS via an adsorption mechanism (Srinivasan et al., 2010).

The oil content in the dried fungal biomass grown on TS was in the range of 46 ± 2.0 % (wt of oil per 100 g of dry biomass). The lipid content in the fungal cells was found to be the highest at the stationary phase of growth (between day 2 and day 3) under nitrogen limited conditions (Fig. 3b). An average fungal biomass yield of 20 g/L from TS with an oil content of 46% (db) led to an oil yield of 9.2 g per liter of TS after 3 d of incubation. Since TS originally has an oil concentration of ~4.8 g/L, fungal processing led to a 92% increase in oil production. When *M. circinelloides* was grown on YM broth under the same culture conditions with only glucose as the carbon source, the biomass yield obtained was 5.0 ± 0.5 g/L (db) and the oil content was 20.0 ± 1.5% or ~ 1 g/L (Table 4).

Table 4. Biomass and oil yields after growing *Mucor circinelloides* in thin stillage (TS) and YM broth for 48 h, at pH 6, 37 ℃ and 7 SLPM aeration rate in a 6-L airlift bioreactor with a working volume of 5-L. Data are means ± SD (n=3)

Parameters	Growth media	
	YM	TS
Biomass yield (g/L)	5.0 ± 0.5	20.0 ± 0.5
Biomass productivity (g/L/day)	2.5 ± 0.25	10.0 ± 0.25
Oil content in fungal cells (%)	20.0 ± 1.5	46.0 ± 2.0
Oil yield (g/L)	1.0 ± 0.2	9.2 ± 1.0
Oil productivity (g/L/day)	0.5 ± 0.1	4.6 ± 0.5
PUFA content in fungal oil (% total lipids)	33.2 ± 1.2	51.4 ± 2.6

Crude glycerol was supplemented to TS on the fourth day of fungal cultivation when the fungal cells were seen to reach the stationary phase, possibly due to nitrogen exhaustion. After supplementation, cells were allowed to grow till Day 6 and their oil content showed a 41% increase as compared to the control cells. This indicated the future possibility of recycling the crude glycerol generated during biodiesel production from fungal oil, and using it as a supplemental carbon in the fungal cultivation process in TS. With the current rapid growth in biodiesel production, crude glycerol, the primary byproduct, is being generated in surplus amounts. Due to the presence of impurities, it has restricted applications and requires value-addition for the sustainability and profitability of biodiesel plants (Nityavardhana and Khanal, 2011). Thus, in our study we selected crude glycerol

due to its easy availability, inexpensive nature and existing evidence of its consumption by fungal strains (Nityavardhana and Khanal, 2011).

4.4.7 Oil characterization

The nomenclature used in this study for the oil samples extracted from fungal biomass grown on YM and TS, and from TS itself, was Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil} respectively. Table 5 shows the major lipid class distribution in Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil} oil samples. Triacylglycerol (TAG) was found to be the most dominant class (64.5 wt%) in TS_{oil} sample followed by free fatty acids (FFA) (6.4 wt%), as expected in a plant - based oil. Interestingly, fungal oil from cells grown on YM where glucose was the only carbon source (Mucor-YM_{oil}) also contained TAGs as the dominant lipid class (34.2 wt%). But when grown on a substrate containing a mixture of carbohydrates and oil, as found in TS, Mucor oil (Mucor-TS_{oil}) contained FFA as the major lipid class (38.4 wt%). This also indicated the lipase activity of *M. circinelloides*, which may have hydrolyzed the TAGs in corn oil leading to the high FFA content in the Mucor-TS_{oil} samples. The FAME content of each lipid class in the oil samples did not add up to a 100% since the unsaponifiable lipids and a large portion of the glycolipids and phospholipids were not converted to FAME but these non-acyl portions did contribute to the total oil weight. The FAME content (in wt%) of Mucor-YM_{oil}, and Mucor-TS_{oil} samples was almost the same but the lipid class distribution was quite different. All the samples contained unidentifiable TLC bands, which were likely to be carotenoids, hydrocarbons, cholesterol or other sterols; and some polar complex lipids. Such lipid materials could not have been converted to

FAME if their TLC bands had been included in the transesterification step. Some unknown TLC bands which were not included in the transesterification process represented a non-negligible quantity according to the band width shown on the TLC plate.

Table 5. Lipid class composition of oil extracted from fungal cells grown on YM broth (Mucor-YM_{oil}) and thin stillage (Mucor-TS_{oil}), and from thin stillage alone (TS_{oil}). The different lipid classes were separated by TLC and their quantities were measured by GC. Data are means ± SD (n=2)

Lipid composition (wt %)	Mucor-YM_{oil}	Mucor-TS_{oil}	TS_{oil}
TAG	34.2±0.7	14.7±1.8	64.5±4.3
FFA	4.2±0.5	38.4±6.1	6.4±0.8
DAG	1.5±0.1	8.4±1.1	1.1±0.3
Polar	4.1±0.3	3.2±0.4	0.4±0.1
Total lipids (wt %)	44.0±0.4	64.7±2.3	72.4±1.4
FAME (wt %)	69.7±5.3	70.0±4.3	87.5±13.8

Table 6 illustrates the fatty acid composition of the three oil samples, Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil}. The fatty acid composition of Mucor-TS_{oil} matched closely with that of TS_{oil} and further proved that the nature of the oil present in the growth medium greatly influences the fatty acid composition of the intracellular microbial oil. The fatty acid with the highest concentration in Mucor-YM_{oil} sample was C18:1 (Oleic acid) at 24.4 ± 0.7 wt%; while Mucor-TS_{oil} and TS_{oil} samples contained C18:2 (linoleic acid) as the primary fatty acid at 50.0 ± 1.6 wt% and 52.5 ± 0.2 wt%

concentrations respectively. This is similar to the corn oil FAME composition with C18:2 at ~57% and C18:1 at ~28% (Majoni et al., 2010). Mucor-YM_{oil} sample contained certain unique saturated fatty acids like C13:0 (tridecylic acid), C14:0 (myristic acid) and C15:0 (pentadecylic acid) and a monounsaturated fatty acid C16:1 (palmitoleic acid), which were absent in the other two oil samples. Another interesting finding was the presence of linolenic acid (C18:3) in Mucor-YM_{oil}, (17.5 ± 0.1 wt %) which was much higher than that in Mucor-TS_{oil} (1.4 ± 0.1 wt %) and TS_{oi} (not detected). The linolenic acid in the Mucor-YM_{oil} was further confirmed to be of the gamma form. This again proves that this microbe is able to synthesize lipids from media that do not contain acyl compounds, and the *de novo* synthesis and assimilation from the environment produce lipids with different composition. The presence of linolenic acid (C18:3) in fungal-based lipids is well documented (Chen & Liu, 1997; Kavadia et al., 2001) and fungal oil is known to be a rich source of gamma linolenic acid (GLA), or omega 3, a PUFA of nutritional and dietary importance (Ratledge, 2004; Kavadia et al., 2001). Overall, fungal oil grown on TS was not found to be ideally suitable for biodiesel production due to its high FFA content and low percentage of saturated fatty acids. But it could have applications in the production of certain high-value fatty acids like linoleic and oleic acids. With encouraging fungal biomass yields from TS, other oleaginous fungal strains could be tested on TS as a future study, for the production of high-value fatty acids such as GLA.

Table 6. Total lipid fatty acid composition of oil extracted from fungal cells grown on YM broth (Mucor-YM_{oil}) and thin stillage (Mucor-TS_{oil}), as well as oil from thin stillage alone (TS_{oil}). Compositional analysis was done by GC with prior transesterification to FAME's. Data are means ± SD (n=2)

Lipid class	Total fatty acid composition (wt %)		
	Mucor-YM_{oil}	Mucor-TS_{oil}	TS_{oil}
C 13:0	12.5 ± 1.4	NA	NA
C 14:0	2.3±0.1	NA	NA
C15:0	0.6±0.0	NA	NA
C16:0	15.8±0.4	15.7±1.0	15.0±0.2
C16:1	6.3±0.0	NA	NA
C18:0	4.0±0.2	2.3±0.1	2.2±0.0
C18:1	24.4±0.7	29.6±0.5	28.7±0.2
C18:2	15.7±0.2	50.0±1.6	52.5±0.2
C18:3	17.5±0.1	1.4±0.1	NA
C20:0	0.6±0.0	1.2±0.1	1.5±0.1
C22:0	0.4±0.0	NA	0.1±0.1

4.5 Conclusions

This work provided four main findings – 1) an efficient method of converting thin stillage into oil and animal feed with the effluent TS having much lower organic load facilitating water reclamation; 2) fungal growth and morphology was affected by the solids content in TS. The highest biomass and lipid yields were obtained when TS had a total solids content of 5-7% (20.0, 9.2 g/L); 3) fatty acid composition of the fungal lipids was influenced by the nature of the growth medium (YM/TS); and, 4) both YM and TS-derived fungal oil contained high-value omega-3 and omega-6

PUFAs. Overall, oleaginous fungal cultivation added value to thin stillage and would help to boost the profitability of the corn-ethanol industry.

4.6 Acknowledgement

The authors would like to thank the Iowa Energy Center for partial funding of this research and Lincolnway Energy, Nevada, IA for providing thin stillage for this study. We appreciate the lab facilities provided by the Dept. of Food Science and Human Nutrition and Iowa State University's Center for Crops Utilization Research (CCUR). We are also grateful to the National Science Foundation for grant support of part of this work. We would also like to acknowledge the help given by Bill Colonna, Carol Ziel, Dr. Mary Rasmussen and Dr. Supriyo Das at the various stages of this research.

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CHAPTER 5: HETEROTROPHIC/MIXOTROPHIC CULTIVATION OF OLEAGINOUS *CHLORELLA VULGARIS* ON INDUSTRIAL CO-PRODUCTS

A paper submitted to *Journal of Algal Research*

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5.1 Abstract: Among many challenges faced in the commercial cultivation of microalgae, low-cost water and nutrients availability is crucial. Our study aimed at testing and optimizing two agro-industrial co-products, dry-grind ethanol thin stillage (TS) and soy whey (SW), as nutrient feedstock for mixotrophic/heterotrophic microalgal cultivation. Heterotrophic growth of *Chlorella vulgaris* was first optimized in a Bioscreen turbidimeter and 250 mL Erlenmeyer flasks, then scaled up to a 6-L stirred bioreactor. Intracellular oil was extracted from dried microalgal biomass by ultrasonication and solvent extraction treatments for yield comparison, and fatty acid (FA) profile. Algal biomass yields (dry basis, db) from TS, SW and control modified

basal medium (MBM) after 4 days of incubation at mixotrophic conditions in the bioreactor were 9.8, 6.3 and 8.0 g/L with oil content at 43, 11, and 27 % (w/w) respectively. FA profile of oil samples were found to vary and depend on growth media characteristics. *C. vulgaris* when grown on TS and MBM produced oil richer in linoleic (omega 6 FA) and linolenic acids (omega 3 FA), respectively. This research highlights the potential of two agro-industrial co-products as microalgal growth media with consequent production of high-value microalgal oil and biomass.

Keywords: Heterotrophic/mixotrophic, oleaginous algae, *Chlorella vulgaris*, industrial co-products

5.2 Introduction

Microalgae are a source of several products useful to humans, ranging from carbohydrates, essential fatty acids, pigments, food supplements, fertilizer, pharmaceuticals, and biofuels [1]; and efforts are underway to develop more economical mass cultivation methods. Availability of water and nutrients is one of several challenges facing algaculture. Heterotrophic and mixotrophic algae are known to grow much faster with higher cellular oil content than photoautotrophic cells [2, 3]. However, they require organic carbon sources like glucose or acetate for growth, responsible for 80% of the medium costs [4]. In order to reduce microalgal production costs, it is imperative to find cheap organic substrates that meet the nutritional requirements of oleaginous algae. A few studies in this direction have used cassava starch hydrolysate [5] and corn powder hydrolysate [6] as glucose

substitutes resulting in higher lipid yields. The key is to maintain both lipid yield and growth rates at or near the levels found on glucose while decreasing the costs by using cheaper substrates. The current study investigates two agro-industrial co-products streams, thin stillage (TS) and soy whey (SW) in their ability to support microalgal growth compared to a synthetic medium MBM (Modified Basal Medium) containing glucose as the carbon source.

Wet processing of soybean meal is commonly used for the production of soy protein isolates, which is used as energy supplement, and food ingredient. Soy whey is the leftover liquid after the iso-electric precipitation (pH 4.5) of soy protein from the crude extraction of defatted soy flakes. Large volumes of this liquid waste require energy-intensive steps for concentration and are finally discarded, leading to serious waste disposal problems [7]. Therefore, value-addition to this co-product stream is necessary to minimize pollution and maximize resource utilization. On a dry basis, soy whey typically contains 3.6–4.4% nitrogen (50% of which is protein nitrogen), 25–35% soluble sugars, and substantial amounts of phosphorus, minerals and numerous trace elements essential for microalgal growth [7, 8]. In a different study [9], soy whey was utilized as a growth medium for cultivating fastidious lactic acid bacteria. We hypothesize that the high-nutrient content of soy whey could also support the growth of microalgae.

The second microalgal growth medium used in this study was a by-product from the dry-grind corn ethanol process, called thin stillage (TS). Every L of ethanol generates ~ 5 Liters of stillage (Rasmussen et al., in press). The stillage contains a

substantial amount of the nutrients present in corn. It is centrifuged to produce a liquid (called thin stillage) and a solid fraction (called wet distillers grains). A part of the thin stillage (TS) is recycled back as backset for liquefaction of ground corn [10], while the rest goes through multiple effect evaporators requiring substantial amounts of energy to make a “syrup” known as condensed distiller’s solubles (CDS) [11]. CDS is usually mixed with wet (distillers’ wet grains with soluble; DWGS) or dry (distillers’ dry grains with soluble; DDGS) forms [11], both of which are sold at low margins [10]. Lately, the syrup has suffered a decline in demand due to its low nutritional value, and is sold for very low prices or even given away (Rasmussen, et al., in press). Corn ethanol production in the United States has doubled from 6,500 in 2007 to 13,230 MGY in 2010, leading to increased volumes of stillage generation [12]. It is imperative to find new applications for this co-product stream in order to maintain the economic viability of this industry [13]. Initial experiments using unsettled TS indicated that the microalgal cells were unable to grow in the presence of the high solids in TS. Upon storage at refrigeration temperatures, solids in TS (varying between 6-8%) tend to settle, leaving a clear liquid with the dissolved nutrients on the top. This clear liquid contained high amounts of organic materials (~50 g/L soluble COD) including sugars, nitrogen and other micronutrients (Rasmussen et al., in press; Mitra et al., in press) and could be used as a growth medium for microalgal cultivation.

The research objectives were to-

1) Optimize culture parameters for maximal growth of *Chlorella vulgaris* on TS and SW in microtiter plates, shake-flask, and 6-L stirred bioreactor levels,

2) Optimize oil extraction methodology using ultrasonication and organic solvents, and, 3) Determine the effect of culture media (SW, TS and MBM) characteristics on the fatty acid profile of intracellular microalgal oil.

5.3 Materials and Methods

5.3.1 Microorganisms and media

An axenic culture of *Chlorella vulgaris* was obtained from the Carolina Biological Supply Company (Burlington, NC). The microalgal culture was propagated and maintained in MBM that contained glucose, 10 g; yeast extract (Difco Laboratories, Lawrence, KS, USA), 6 g; KH_2PO_4, 1.25 g; $MgSO_4.7H_2O$, 1 g; EDTA, 0.5 g; H_3BO_3, 114.2 mg; $CaCl_2.2H_2O$, 111 mg; $FeSO_4.7H_2O$, 49.8 mg; $ZnSO_4.7H_2O$, 88.2 mg; $MnCl_2.4H_2O$, 14.2 mg; $CuSO_4.5H_2O$, 15.7 mg; and $Co(NO_3)_2$.$6H_2O$, 4.9 mg per liter [14].

Defatted soybean flakes were obtained from Cargill, Inc., (Cedar Rapids, IA), packed in airtight plastic bags and stored at 4 ℃ until further use. The defatted soy flakes contained 57.3% (dry basis) crude protein and had a protein dispersibility index of 93.8. The soy whey medium was prepared in the laboratory from the defatted soy flakes following the procedure described elsewhere [9]. It contained 1,210 mg/L of total nitrogen, 7,575 mg/L of crude protein, 7.14 mg/mL sucrose, 6.56 mg/mL stachyose, 0.84 mg/mL galactose, and 0.74 mg/mL glucose [9].

Corn thin stillage was obtained from Lincolnway Energy (Nevada, IA, USA), a local dry-grind corn-ethanol plant. Samples were collected in sterile carboys and

stored at 4℃ for a week prior to use. The pH of fresh TS varied between 3.8 and 4.7, the total and reducing sugar contents averaged 17 and 6 g/L, respectively, suspended solids were 20–30 g/L, the TCOD averaged 90 g/L while the SCOD (soluble COD) was 55 g/L and the total nitrogen content was 6 g/L (Rasmussen et al., in press; Mitra et al., in press). After a week in storage, the suspended solids in TS were found to settle. The supernatant from settled TS (0.1- 0.2 g/L suspended solids) was sterilized and used as the second growth medium for *C. vulgaris* in the current study. The TS supernatant was also termed as 'TS' and its chemical characteristics are given in Table 1.

Table1. Chemical characteristics of Thin Stillage and Thin Stillage supernatant. All values are means ± SD, n = 3

Parameter	Thin Stillage	Thin Stillage supernatant*
pH	4.2 ± 0.3	4.1 ± 0.3
Total Suspended solids (g/L)	19 ± 3	0.1 ± 0.05
Total dissolved solids (g/L)	41 ± 3	40 ± 2
Total COD (g/L)	80 ± 6	45 ± 5
Soluble COD (g/L)	48 ± 6	45 ± 5
Total sugars (g/L)	17 ± 2	12 ± 1.5
Total nitrogen (g/L)	5.5 ± 1.2	1.5 ± 0.1

* Suspended solids in TS were found to settle after a week in storage. The supernatant from settled TS was sterilized and used

5.3.2 *Inoculum preparation*

Colonies of *C. vulgaris* from the original agar slant received from the supplier were used to inoculate 100 mL of sterile MBM medium (pH 6.8) in 250-mL Erlenmeyer flasks. Flasks were incubated on an orbital shaker set at room temperature (~25℃), under a constant fluorescent light (luminance 880 lux) at 150-rpm agitation speed for 3 days. Twenty μgmL^{-1} of chloramphenicol (Sigma Chemical, St. Louis, MO) was added to the medium to prevent possible bacterial contamination during incubation.

5.3.3 Culture conditions

Three types of culture systems were used: 1) heterotrophic culture of *C. vulgaris* in micro-titer plates using an automated turbidimeter (Bioscreen; Growth Curves, Inc., Piscataway, NJ); 2) heterotrophic and mixotrophic batch cultivation of *C. vulgaris* in 200-mL Erlenmeyer flasks; and 3) mixotrophic batch cultivation of *C. vulgaris* in a 6-L bench-top stirred bioreactor. The details of each culture system are described in sections 5.3.4 – 5.3.6.

5.3.4 Microtiter Plate Rapid Bioassay

The Bioscreen C Microbiology Reader (Growth Curves Inc., Piscataway, NJ) was used for this study to evaluate the effect of different culture parameters on the microalgal growth rates. The unit is a combined incubator and microplate reader that enabled collection of high-resolution absorbance at 600 nm for up to 200 individual wells (well capacity 400 µL). The effect of pH was tested by growing *C. vulgaris* in

MBM, SW, and TS at pH levels of 5, 6, 6.8, 7.5 and 8 maintained via 0.1 M phosphate buffer addition. The buffers were 100 mM in strength and were prepared at the desired pH levels by the addition of required quantities of mono and di-sodium phosphate salts. The effect of supplementing the media with carbon, phosphorus and nitrogen sources in the form of glucose, KH_2PO_4 and KNO_3 respectively, was also studied. Glucose was added in varying concentrations (0.5 to 4%; w/v) while KH_2PO_4 and KNO_3 were added at 0.13 and 0.24%, w/v respectively. The nutrient solutions were filter-sterilized using a Millipore nylon membrane filter of pore size 0.2 µm (Millipore Corp. Billerica, MA, USA) before addition to the media. The effect of media sterilization method (filtration vs. heat) on growth yields was also tested. Filter sterilization was done using a Millipore nylon membrane filter of pore size 0.2 µm, while heat sterilization was done by autoclaving the media at 121 ℃ for 15 min. To each well, 270 µL of the nutrient media and 30 µL of the inoculum were added, which had the average initial absorbance of ~0.2 at 540 nm. The inoculated plates were placed in the Bioscreen and incubated under heterotrophic conditions, at 28 ℃ for 5 days. Abs_{540} were recorded at 30 min intervals with automatic shaking for 5 s before each reading to ensure proper mixing of well contents. All treatments were evaluated in replicates of five (n=5). Media conditions in the Bioscreen corresponding to highest cell densities were chosen and scaled-up in shake flasks.

5.3.5 Shake flask culture

C. vulgaris was grown both heterotrophically and mixotrophically on 100 mL of sterile culture media (initial pH of 6.8) in 250-mL Erlenmeyer flasks on an orbital shaker with 150-rpm agitation speed at 28 ℃ for 4 day s. Mixotrophic algal cells were cultured under a constant fluorescent light intensity of 880 lux. Heterotrophic cultivations were done in the dark. The inoculum size was 10% (v/v) for both types of cultivation. The media were not buffered and were heat sterilized (121℃, 15 min) based on Bioscreen optimization results. Periodic samples were taken from the flasks to determine the cell density and oil yields, which were then used to calculate the biomass and oil productivities.

5.3.6 *Bioreactor study*

Microalgal batch cultivations were performed and operation conditions optimized in a 6-L bench-top stirred bioreactor. The bioreactor was sterilized by autoclaving for 45 min at 121℃, and filled with the respective nutrient feedstock (MBM, SW or TS) to a working volume of 3 L. The reactor was placed under a white fluorescent light with a constant luminance of 880 lux. The microalgal cultivation was carried out for 4 days at 28 ℃, 180 rpm and aeration rate of 0.7 SLPM (standard L per min; .2 vvm). A constant pH of 6.8 was maintained in the bioreactor with acid (hydrochloric acid, 0.1 N) and alkali (sodium hydroxide, 0.1 N) dosing. Variation in biochemical composition of the media including carbohydrates, nitrogen, and SCOD was monitored during the culture process via periodic sampling and testing. When the microalgal culture reached a stationary growth phase marked by steady absorbance readings, the entire culture broth was harvested by centrifuging at

12,000 × *g* for 15 min, and freeze-dried. The dried microalgal biomass samples were then analyzed for their chemical composition and intracellular oil content.

Analytical methods

5.3.7 Growth analysis

A standard curve was prepared by plotting cell dry weight (CDW) values (in g/L) against corresponding absorbance (Abs_{540}) readings (Spectronic 20 Genesys, Thermo Electron, Cambridge, UK). For CDW determination, algae preparations with known absorbance were centrifuged at 12,000×*g* for 15 min, at 4℃, washed twice with sterile saline (0.85% (w/v) NaCl) and dried to a constant weight at 80 ℃. A linear regression fit was obtained for CDW as a function of Abs_{540}, with R^2 value of 0.99. The measured Abs_{540} values were converted to CDW values using this regression equation. The CDW values were plotted against time (hours) to obtain the growth curves. Biomass productivity (mg of biomass.L^{-1}.d^{-1}) was calculated based on the growth rate (mg of biomass.d^{-1}) derived from the growth curves.

5.3.8 Chemical composition of C. vulgaris cells

Proximate analyses of dried mixotrophic C. vulgaris cells cultivated on SW and TS in a 6-L bioreactor were done. Total crude protein was measured using an Elementar Vario Max Carbon Nitrogen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), based on the AOAC official method AOAC 990.03 [15]. The total carbohydrate and fat measurements were performed by Dairyland Laboratories Inc., Arcadia, WI. Starch was measured by amyloglucosidase/ α-

amylase method [16]; while an amylase neutral detergent fiber method [17] and phenol-sulfuric acid colorimetric assay [18] were used to measure the fiber and soluble carbohydrates content, respectively. Total fat was measured using the SoxCap 2047 with Soxtec extraction systems (based on AOAC Official Method 920.39 ether extract method for crude fat) [19]. The ash content was determined as described in the Standard methods for the examination of water and wastewater [20].

5.3.9 Intracellular microalgal-oil extraction using high-power ultrasonication and organic solvents

A Branson 450 Series bench-scale ultrasonics unit (Branson Ultrasonics, Danbury, CT) with a maximum power output of 400 W and a frequency of 20 kHz was used. The ultrasonic cell-disruptor horn used was a tapered microtip titanium horn with a flat 1/8" (3mm) diameter face. The freeze-dried microalgal cells were subjected to ultrasonic waves in the presence of organic solvents in order to mechanically disrupt the cell wall and enable extraction of the intracellular oil by the solvents. All sonications were done at amplitude of 494 μm_{pp} (peak to peak). The solvent system comprised methanol and toluene at 1:2 ratios (Mitra et al, in press). Many other solvents were tested and the methanol-toluene system was selected based on highest extraction efficiency (data not shown). Toluene was tested as a substitute for chloroform used in the standard Folch method of oil extraction [21] due to hazards associated with chloroform. One gram of dried biomass was mixed with 20 mL of the solvent system and sonicated at 70% amplitude (494 μm_{pp}) for 3 min.

Three min sonication period was selected after testing 0.5, 1, 2, 3, 4 and 5 min time intervals (data not shown). The samples were kept in an ice bath during the ultrasonic process to prevent temperature rise. After each sonication-extraction, anhydrous magnesium sulfate was added to remove any water present and the mixture was filtered using a Whatman no. 1 filter paper. The filter paper was rinsed with toluene to wash away any residual oil sticking to the paper. The solvents were removed from the filtrate using a rotary evaporator at 65℃ (Buchi rotavapor R124) and the weight of the oil was determined gravimetrically. The lipid concentration was calculated as percent oil yield (g of extracted oil per 100 g of biomass). The lipid productivity (g L^{-1} d^{-1}) obtained from each culture medium was determined as the product of biomass productivity (g L^{-1} d^{-1}) and lipid content (g of oil per g of biomass) as follows: $P_L = C_L \times \Delta CDW / t$; where P_L is the lipid productivity, ΔCDW is the accumulated dry cell weight from inoculation to harvest, t is the overall culture time, and C_L is the lipid content.

As a control, the oil content (in wt %) in the microalgal biomass was measured by the standard modified Folch method [22]. The efficiency of the sonication method of cell disruption was compared to other mechanical shearing techniques like magnetic stirring (with glass beads) and rotary mixing, using the same solvent system and the same batch of microalgal biomass. The oil content of TS was determined by freeze-drying 1 L of TS and extracting the corn oil from the dried stillage solids using the sonication-solvent method explained earlier**.** SW did not have any oil on it as it was extracted from defatted soy flakes (>99.5% oil removal).

5.3.10 Fatty acid analysis of intracellular lipids

Dairyland Laboratories Inc., WI, performed the fatty acid analyses of the oil samples. The fatty acids were esterified into methyl esters in a one step procedure using chloroform and methanolic HCl. The methyl esters were detected by gas chromatography (GC) using an Agilent 7820 GC with a packed column and FID (flame ionization detector).

5.3.11 Chemical analyses of culture broth from bioreactor study

Samples collected periodically from the 6-L stirred bioreactor were analyzed for soluble sugars, acids, ethanol, total nitrogen (crude protein) and soluble COD (SCOD), after removal of the microalgal cells by centrifugation. The soy sugars were measured using a Varian high-performance liquid chromatography (HPLC), autosampler: model 410, solvent delivery module: model 210, with a Bio-Rad HPX-87P column for carbohydrates, a guard column, and equipped with a Varian's refractive index (RI) detector- 356_LC. The sample volume was 20 µL; eluent used was filtered (Millipore nylon membrane filter, pore size 0.2 µm), and degassed HPLC grade water at a flow rate of 0.6 mL/min. The column temperature was 85℃, and run time was 22 min. Standards used were glucose, galactose, fructose, sucrose, stachyose, and raffinose prepared at a concentration of 5 mg/mL in deionized water. The corn sugars in the TS and glucose in MBM were measured by a Waters Model 401 RI detector, equipped with a column heater, auto-sampler, and computer controller. A Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad Chemical

Division, Richmond, CA, USA) was used with 0.012 N sulfuric acid as the mobile phase with a flow rate of 0.8 mL/min, injection volume of 20 µL, and a column temperature of 65℃. Total crude protein was measured as described earlier. SCOD in the culture supernatants was measured using a Hach COD kit (COD Vials 200-15,000 mg/L, High Range Plus, Hach Company, Loveland, CO, USA). Total and suspended solids in TS were measured as described in Standard Methods for the Examination of Water and Wastewater (2005) [20].

5.3.12 *Statistical Analysis*

The Analysis of Variance (ANOVA) was performed using JMP 8.0.2 (Cary, NC, USA), to determine significant difference among the different treatments. Least Significant Means Differences (LSD) were calculated at $p = 0.05$ by Student's t test. All treatments were carried out in duplicates and results are shown as the means of two replicates ± standard deviation (SD).

5.4 Results and Discussion

5.4.1 Heterotrophic growth of C. vulgaris in soy whey and thin stillage using the Bioscreen

The 100-wells in the micro-titer plates enabled optimization of several culture conditions in the same experiment facilitating faster and more reliable results. As seen in Fig.1a, the microalgal growth rate (average change in absorbance readings in unit time in the log phase) in the dark was fastest on the control medium MBM,

followed by filtered TS (FTS) and filtered SW (FSW). The highest final cell titer, however, was achieved in FTS. A statistically significant difference in growth was observed between FSW and FTS and between FSW and MBM ($p<0.05$), while no significant difference was observed between FTS and MBM ($p>0.05$). The cells reached their stationary phase faster on MBM probably due to carbon limitation having glucose as the only carbon source. The longer log phase in case of FTS could be due to the presence of more than one type of available sugars (glucose, fructose, and maltose). On the other hand, adaptation time of the microalgae to the nutrients could explain the longer lag phase in FTS and FSW as compared to MBM. Since the majority of the carbohydrates in SW were oligosaccharides like sucrose, stachyose and raffinose with much lower concentrations of simple sugars (glucose, fructose, and galactose) [9], the final cell titer in SW was lowest among the three media. Although *C. vulgaris* was found to be able to metabolize stachyose (as discussed in Section 5.4.5), the rate was comparatively lower. Mixed substrate utilization is an important aspect of microbial metabolism, which dictates microbial growth rates in complex media [23]. *C. vulgaris* growth curve in FTS and FSW showed a diauxic pattern (Fig. 1a), indicating the metabolic shift of the microalgal cells from simple carbon sources to more complex ones after the exhaustion of the simple sugars. A similar physiological behavior has been reported for brown algae (*Laminaria hyperborea*) [23]. Change in concentration of individual sugars in TS and SW during microalgal growth in the bioreactor was measured by HPLC and has been discussed in section 5.4.5.

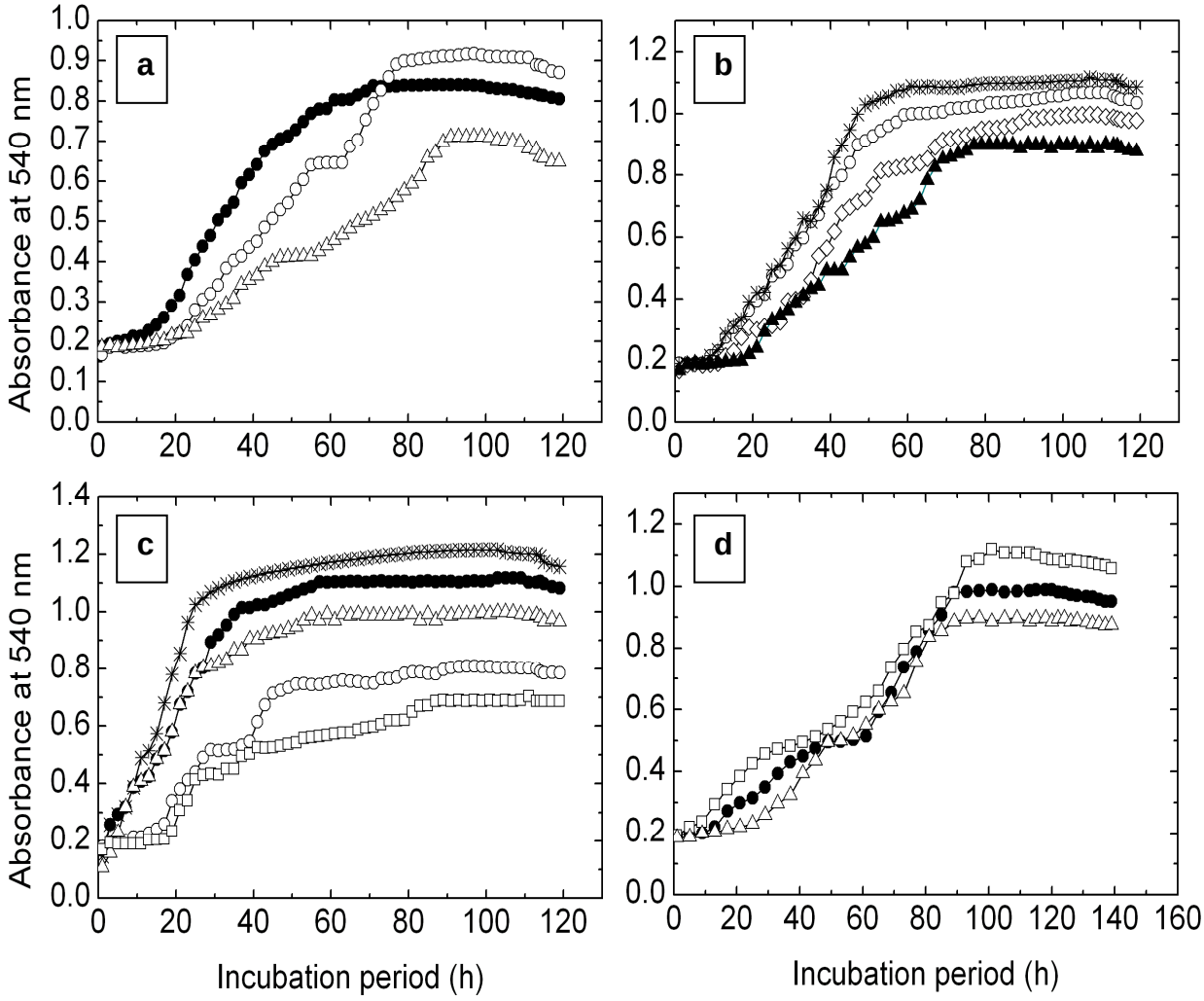


Figure 1. Heterotrophic growth of *C. vulgaris* in MBM (Modified Basal Medium), SW (Soy Whey) and TS (Thin Stillage) culture media, incubated for 5 days using a Bioscreen turbidimeter.

(1a) Growth of *Chlorella* in MBM, TS and SW: *Closed circle* – MBM, *open circle* – FTS, *open triangle* – FSW;

(1b) Effect of sterilization method and addition of phosphate buffer to TS at pH6.8 on growth of *Chlorella*: *Asterisk* – ATS w/ buffer, *open circle* – ATS w/o buffer, *open diamond* – FTS w/ buffer, *closed triangle* – FTS w/o buffer;

(1c) Effect of sterilization method, glucose supplementation and addition of phosphate buffer to SW at pH6.8 on growth of *Chlorella*: *Asterisk* – ASW w/

buffer & 1%glucose, *closed circle* – ASW w/ buffer, *open triangle* – ASW w/o buffer, *open circle* – FSW w/ buffer, *open square* - FSW w/o buffer; (1d) Effect of glucose and nitrogen (KNO_3) supplementation to TS on the growth of *Chlorella*: *Open square* – FTS w/ glucose & KNO_3, *closed circle* – FTS w/ KNO_3 , *open triangle* – unsupplemented TS. Data are means, n=3.

5.4.2 Effect of sterilization method and pH

The method of sterilization employed for the media significantly affected the microalgal growth on SW but not on TS. Pre-treatment of SW via autoclaving resulted in significantly higher ($p<0.05$) cell densities than filter sterilization. There was no significant difference ($p>0.05$) between autoclaved TS (ATS) and FTS (Fig.1b), while Fig. 1c shows a significant difference in the microalgal cell densities ($p<0.05$) between autoclaved SW (ASW) and FSW. Soy is known to contain protease inhibitors (Mitra et al., 2010), which could prevent protein assimilation by the microalgal cells via inhibition of their protease enzyme. Heat denaturation of endogenous soy protease inhibitors could have led to better growth of the microalgal cells in ASW as compared to FSW. Therefore, TS and SW were autoclaved for the shake-flask and bioreactor studies. Buffering ATS and ASW with pH 6.8 phosphate buffer helped to extend the log phase of the microalgal growth curve (Figs.1b&c). The final cell concentrations significantly increased in ASW on buffer addition ($p<0.05$). However, no significant effect ($p>0.05$) was observed in case of ATS due to the presence of lactic acid, which gave it an inherent buffering capacity.

5.4.3 Effect of nutrient supplementation

Supplementing ATS and ASW with 0.5 to 3% (w/v) glucose led to a significant rise ($p<0.05$) in microalgal growth rate, and final cell concentrations. However, increasing sugar content beyond the 3% critical concentration did not increase biomass yields further. In fact, glucose concentrations at 4% and above resulted in lower cell titers probably due to possible substrate inhibition. The negative effect of high glucose concentrations on algal cell growth was also reported by Arroyo et al. (2010). A significant rise ($p<0.05$) in cell concentration was observed in ATS supplemented with nitrogen in the form of KNO_3 (Fig. 1d), while no significant change occurred in case of ASW (data not shown). Cells were seen to reach stationary phase on unsupplemented and KNO_3-supplemented ATS after around 85 h and 100h, respectively. This indicated nitrogen limitation in unsupplemented ATS after about 80-85 h of microalgal growth. This information is vital for microbial-oil production since microalgae are known to synthesize more storage lipids in nitrogen-limited or stressed conditions in presence of available sugar source [24].

5.4.4 Comparison of heterotrophic and mixotrophic growth and oil production in shake flasks

The shake-flask study indicated that biomass yields in mixotrophic conditions were significantly ($p<0.05$) higher than heterotrophic conditions for the three media studied (Fig. 2a). Biomass yields of 7.1 and 5.6 g/L were obtained from MBM at mixotrophic and heterotrophic conditions, respectively. Biomass yields from SW were 5 and 2.5 g/L while TS gave 8.3 and 5.8 g/L at mixotrophic and heterotrophic conditions, respectively. Greater cell densities under mixotrophic conditions could be

due to higher energy availability contributed by aerobic respiration coupled with catabolism of carbohydrates present in the medium along with photosynthesis. Synergism between light and organic substrate/s has been reported by Cid et al. [25]. The occurrence of pale green color in the mixotrophic cells (Fig. 2b) as compared to the yellow color of the heterotrophic cells, further confirmed the active photosynthetic metabolism of the microalgal cells under mixotrophic conditions.

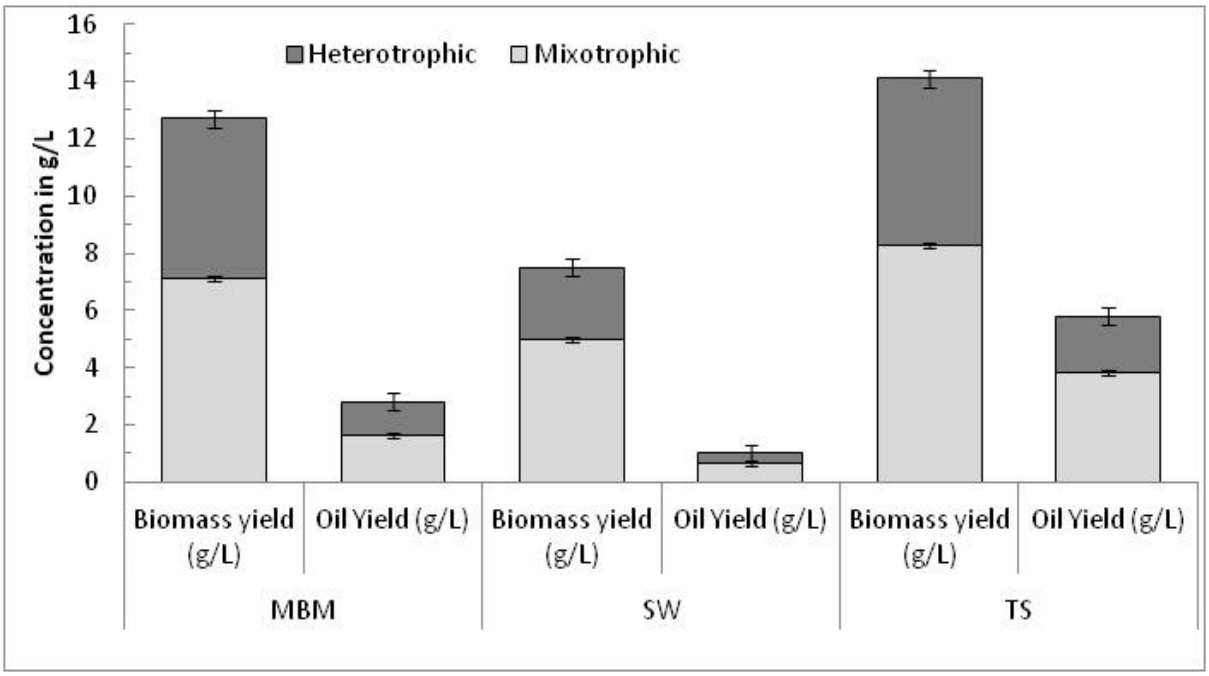
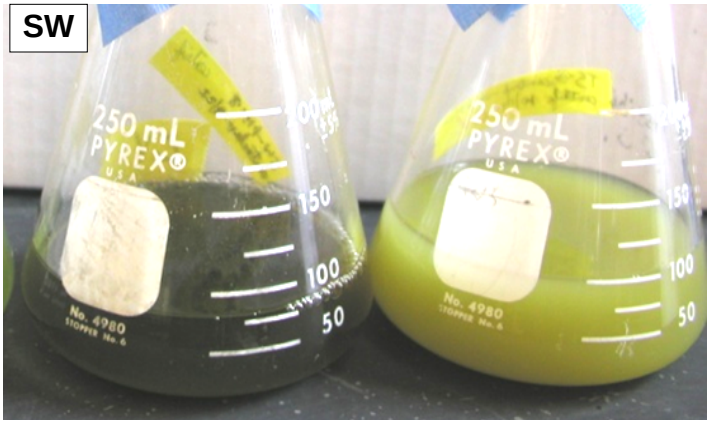
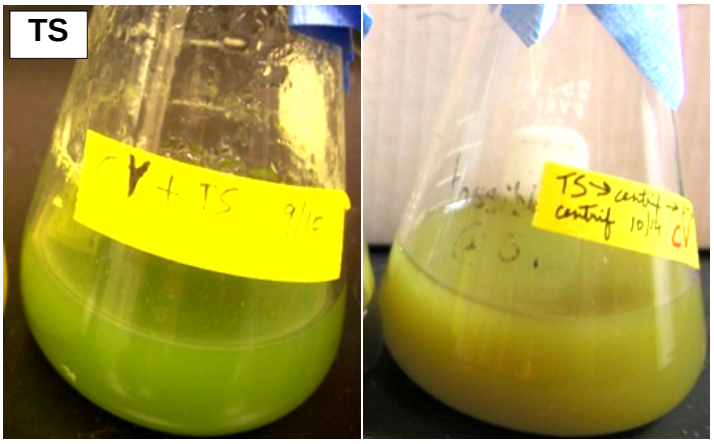


Figure 2a. Biomass and oil yields from *C. vulgaris* grown in sterile MBM (Modified Basal Medium), SW (Soy Whey) and TS (Thin Stillage). Batch cultivation was done in 250 mL Erlenmeyer shake flasks for 4 days at 28 ℃, 150 rpm agitation, under heterotrophic (in dark) and mixotrophic (constant fluorescent light with luminance of 880 lux) conditions. Data are means ± SD (n=3)



Mixotrophic Heterotrophic



Mixotrophic Heterotrophic

Figure 2b. Heterotrophic and mixotrophic growth of *C. vulgaris* in SW (Soy Whey) and TS (Thin Stillage). Batch cultivation for 4 days, at 28 ℃ and 150 rpm agitation. Mixotrophic cells were grown under a constant fluorescent light with luminance of 880 lux, while heterotrophic cells were grown in the dark. Note the color difference between the green mixotrophic cells indicating photosynthetic activity, as opposed to the brownish-yellow heterotrophic cells.

The lipid yields (g/L) were calculated by multiplying the lipid content in the cells (wt %) and the biomass yield (g/L). Mixotrophic microalgal cells were found to have higher lipid contents and consequently gave higher lipid yields than

heterotrophic cells (Fig.2a). Liang et al. [26] also reported higher oil productivity in mixotrophic conditions for *C. vulgaris* and suggested that increased availability of carbon could have led to increased proportion of storage lipids. Since the energy requirements of the cells are mostly being met by the photosynthetic process, it is possible that the excess carbon in the nutrient media is being directed to intracellular lipid storage unlike heterotrophic mode. Active transport was reported as the primary means by which algae assimilated organic carbon substrates from the environment and this transport system appeared to be regulated by irradiance in many microalgal species [27]. Under low irradiance, these cells have been shown to incorporate a selective range of organic carbon compounds as storage materials, including pyruvates, acetate, lactate, ethanol, saturated fatty acids, glycollate, glycerol, hexoses, and amino acids, as a means of subsidizing their photosynthetic metabolism [27]. In this present study, the mixotrophically grown cells were exposed to a constant low irradiation (880 lux) and were also supplied with a wide range of carbon sources. The low light intensity could have acted as a stress signal to the cells, prompting them to convert the excess available sugars in the media into storage lipids and minimize cell division. This stress-adaptation phenomenon is similar to what has been observed for nitrogen limitation and increased lipid production in other algae [24].

5.4.5 Mixotrophic microalgal growth in 6-L stirred-tank bioreactors and oil recovery

The microalgal growth on TS and SW was compared in a 6-L stirred-tank bioreactor under similar conditions as observed in mixotrophic cells grown in shake-

flasks. Microalgal cell growth profile indicated exponential rise on all three media until the third day, after which the biomass yields remained fairly stationary, except for TS, which showed a slight increase from Day 3 to Day 4 (Fig. 3a). Figure 3b shows the changes in appearance of SW and TS during the mixotrophic growth of *C. vulgaris* incubated for 4 days in stirred bioreactors. The biomass yields from MBM, SW and TS were 8.0, 6.3 and 9.8 g/L and biomass productivities were 2.0, 1.6 and 2.5 $gL^{-1}d^{-1}$ respectively. Highest cell concentrations on TS could be due to the higher concentration of metabolizable sugars in TS compared to MBM and SW. According to Sun et al. [28], *Chlorella* sp. are capable of metabolizing many kinds of monosaccharides (glucose, fructose, galactose, and mannose) as well as disaccharides (lactose and sucrose), however, the growth rates and biomass concentrations were found to be quite different in the various sugars. Glucose and mannose were the best carbon sources followed by fructose. Interestingly, results from the current study also showed that TS, which had higher amounts of glucose than SW supported a higher microalgal growth rate. In addition, the presence of fructose in TS (absent in SW) along with sucrose gave it an advantage over SW and MBM resulting in higher final cell concentrations.

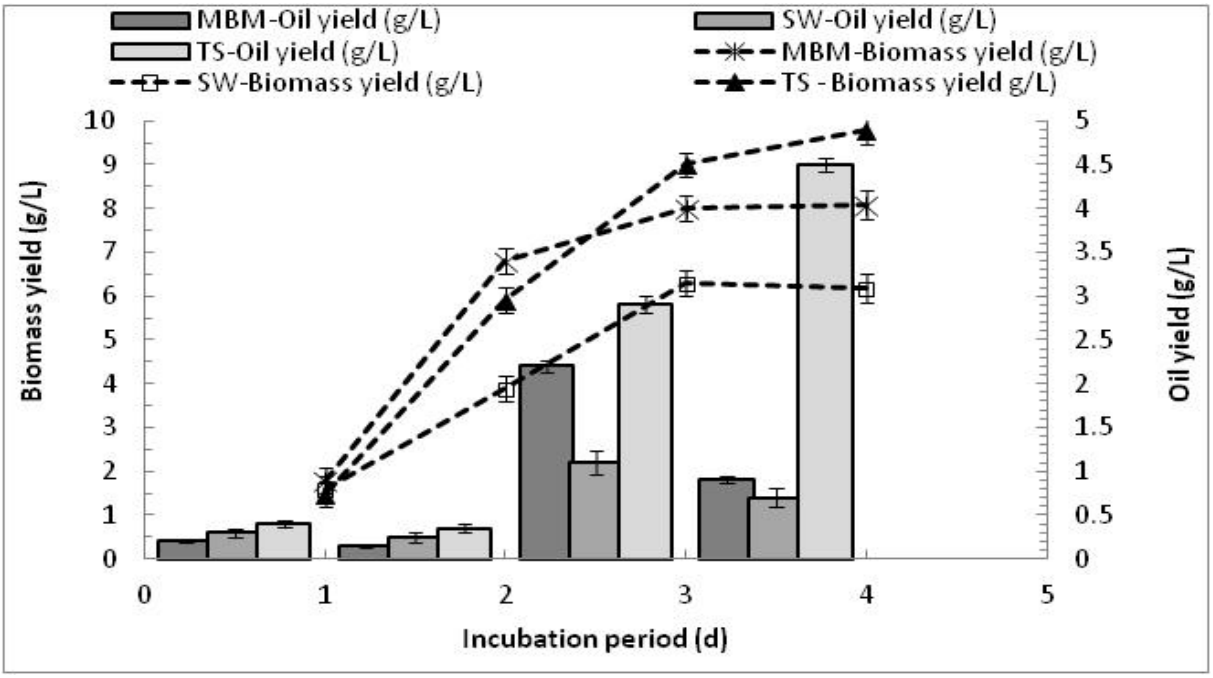


Figure 3a. Biomass and lipid yields from *C. vulgaris* grown on MBM (Modified Basal Medium), SW (Soy Whey) and TS (Thin Stillage) in a 6-L stirred tank reactor. Cells were grown under mixotrophic conditions (880 lux), for 4 days, at 28 ℃, 180 rpm, 0.7 SLPM (standard liters per mi n) aeration rate, working volume of 3 L and 6.8 pH. Inoculum was prepared by inoculating 100 mL of sterile MBM (pH 6.8) using an agar slant and incubating flasks on an orbital shaker set at room temperature (~25℃), under a con stant fluorescent light (luminance 880 lux) at 150-rpm agitation speed for 3 days. Data are means ± S.D (n=3)

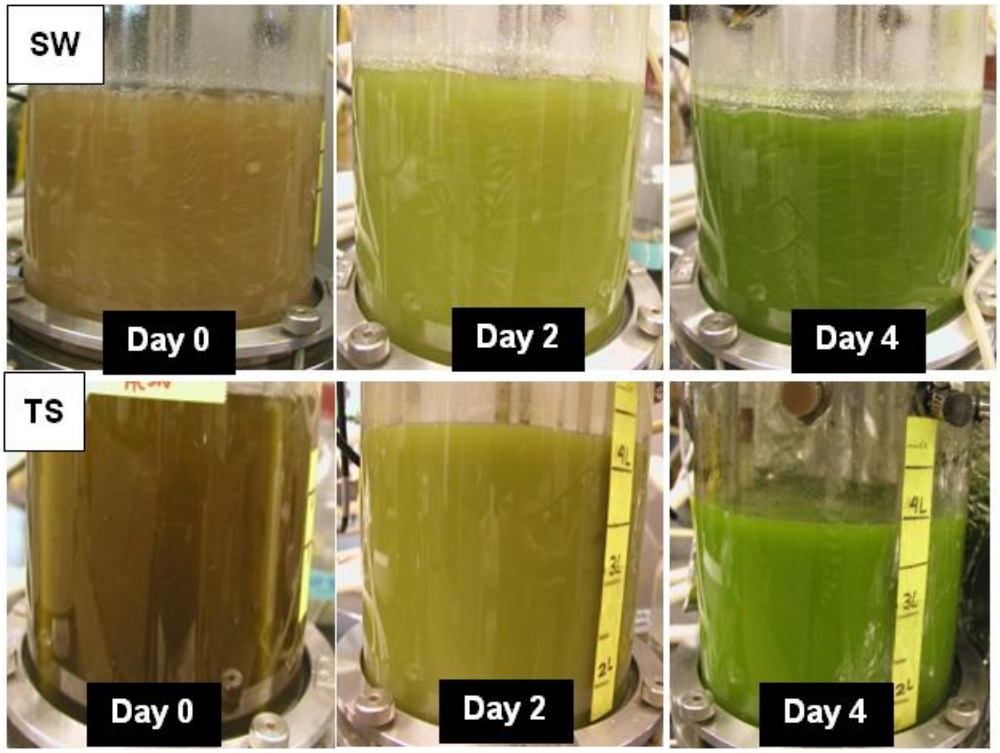


Figure 3b. Mixotrophic batch cultivation of *C. vulgaris* on SW (Soy Whey) and TS (Thin Stillage) in a 6-L stirred tank reactor. Cells were grown under mixotrophic conditions for 4 days, at 28 ℃, 180 rp m, 0.7 SLPM aeration rate, working volume of 3 L and pH 6.8. Inoculum was prepared by inoculating 100 mL of sterile MBM (pH 6.8) using an agar slant and incubating flasks on an orbital shaker set at room temperature (~25℃), und er a constant fluorescent light (luminance 880 lux) at 150-rpm agitation speed for 3 days

A comparison of the biomass and oil production by *C. vulgaris* when grown on TS, SW, and MBM is presented in Table 2. The lipid content (wt %) in the microalgal cells grown on the three media was found to be highest on Day 4, although the lipid yield/ productivity were highest on Day 3 for MBM and SW and on Day 4 for TS (Fig. 3a). This was due to the higher biomass yield/productivity of the microalgal cells on those days. The total lipids in terms of percent dry weight of the cells, recovered

from MBM-, TS-, and SW-derived microalgal biomass were 27, 43, and 11%, respectively. The biomass yield and productivity from TS (9.8 gL^{-1}, 2.5 $gL^{-1}d^{-1}$) was the highest among the three substrates and consequently the oil productivity was also the highest in TS (1.1 $gL^{-1}day^{-1}$).

Table 2. Biomass and oil productivities of *C. vulgaris* grown on MBM, SW and TS in a 6-L stirred tank reactor. Cells were grown under mixotrophic conditions for 4 days, at 28 ℃, 180 rpm agitation, 0.7 SLPM and 6.8 pH. Data are means ± SD, n=3

Growth media[a]	Biomass		Oil		
	Yield (gL^{-1})	Productivity ($gL^{-1}day^{-1}$)	% (g oil/100g biomass)	Yield (gL^{-1})	Productivity ($gL^{-1}day^{-1}$)
MBM	8.0 ± 0.2	2.0 ± 0.05	27.0 ± 1.0	2.2 ± 0.09	0.6 ± 0.05
SW	6.3 ± 0.1	1.6 ± 0.03	11.1 ± 1.1	0.6 ± 0.08	0.2 ± 0.02
TS	9.8 ± 0.3	2.5 ± 0.08	43.0 ± 1.2	4.2 ± 0.3	1.1 ± 0.1

[a]MBM (Modified Basal Medium), SW (Soy Whey) and TS (Thin Stillage)

As seen in Fig. 3c, the monosaccharides in SW, glucose, galactose, and fructose were consumed during early growth period (between Day 0 and Day 2), while the disaccharide, sucrose and oligosaccharide, stachyose were broken down and utilized by the algae only after Day 2. Fig. 3d illustrates the change in soluble sugar concentrations in TS during microalgal growth. Similar to observations with SW, the monosaccharides like glucose and fructose were consumed by Day 2 after which the microalgal cells metabolized maltose, glycerol and other oligosaccharides.

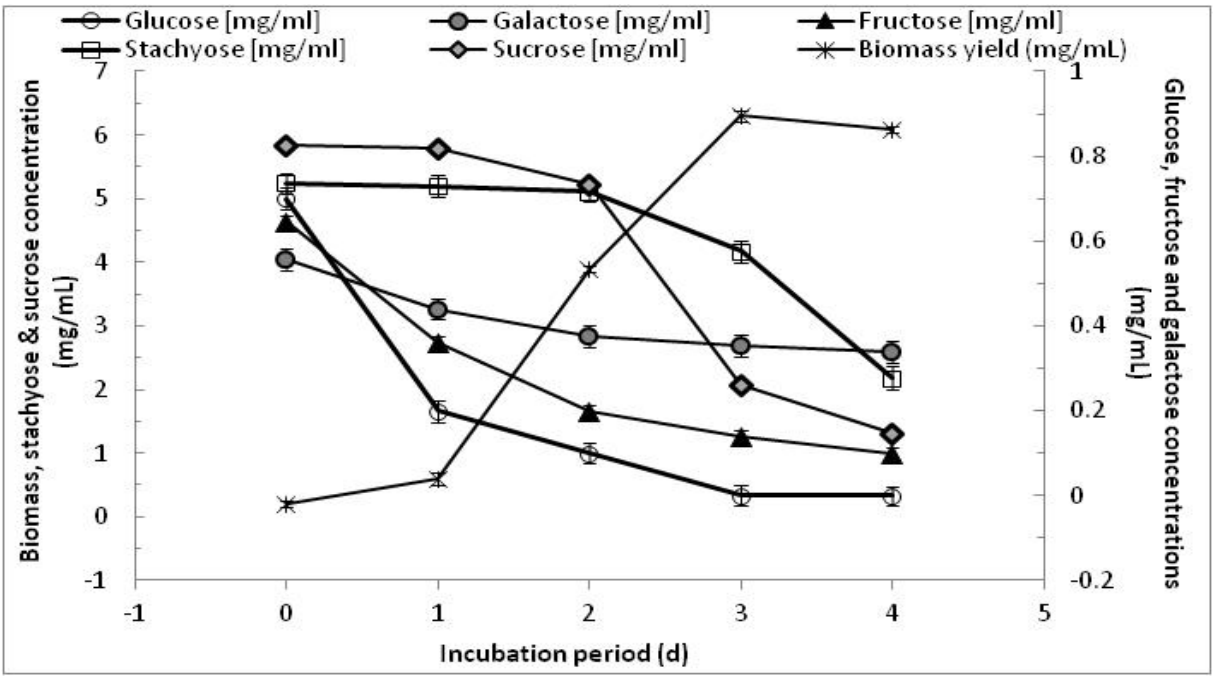


Figure 3c. Change in the concentrations of sugars found in SW (Soy Whey) during the growth of *C. vulgaris* in 6-L stirred bioreactor. Cells were grown under mixotrophic conditions, for 4 days, at 28 ℃, 180 rpm agitation, 0.7 SLPM aeration rate and 6.8 pH. Data are means ± S.D (n=3)

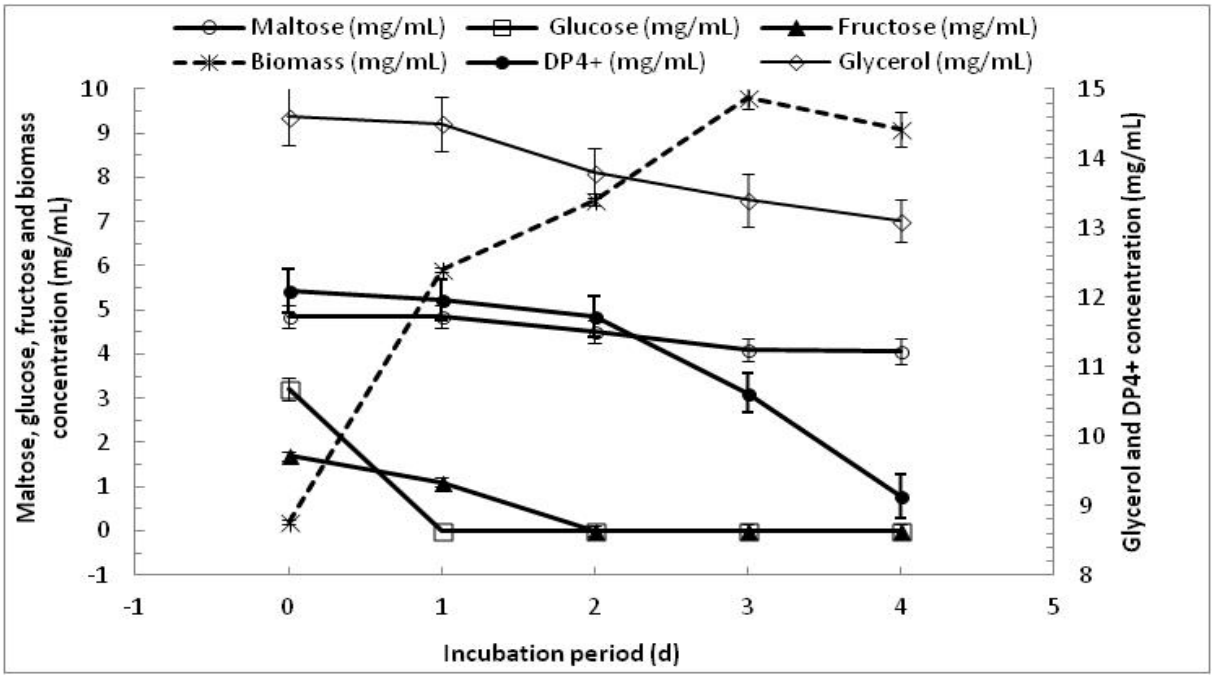


Figure 3d. Change in the concentrations of sugars found in TS (Thin Stillage) during the growth of *C. vulgaris* in 6-L stirred bioreactor. Cells were grown under mixotrophic conditions, for 4 days, at 28 ℃, 180 rpm agitation, 0.7 SLPM aeration rate and 6.8 pH. Data are means ± S.D (n=3)

The total soluble nitrogen (TN) in the culture broth fell sharply from Day 1 to Day 2, of incubation period, which corresponded with the log phase of growth of the microalgal cells. A slight increase in TN concentration towards the end of the growth curve could be due to the release of intracellular contents from dead microalgal cells. The microalgal growth reached its stationary phase when the nitrogen content in the media (SW and TS) reached its lowest concentration (Figs.3e & f). At this stage, the lipid content in the cells was seen to rise, possibly as a reaction to the stress conditions. During microalgal growth, SCOD was seen to fall by 45 and 29% in SW and TS, respectively (Figs.3e and f).

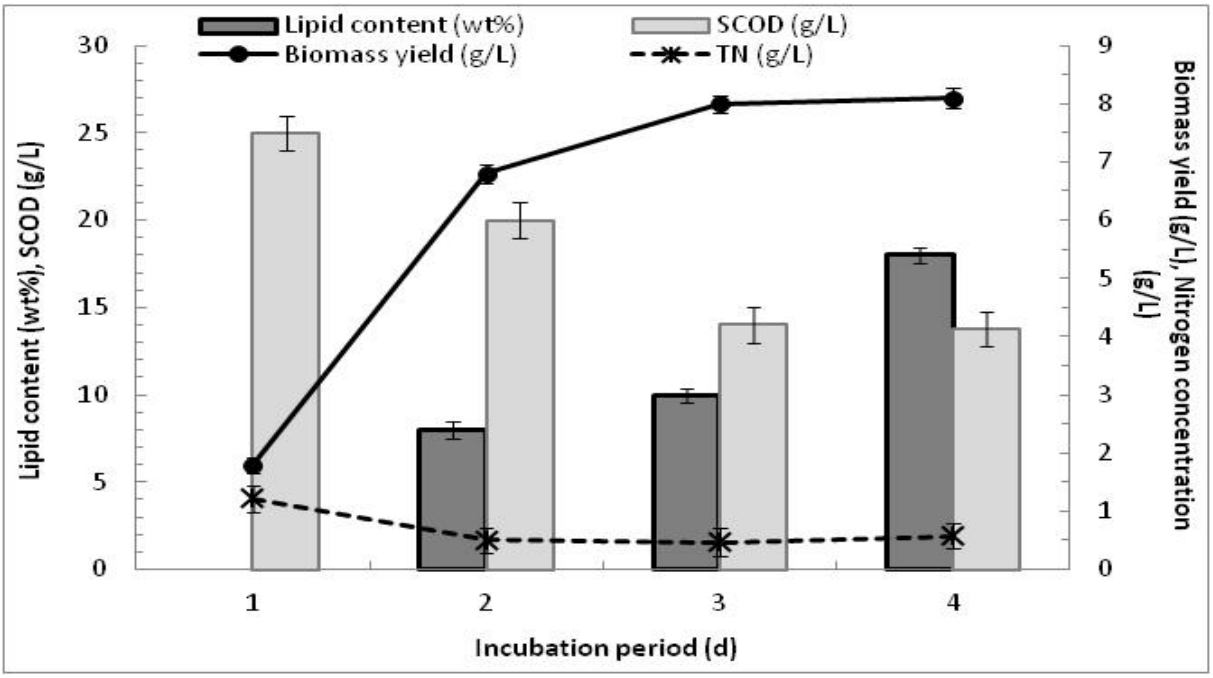


Figure 3e. Change in total nitrogen and SCOD concentrations in SW (Soy Whey) during the growth and lipid production of *C. vulgaris* cells in a 6-L stirred bioreactor. Cells were grown under mixotrophic conditions for 4 days, 28 ℃, 180 rpm agitation, 0.7 SLPM aeration rate an d 6.8 pH. Data are means ± S.D (n=3)

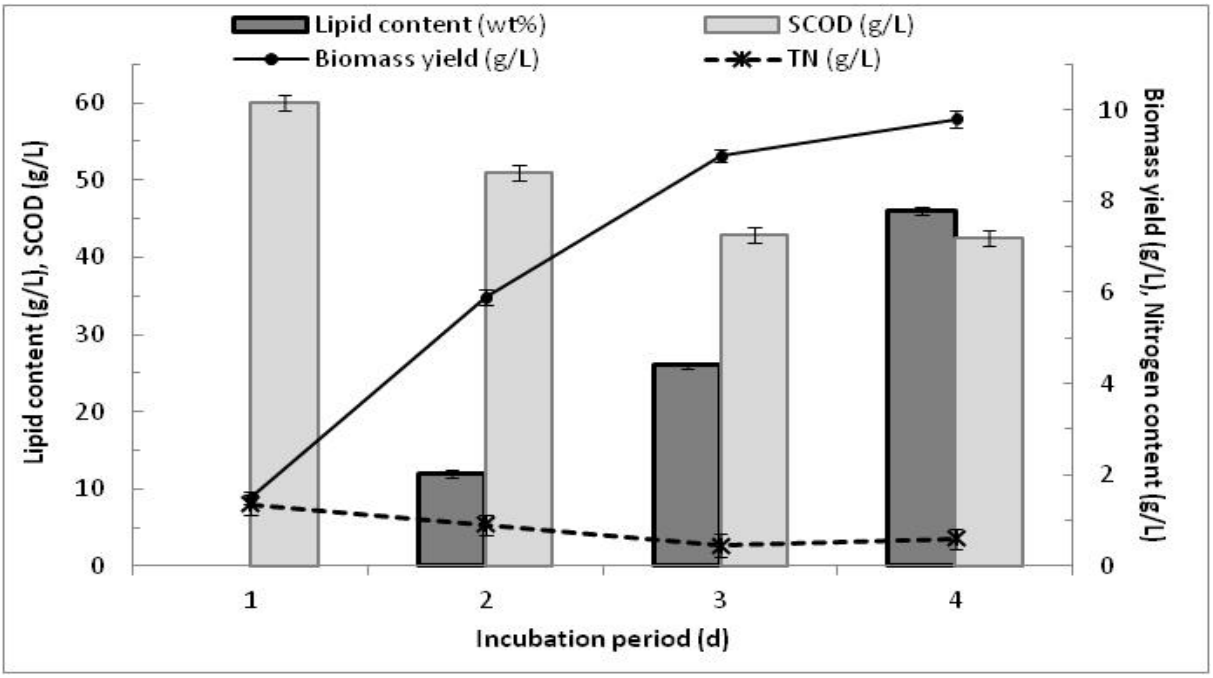


Figure 3f. Change in total nitrogen and SCOD concentrations in TS (Thin Stillage) during the growth and lipid production of C. vulgaris cell in a 6-L stirred bioreactor. Cells were grown under mixotrophic conditions for 4 days, 28 ℃, 180 rpm agitation, 0.7 SLPM aeration rate an d 6.8 pH. Data are means ± S.D. n=3

5.4.5 Chemical composition of mixotrophic cells of C. vulgaris grown in bioreactor

The proximate analysis of the various biomass samples indicated that the chemical composition of *Chlorella* cells was dependent on the nutrient growth medium used. The cells grown in TS had a higher percent of fat than those grown in SW, while cells grown on SW had a higher percent of protein and carbohydrate content (Table 3). The higher oil content observed in cells grown on TS could be a result of higher concentration of metabolizable sugars, nitrogen deficiency and presence of oil, in TS (~0.5 % (w/v). The protein-rich microalgal biomass derived

from TS and SW could have high value as animal feed. The carbohydrates in the biomass after oil extraction could be fermented to produce ethanol, or the biomass could be used via pyrolysis or gasification to generate electricity. The cultivation process also lowers the organic content of TS and SW enabling their recycling on full scale.

Table 3. Chemical composition of *C. vulgaris* cells grown on SW (Soy Whey) and TS (Thin Stillage) in a 6-L stirred bioreactor. Cells were grown under mixotrophic conditions for 4-days at 28 ℃, harvest ed via centrifugation (12,000×*g*, 15 min, 4℃), washed twice with sterile saline (0 .85% (w/v) NaCl) and dried to a constant weight at 80 ℃. Data are means ± SD (n=2)

Component (%) (db)	Cells grown on TS	Cells grown on SW
Moisture	4.49	4.99
Ash	8	9.16
Total carbohydrates	6.64	15.48
Starch	2.62	5.67
Fiber	<0.01	<0.01
Soluble sugars	5.02	9.81
Total fat	32.76	8.95
Total protein	40.47	50.6

5.4.6 Optimization of microalgal oil extraction

Among the various challenges in microalgal cultivation, like strain selection and development, culturing, harvesting, dewatering, cell disruption and oil extraction

etc., the cell disruption step is particularly crucial as the microalgal cell wall is generally thick [29]. The cell wall of *Chlorella vulgaris* is composed predominantly of proteins and saccharides, which prevent the easy release of intracellular lipids [30]. Among the three cell-disruption procedures tested, magnetic and rotary mixing took almost 12 h to produce the same oil yields from the microalgal biomass (4.2 g oil/L of TS, and 0.7 g oil/L of SW) as achieved by the sonication method in 3 min. Sonication was also faster than the modified Folch method [22] which required a homogenization step before solvent addition and ~4 h of mixing/contact times. The oil yield using the standard Folch method [21] was found to average ~4.1 g of oil/ L of TS, and ~0. 64 g of oil/ L of SW, which was quite similar to the yields obtained from the ultrasonication-solvent method. This showed that ultrasonication was effective in breaking the microalgal cell walls and releasing the intracellular lipids for dissolution in solvent. Sonication also reduced the particle size of the microalgal cells allowing a larger surface area of cells to be in contact with the organic solvents. With an attempt to arrive at the oil extraction technique that provided the best balance between oil yields and chemical hazards, results with ultrasonication and toluene/methanol were quite promising.

5.4.7 Effect of substrate characteristics on the fatty acid profile of microalgal oil

The fatty acid composition of microalgal oil was found to vary depending on the chemical characteristics of the growth media. As shown in Table 4, fatty acid composition of oil from *C. vulgaris* grown on TS closely resembled the composition

of corn oil in TS. Certik et al. [31] also suggest that the presence of oil in a microbial growth medium not only increases the lipid accumulation in the microbial cells, but also the composition of the accumulated lipid reflects the length of the carbon chain and structure of the oil source. In this study, among the three media, TS contained ~0.5% (w/v) oil and *C. vulgaris* cells when grown on TS had the highest oil content (43%, w/w). When *C. vulgaris* cells were grown on SW and MBM media which did not contain any oil, the cells were found to have lower oil contents (11 and 27%, w/w). Iassonova et al. [32] also made similar observations, when they found that yeasts varied their cellular lipid composition depending on the carbon sources present in the culture medium.

The nomenclature used in this study for the oil samples extracted from algal biomass grown on MBM, SW and TS was Chlorella-MBM_{oil}, Chlorella-SW_{oil}, and Chlorella-$TS_{oil.}$ Our analyses indicated that each of the oil samples differed from the rest by the presence/absence of one or more fatty acid, even though they were all produced by the same microalgal culture but on different media. For example, myristic acid (C14:0) in Chlorella-SW_{oil} sample and arachidonic acid (C20:0) in Chlorella-MBM_{oil} sample were absent in other oil samples. Palmitoleic acid (C16:1) was found in much higher concentrations in Chlorella-SW_{oil} sample, while stearic acid was quite high in Chlorella-MBM_{oil} sample. On the other hand, polyunsaturated fatty acids (PUFAs) like linoleic (omega 6 fatty acid) and linolenic acids (omega 3 fatty acids), also known as essential fatty acids were found to be high in Chlorella-TS_{oil} (52.2% of total FAs) and Chlorella-MBM_{oil} (13.3%) samples, respectively. Chlorella-SW_{oil} sample was also found to be rich in linoleic acid (33.1%). These

essential fatty acids are an obligatory dietary requirement for humans and animals [33]. Most human diets are deficient in linolenic acids. PUFA's are in great demand for dietary supplements and aquaculture; TS and MBM-derived microalgal oils could be a great source of such high-value products. MBM-derived microalgal oil had the highest percentage (~43%) of saturated FA's while TS-derived microalgal oil had the lowest (~16.5%). This could be an indication that microalgae grown on agro-based co-product substrates like SW and TS may not be a very good feedstock for biodiesel application. Rather, the value of growing algae on agro-waste co-products lies in recovery of high-value constituents from algal biomass, e.g., PUFA, protein etc., based on desired applications.

Table 4. Fatty acid (FA) compositional analysis of microalgal oil. Oil samples were extracted from *C. vulgaris* cells grown on Modified Basal Medium (Chlorella-MBM_{oil}), Soy Whey (Chlorella-SW_{oil}) and Thin Stillage (Chlorella-TS_{oil}), and from thin stillage alone (TS_{oil}). Data are means ± SD (n=2)

Microalgal oil samples	Individual fatty acid (% of total fatty acids)			
	Chlorella-MBM_{oil}	Chlorella-SW_{oil}	Chlorella-TS_{oil}	TS_{oil}
C 14:0	NA	2.5±0.2	NA	NA
C16:0	22.9±0.2	18.7±0.3	13.9±0.2	14.5±0.2
C16:1	2.1±0.0	9.8±0.1	2.9±0.2	NA
C18:0	18.6±0.3	7.2±0.3	2.5±0.1	2.1±0.3
C18:1	21.5±0.4	19.7±0.1	21.4±0.2	25.7±0.4

C18:2	17.7±0.2	33.1±0.1	52.2±0.4	30.7±0.2
C18:3	13.3±0.2	4.7±0.2	4.1±0.3	NA
C20:0	1.3±0.1	NA	NA	1.9±0.2

5.5 Conclusion

High density microalgal cell growth was observed in both TS and SW with the highest biomass and lipid yields obtained from cells grown on TS (9.8, and 4.2 g/L). The microalgal oil samples differed in their fatty acid composition based on the growth substrate composition. The oil showed presence of PUFA's, and the cells were rich in protein. The use of ultrasonics with toluene/methanol solvent system improved lipid extraction efficiency in terms of time and solvent use. Thus, this study demonstrated the use of inexpensive and readily available agro-industrial co-products as mixotrophic microalgal nutrient feedstock, that could reduce the need for fresh water and external nutrients.

5.6 Acknowledgement

The authors would like to thank Lincolnway Energy, Nevada, IA and Iowa State University's Center for Crops Utilization Research (CCUR), for providing thin stillage and soy flakes respectively. We appreciate the lab facilities provided by the Dept. of Food Science and Human Nutrition and CCUR for this research. We would also like to acknowledge the help given by Priyanka Chand, Dr. David Grewell, Dr.

Venkat Reddy Chintareddy, Dr. John Verkade, Dr. Bill Colonna, Carol Ziel, Dr. Mary Rasmussen, Dr. Zhiyou Wen, Dr. Yingjun Wang, Dr. Martin Spalding, Dr. Byron F. Brehm-Stecher and Dr. Supriyo Das at the various stages of this research.

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CHAPTER 6: SYNERGISTIC ACTION OF PLANT ESSENTIAL OILS AND ORGANIC ACIDS AND THEIR GROUP/STRAIN-SPECIFIC ANTIMICROBIAL ACTIVITY AGAINST FOOD-RELATED PATHOGENS

A paper to be submitted to *Journal of Food Protection*

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6.1 Abstract: Plant essential oils (EOs) are a versatile group of natural antimicrobials which can be used in both food and feed for the safety of both humans and animals. Among the sixteen EOs tested for their antimicrobial potency against a broad panel of food-related pathogenic strains, cassia oil was found to be the most effective against *Staphylococcus aureus* 25923 (Minimum Inhibitory Concentration, MIC = 0.02%), while redistilled oregano was most potent against *Escherichia coli* 25922 (MIC=0.03%) and cinnamon had the lowest MFC against *Candida albicans* 10231(Minimum Fungistatic Concentration, MFC=0.01%). Among the seven most active EOs, cassia and redistilled oregano (RO) oils were most active with equal

potency against Methicillin Resistant *Staphylococcus aureus* BAA-44 (MRSA), and Vancomycin Resistant *Enterococcus faecalis* 700802 (MIC=0.06% and 0.13%, respectively), while cassia, RO and cinnamon oils were most inhibitory towards *Rhodococcus equi* (MIC=0.13%).Certain EOs showed group and/ or strain-specific activity. Tsuga oil was inhibitory only against *C. albicans;* while bergamot, manuka and sandalwood oils were inhibitory only against *S. aureus.* Also, some EOs such as cinnamon, RO and cassia exhibited broad spectrum activity. Also, cinnamon oil exhibited strain-specific activity being highly inhibitory against *S. aureus* 25923 but not very effective against *S. aureus* BAA44. Synergistic interaction between the oils and acids was in general observed more against bacteria as compared to the yeast. Malic and citric acid were the most active acids showing synergistic interactions with mountain savory, RO, and cinnamon oils against *S. aureus* 25923 and with cassia, RO, and cinnamon oils against *E. coli* 25922*.* The only EO-acid combination which demonstrated synergism against *C. albicans* 10231 was lemon myrtle-citric acid. In sum, our results successfully demonstrated the advantages of the hurdle concept, to ensure better food safety and quality with minimal processing.

Keywords: Plant essential oils, organic acids, synergism, antimicrobial activity, foodborne pathogens

6.2 Introduction

Microbiological safety as well as quality are major factors in overall consumer acceptance of food products, and therefore are essential not only to public health, but to the ultimate economic soundness of the food industry. Increasing consumer

awareness has led to high demands for minimally processed foods with natural preservatives /antimicrobials (Roller, 2003). Organic acids and EOs are natural antimicrobials with GRAS (Generally Regarded as Safe) status (Tamblyn and Conner, 1997; Kabara, 1991) and a strong track record of food-based and other applications. Several studies have confirmed the antimicrobial properties of EOs in both model food systems as well as in real food matrices (Tsigarida et al, 2000). However, due to the inherent nature of EOs, their use in foods may be limited by flavor, since potent doses usually exceed acceptable levels from an organoleptic standpoint (Fisher et al., 2007). The concentrations at which the EOs are cidal in their action are too high and above the tolerable taste threshold. Therefore, this drawback restricts their application to only inhibition of the microbes (Brul and Coote, 1999). If their inhibitory powers could be reinforced with the supplementation of small amounts of another antimicrobial, a balance could be reached between sensory acceptability and antimicrobial efficacy.

The wide use of organic acids in foods and their unique structural properties that provide them with their versatile functionalities make them important elements in multi-component antimicrobial compositions. Microbial growth inhibition by weak acid preservatives has been proposed to be due to a number of different mechanisms including, membrane disruption, stress on intracellular pH homeostasis and the accumulation of toxic anions (Ricke, 2003). The mode of action of EOs is mainly due to their hydrophobicity, enabling them to penetrate the lipid layers in bacterial cell membranes and disrupt them causing cell lysis (Knobloch et al., 1986;

Sikkema et al., 1994). Antimicrobial efficiency of EOs is known to increase at low pH (Burt, 2004), hence combining EOs with weak organic acids is known to increase their antimicrobial efficacy through synergistic interactions. Dimitrijevic et al. (2007) used commercial essential oils of thyme, rosemary, and oregano and tested them in combination with lactic acid against *Listeria monocytogenes.* They found that only the lower concentrations of oils behaved synergistically with the acids. Friedly et al. (2009) reported that organic acids, citric and malic significantly reduced the MIC of citrus oil fractions.

The simultaneous application of several different antimicrobial treatments in foods is referred to as the “hurdle” technology. Leistner and Gorris (1995) developed this concept where combinations of preservation techniques are used to prevent the survival and re-growth of pathogens in food. When two or more antimicrobials are added to food, they can interact either synergistically, additively or antagonistically. Synergistic reactions enable the addition of lower concentrations of each of the antimicrobials with the same effectiveness. In our study we investigated this new wave of food preservation that ensures maximum safety while producing minimal impact on the food quality by exploring the interaction between two well-known antimicrobial agents, EOs and organic acids. Though EO-acid synergism is now an established phenomenon, to our knowledge, only a few studies with selected oils and acids have been performed so far. Our research is a preliminary investigation involving sixteen different EOs, five different acids, and six pathogenic strains, thus allowing for a broader platform where almost 35 different EO-acid combinations

were studied. The test cultures used in this study have been selected to represent the most commonly found microbial types including *S.aureus, E.coli* and *C.albicans*; as well as a few difficult to treat pathogens like *R.equi,* MRSA, and VRE. *R.equi* is a Gram positive coccobacillus responsible for causing pneumonia in foals and other infections in domesticated animals like goats and pigs. It is also emerging as an important opportunistic pathogen in immunocompromised patients, especially those with acquired immunodeficiency syndrome (AIDS). Although it requires fastidious growth conditions in labs (media supplementation with cofactors and specific nutrients), in nature it is a serious cause of infections. The infections caused by MRSA in humans and animals are very difficult to treat. The particular strain of MRSA used in this study is known to be resistant to gentamycin, ampicillin, methicilin, erythromycin, oxacillin, penicillin and tetracycline. Similar to MRSA, infections caused by VRE in humans and animals are also difficult to treat. The strain used in this study is resistant to gentamycin and vancomycin.

This study had three main objectives-

1) To measure the MICs and MBCs of sixteen individual essential oils against a representative panel of select microbial cell types, which included a Gram-negative bacterium, *Escherichia coli* ATCC 25922; a Gram-positive bacterium, *Staphylococcus aureus* ATCC 25923; and a pathogenic yeast, *Candida albicans* ATCC 10231. This would enable an initial screening of the most broadly active oils.

2) To broaden the microbial test group and measure the antimicrobial effectiveness of the most active oils from the initial screen against two drug-resistant pathogenic bacteria, MRSA, VRE and an important veterinary pathogen, *R. equi*.

3) To determine whether the selected essential oils interacted synergistically, additively or antagonistically when combined with five different food-grade organic acids. The antimicrobial action was tested against three microbial types - Gram-negative *E.coli* ATCC 25922; Gram-positive *S. aureus* ATCC 25923; and *C.albicans* ATCC 10231.

6.3 Materials and Methods

6.3.1 Microbial strains and culture conditions

The test cultures, *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, *Candida albicans* ATCC 10231, Methicillinn resistant *Staphylococcus aureus* BAA44 (MRSA; originally isolated from a hospital in Lisbon, Portugal), Vancomycin Resistant *Enterococcus faecalis* ATCC 700802 (VRE; originally isolated from a human bloodstream infection, St. Louis, MO), and *Rhodococcus equi* ATCC 6939 (originally isolated from a lung abscess in a foal); were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). MRSA, VRE, *S. aureus* 25923 and *E. coli* 25922 were grown in Tryptic Soy Broth (TSB, BD Diagnostics Systems, Sparks, MD). *R. equi* 6939 was grown in Heart Infusion Broth w/ Fildes supplement (BHI + Fildes; Difco Laboratories), while *C. albicans* was grown in Yeast Mold broth (YM, BD Diagnostics Systems, Sparks, MD). Fildes is a nutritional

supplement that provides important cofactors associated with the host, including blood components and is necessary for the good growth of *R. equi*.

All strains were grown aerobically at 35ºC for 18 hours and enumerated via plating onto Tryptic Soy Agar (TSA; *S. aureus* 25923*, E. coli* 25922, MRSA and VRE) or YM agar (*C. albicans* 10231) or blood agar (TSA + 8% horse blood; *R. equi* 6939). Initial enumeration experiments indicated that a 0.1 absorbance reading at 600 nm corresponded to approx. 10^8 CFU/mL. Based on this, the Abs_{600} of the 18 h cell suspensions was adjusted to 0.1 using sterile growth medium as the diluent. Serial dilutions were made to guide the cell concentration of the inocula to 10^6 CFU/mL in order to obtain the desired cell concentration of 10^5 CFU/mL in the Bioscreen test wells.

6.3.2 Essential Oil extracts

A 1% (v/v) stock solution of the 16 EOs was prepared in TSB (or YM for *C. albicans*) and a homogenous emulsion was visible which was found to be stable for ~ 24 h. Stepwise, two-fold dilutions of the oils were done according to the Clinical Laboratory Standards Institute's approved protocol for antimicrobial micro-dilution testing. A range of seven oil concentrations - 0.5, 0.25, 0.13, 0.063, 0.031, 0.016 and 0.008% (w/v), respectively, were prepared in the wells of the Bioscreen micro-titer plate using the appropriate growth medium. The MIC and MBC values of all the oil samples were obtained against *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and

C. albicans ATCC 10231 (as explained in sections 6.4.1 - 6.4.2). Based on the initial MIC data seven of the most effective EOs, viz. lemongrass, lemon myrtle, manuka, mountain savory, cassia, redistilled oregano, and cinnamon were selected. These were tested for their antimicrobial effectiveness against two drug-resistant "super bugs", VRE and MRSA. Also, as a prelude to *in vivo* testing of essential oils in animals to obtain healthy, antibiotic-free meat products, the in-vitro efficacy testing of the selected oils was also done against a veterinary pathogen, *R. equi*. The seven selected EOs were further mixed at their minimum inhibitory and sub-inhibitory concentrations with five organic acids to test for synergistic/ additive effects. The EOs and organic acids tested in this study have been listed in Table 1.

Table 1 Nomenclature of EOs and organic acid samples

Essential oils*	Organic acids
Lemongrass oil	Butyric
Lemon Myrtle	Citric
Mountain Savory	Ascorbic
Cumin Oil	Malic
Tsuga Oil	Tartaric
Citrus Hystrix Oil	
Tea Tree Oil	
Bay Laurel Oil	
Bergamot Oil	
Dalmation Sage Oil	
Manuka Oil	
Sandalwood Oil	

Cassia Oil
Redistilled Oregano
Cinnamon Oil
Clove Oil

* EOs were obtained from Van Beek Natural Sciences Inc.

6.3.3 Organic acids

Five food-grade organic acids, viz. butyric, citric, ascorbic, malic, and tartaric were tested for their antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *C. albicans* ATCC 10231. A 5% (v/v) stock solution of the individual organic acids was prepared in sterile distilled water (DW). Stepwise, two-fold dilutions of the acids were then made in sterile DW, according to the Clinical Laboratory Standards Institute's approved protocol for antimicrobial micro-dilution testing. Acid concentrations ranging from 2.5 to 0.01% were tested in the Bioscreen to evaluate the MIC and MBC values against the test pathogens. After evaluation of their MIC values, acids were used at their minimum inhibitory and sub-inhibitory concentrations in combination with the EOs to test for synergistic/additive effects in their interactions.

6.3.4 Determination of Minimum Inhibitory Concentrations using the Bioscreen

The Bioscreen C Microbiology Reader (Growth Curves Inc., Piscataway, NJ) was used for this study to evaluate the MICs of the antimicrobial samples. The unit is

a combined incubator and micro-plate reader that enabled collection of high-resolution absorbance data for up to 200 wells in each experiment with individual well capacity of 400 µL. As described above, the antimicrobial extracts (EOs, acids and oil-acid combinations) were added to an appropriate growth medium and stepwise dilutions were made using the same medium to obtain a series of media containing different, known levels of the extracts in the Bioscreen wells. Test culture suspensions were inoculated to give a final concentration of 10^5 CFU/well. The positive control wells contained the growth media and test cultures and no antimicrobial extract. Test cultures were absent in the negative control wells which contained only extracts and growth media. All tests were performed in triplicate wells and all Bioscreen experiments were run in duplicates. The Bioscreen plates were incubated at 30ºC. The absorbance readings were taken every 15 min for 24 h at 600 nm. To ensure cells were evenly suspended at the time absorbance was collected, the instrument was set to automatically shake the test plate for 30 sec prior to each reading. At the end of the experiment, all absorbance readings were plotted against time. The MIC for each antimicrobial extract was defined as the minimum concentration at which no detectable growth or increase in absorbance was observed for 24 hours. The seven most active oils with the lowest MIC values were further mixed with the five organic acids to prepare 35 oil-acid combinations. The oils and acids were mixed at both their individual inhibitory as well as sub-inhibitory concentrations and the MIC of the oil-acid combinations was determined using the Bioscreen.

6.3.5 Determination of Minimum Bactericidal Concentrations (MBCs) by track plating on agar plates

To establish the MBC, 10µl of well contents from each dilution series was plated onto agar plates and incubated at 30ºC for 24 hours. The MBC was defined as the antimicrobial extract concentration that achieved 99.9% killing - demonstrated by absence of growth on the agar plates. The MBC values of the individual oils and organic acids along with the acid-oil combinations were measured.

6.3.6 Determination of cell counts by plating

After 24 h absorbance data collection from the Bioscreen, the well contents from the micro-titer plates were used to measure the final cell counts of the test cultures after the 24 h contact time with the antimicrobials. Serial dilutions of the well contents were done in buffered peptone water (1% peptone, final pH: 7.2 ± 0.2 at 25℃) and 100 µL of each dilution was spread-plated on suitable agar media (TSA for *E. coli* and *S. aureus*, YM agar for *C. albicans*). Plates were incubated at 35 ℃ and colonies were counted after 16 h of incubation.

6.3.7 Effect of pH on the MIC of EOs and Organic acids

To confirm that the antimicrobial activity of the essential oils and organic acids was not solely a pH effect, the pH of the oil and acid samples both individually and as mixtures were recorded at their MIC values.

6.3.8 Fractional Inhibitory Concentrations (FICs)

Dilution results for combined testing of antimicrobials can be interpreted using Fractional Inhibitory Concentrations (FICs). The FIC of the individual oils was calculated as the ratio of the MIC of the oil when combined with an acid, divided by the MIC of the oil alone. The FIC of the individual acids was calculated likewise. The FIC of the oils and acids in an inhibitory combination was calculated by adding their individual FICs to give a total 'FIC_{index}'. A FIC_{index} near 1 indicated additivity, whereas <1 indicated synergy and >1 indicated antagonism.

6.3.9 Statistical Analysis

The Analysis of Variance (ANOVA) was performed using JMP 8.0.2 (Cary, NC, USA), to determine significant difference among the different treatments. Least Significant Means Differences (LSD) were calculated at $p = 0.05$ by Student's t test. All treatments were carried out in triplicates and results are shown as the means of three replicates.

6.4 Results and Discussion

6.4.1 MICs of individual EOs

Among the 16 essential oil extracts tested, each one of them was inhibitory against one or more of the pathogenic cultures at concentrations of 0.5% or below. The antimicrobial activity was found to be group specific. Tsuga oil was inhibitory only against *C. albicans* (0.5%) as shown in Fig. 1. While Bergamot Oil (0.5%), Manuka Oil (0.06%) and Sandalwood oils (0.25%) were inhibitory only against *S. aureus* (Table 2). Cinnamon, redistilled oregano and cassia oils exhibited broad spectrum activity. Cassia and cinnamon oils had the highest inhibition ($p<0.05$) against *S. aureus*. Cinnamon oil was the most potent ($p<0.05$) among the oils against *C. albicans* and *E. coli* with MICs of 0.004% and 0.02%, respectively (Fig. 2). These results are summarized in Table 2.

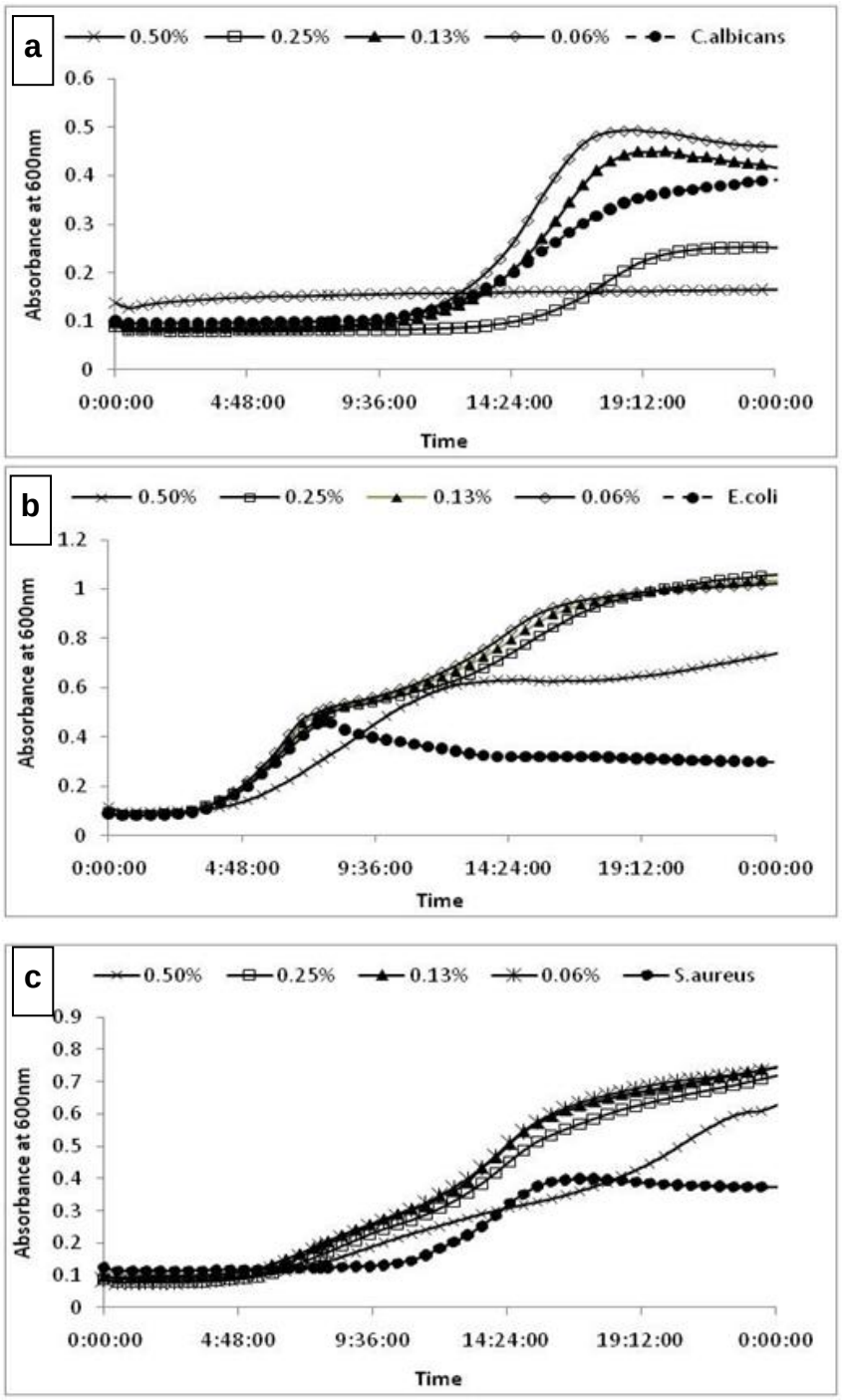

Figure1. Group-specific antimicrobial activity of tsuga oil against *C. albicans* 10231 (a). Inhibition was not observed against *E. coli* 25922 (b) or *S. aureus* 25923 (c). The cultures were grown in micro-titer wells and absorbance

readings were taken using a Bioscreen for 24h at 30 ℃ in the presence of different concentrations of Tsuga oil. All values are means of three replicates

Table 2. MICs and MBCs of individual essential oils and acids against *E. coli* 25922, *S. aureus* 25923, and *C.albicans* 10231; determined via turbidimetry (Bioscreen C instrument) and plating, respectively. All values are means of three replicates

Essential oils	*S.aureus*		*E.coli*		*C.albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC
Lemongrass	0.25%	0.25%	0.5%	0.5%	0.13%	0.125%
Lemon Myrtle	0.25%	0.25%	0.25%	0.5%	0.06%	0.25%
Manuka	0.06%	0.25%	>0.5%	NT	>0.5%	NT
Mountain savory	0.13%	0.25%	0.13%	0.13%	0.13%	0.25%
Cassia	0.02%	0.25%	0.25%	>0.5%	0.008%	0.063%
Redistilled Oregano	0.03%	0.063%	0.03%	0.06%	0.02%	0.063%
Cinnamon	0.06%	0.063%	0.13%	0.13%	0.008%	0.031%
Cumin	0.5%	>0.5%	>0.5%	NT	0.5%	0.5%
Tsuga	>0.5%	NT	>0.5%	NT	0.5%	>0.5%
Citrus Hystrix	0.25%	0.5%	>0.5%	NT	0.25%	0.5%
Tea Tree	0.5%	0.5%	0.5%	>0.5%	0.5%	0.5%
Bay Laurel	0.5%	>0.5%	0.5%	>0.5%	0.5%	0.5%
Bergamot	0.5%	>0.5%	>0.5%	NT	>0.5%	NT
Dalmation sage	0.5%	>0.5%	>0.5%	NT	0.5%	>0.5%
Sandalwood	0.25%	0.25%	>0.5%	NT	>0.5%	NT
Clove	0.5%	>0.5%	0.5%	>0.5%	0.25%	0.25%

Organic acid	*S.aureus*		*E.coli*		*C.albicans*	
Tartaric	0.63%	0.625%	0.31%	0.625%	1.25%	>2.5%
Malic	1.25%	>2.5%	0.63%	>2.5%	1.25%	>2.5%
Butyric	0.31%	0.63%	0.31%	0.63%	0.01%	0.02%
Citric	1.25%	>2.5%	1.25%	>2.5%	>2.5%	NT
Ascorbic	1.25%	>2.5%	1.25%	>2.5%	>2.5%	NT

NT: MBC was not tested for samples with high MIC values

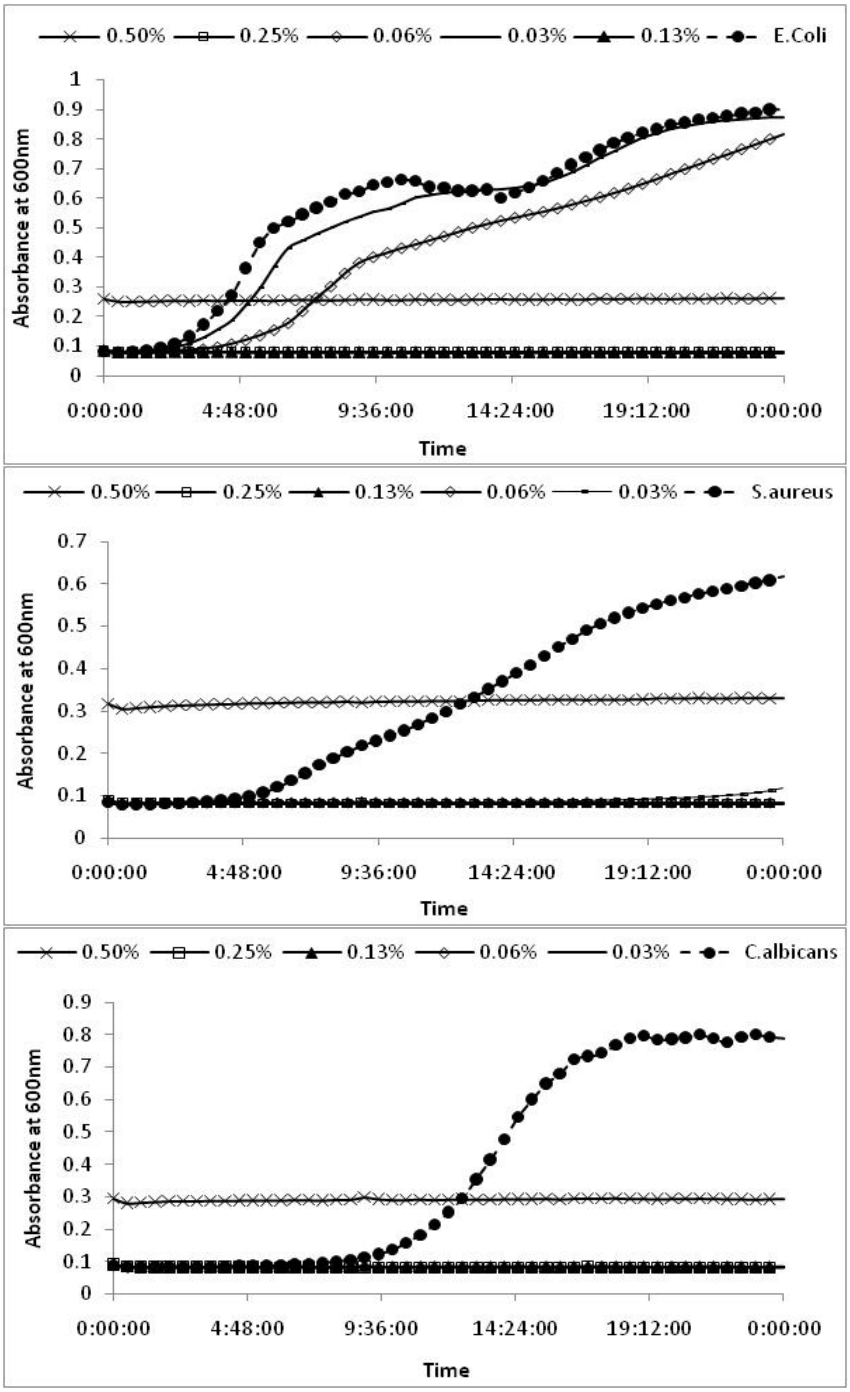

Figure 2. Antimicrobial activity of cinnamon oil against *C. albicans* 10231, *E. coli* 25922 and *S. aureus* 25923. The cultures were grown in a micro-titer well and absorbance readings were taken using a Bioscreen for 24h at 30 ℃ in the presence of different concentrations of cinnamon oil. All values are means of three replicates

Among the 7 selected oils with the lowest MIC values, cassia and RO oils had the lowest MICs ($p<0.05$) of 0.13 and 0.63% among the oils, when tested against VRE and MRSA, respectively (Table 3). Strain specific activity was observed in some of the oils. Cinnamon oil for example, was very effective in inhibiting *S.aureus* 25923 (MIC = 0.06%) but was not very effective against *S.aureus* BAA44 (MIC = 0.25%). Among the different pathogenic strains tested, the animal pathogen *R. equi* was the most resistant to the antimicrobial action of the EOs tested. Cassia, redistilled oregano and cinnamon oils were the most effective among the oils with a MIC of 0.13% (Table 3). The effect of EOs obtained from *Guatteriopsis* spp. (Magnolia family); belonging to the family of Annonaceae, was tested against *R. equi* in a study by Costa et al. (2008). They found that oil from all the species were potent inhibitors and their main components were α-eudesmol, β-eudesmol, γ-eudesmol, α-pinene, β-pinene and caryophyllene oxide (found in ginger, basil, other herbs and spices). Thus, in future studies, some of these pure compounds could be tested in combination with cassia, RO or cinnamon oils (found most effective in this study) for their enhanced efficacy in inhibiting *R. equi.*

Table3. MICs and MBCs of individual essential oils against Methicillin-Resistant *S. aureus* BAA44 (MRSA), Vancomycin-Resistant *E. faecalis* 700802 (VRE), and *R. equi* 6939; determined via turbidimetry (Bioscreen C instrument) and plating, respectively. All values are means of three replicates

Essential Oils	MRSA		VRE		*R.equi*	
	MIC	MBC	MIC	MBC	MIC	MBC
Lemongrass	0.25%	0.25%	>0.5%	0.25%	>0.5%	>0.5%
Lemon Myrtle	0.125%	0.25%	>0.5%	0.5%	>0.5%	>0.5%
Manuka	0.25%	>0.5%	0.25%	>0.5%	0.5%	0.5%
Mountain savory	0.25%	0.25%	0.25%	0.25%	0.5%	>0.5%
Cassia	0.063%	0.25%	0.125%	0.25%	0.125%	0.125%
RO[a]	0.063%	0.25%	0.125%	0.25%	0.125%	0.25%
Cinnamon	0.25%	0.25%	0.25%	0.25%	0.125%	0.125%

a: RO, Redistilled Oregano oil

6.4.2 MBCs of EOs

The MBC data reflected the bactericidal activities, rather than the bacteriostatic or combined bactericidal and bacteriostatic activities reported in the MIC experiments. Therefore, MBC values did not always coincide directly with MIC values. However, the same general trends in activity were preserved between MBC and MIC experiments,

Among the 16 oils, 13 were bactericidal in action with a MBC at or below 0.5% against one or more of the pathogenic cultures tested. A few oils were group-specific in their activities. Cumin, clove and bay laurel oils were bactericidal in action only against *C. albicans* at 0.5%, 0.25% and 0.5% concentrations, respectively. Manuka and sandalwood oils at 0.25% concentration were bactericidal only against *S. aureus.* Cinnamon and redistilled oregano oils were the most potent with the lowest MBCs ($p<0.05$) amongst all the oils against the three test cultures (Fig. 3). Redistilled oregano oil was bactericidal against all the three pathogenic cultures at 0.06% concentration.

Except for manuka oil, all the rest 6 selected oils were equally potent (MBC= 0.25%) against both VRE and MRSA. Cassia and cinnamon oils were the most potent ($p<0.05$) against *R. equi* 6939 at 0.125% bactericidal concentrations. The MBC values of all the oils are summarized in Tables 2 and 3.

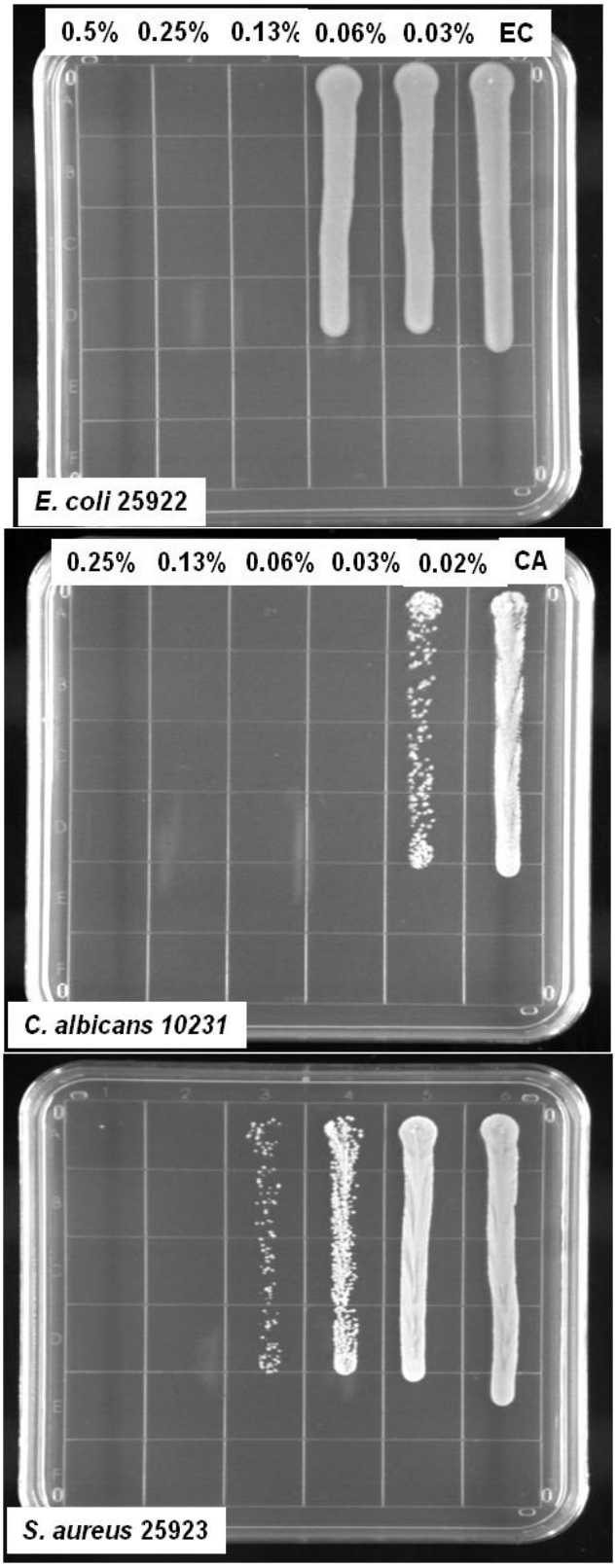

Figure3. Bactericidal activity of cinnamon oil against *C. albicans* 10231, *E. coli* 25922 and *S. aureus* 25923. Cidal action was observed by exposing the cells to different concentrations of the oil and incubating the mixture for 24 h in a Bioscreen and then plating the well contents on YM agar plates and checking for growth/no-growth after overnight incubation of plate at 35 ℃. Minimum concentration of cinnamon oil which caused complete killing of cells (MBC) was 0.06%, 0.13% and 0.03%, against *S. aureus*, *E. coli* and *C. albicans,* respectively. All values are means of three replicates

6.4.3 MICs and MBCs of organic acids

Among the five acids, butyric acid was the most effective against *S. aureus* with the lowest ($p<0.05$) MIC of 0.31% and MBC of 0.63%. The acids showing highest inhibition against *E. coli* were butyric and tartaric acids at 0.31% MIC and 0.63% MBC. In case of *C. albicans*, butyric acid had the lowest ($p<0.05$) MIC at 0.01% and MBC at 0.02%. The MIC and MBC values of the acids have been summarized in Table 2.

6.4.4 Antimicrobial activity of EO-acid combinations

When the oils and acids were mixed at their sub-inhibitory concentrations, certain combinations interacted synergistically, lowered their MICs and caused complete inhibition of the test microbe (Table 4). Manuka at 0.03% and tartaric acid

at 0.31% concentrations were unable to inhibit *S. aureus* when used alone. But when combined together, complete inhibition was observed (Fig. 4). Citric and malic acids were the most active, interacting synergistically with cassia, redistilled oregano, and cinnamon oils against *S. aureus.* In a study by Friedly et al. (2009), similar synergistic behavior by citric and malic acids was observed with citrus oil fractions against *Listeria monocytogenes* and *Listeria innocua*. Citric and malic acids also showed synergism with mountain savory, RO and cinnamon oils against the Gram negative bacterium, *E. coli*. The possible explanation for the successful synergistic behavior seen in case of citric acid could be due to its chelating property (Russel, 1991) that could have potentiated the antimicrobial effect of the EOs. Similar observations have been reported in other studies. Helander et al. (1997) discussed the permeabilising effect of citric acid on the outer membrane of Gram negative bacteria. Shibasaki and Kato, 1978 found that citric acid was able to increase the potency of monolaurin against Gram negative bacteria.

Table 4. Antimicrobial activity of essential oil and acid pairs mixed at their individual sub-inhibitory concentrations and tested for synergism in their action against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231 using the Bioscreen. All values are means of three replicates

Essential oil + Organic acid Pairs	Minimum Inhibitory Concentrations (MICs)		
	S. aureus	*E. coli*	*C. albicans*
Lemongrass+ Tartaric	NA	NA	0.06%+0.6%
Lemon Myrtle+ Tartaric	NA	NA	0.03%+0.6%
Manuka+ Tartaric	0.03%+0.31%	NA	NA
Mountain savory+ Tartaric	NA	0.06%+0.16%	0.06%+0.6%
Cassia+ Tartaric	0.008%+0.31%	NA	NA
Redistilled Oregano+ Tartaric	0.016%+0.31%	0.016%+0.16%	0.008%+0.6%
Cinnamon+ Tartaric	0.008%+0.31%	0.008%+0.16%	0.002%+0.6%
Lemongrass+ Malic	NA	NA	NA
Lemon Myrtle+ Malic	NA	NA	NA
Manuka+ Malic	0.03%+0.63%	NA	NA
Mountain savory+ Malic	NA	0.06%+0.31%	0.06%+0.63%
Cassia+ Malic	0.008%+0.63%	NA	NA
Redistilled Oregano+ Malic	0.016%+0.63%	0.016%+0.31%	NA
Cinnamon+ Malic	0.008%+0.63%	0.008%+0.31%	NA
Lemongrass+ Butyric	NA	NA	NA
Lemon Myrtle+ Butyric	NA	NA	NA
Manuka+ Butyric	0.03%+0.16%	NA	NA
Mountain savory+ Butyric	NA	NA	0.06%+0.005%
Cassia+ Butyric	NA	NA	NA
Redistilled Oregano+ Butyric	NA	NA	NA
Cinnamon + Butyric	NA	NA	NA
Lemongrass+ Citric	NA	NA	NA
Lemon Myrtle+ Citric	NA	NA	0.03%+2.5%
Manuka+ Citric	0.03%+0.63%	NA	NA
Mountain savory+ Citric	NA	0.06%+0.63%	0.06%+2.5%
Cassia+ Citric	0.008%+0.63%	NA	NA
Redistilled Oregano+ Citric	0.016%+0.63%	0.016%+0.63%	NA

Cinnamon+ Citric	0.008%+0.63%	0.008%+0.63%	NA
Lemongrass+ Ascorbic	NA	NA	NA
Lemon Myrtle+ Ascorbic	NA	NA	NA
Manuka+ Ascorbic	NA	NA	NA
Mountain savory+ Ascorbic	NA	0.06%+0.63%	0.06%+2.5%
Cassia+ Ascorbic	0.008%+0.63%	NA	NA
Redistilled Oregano+ Ascorbic	NA	0.016%+0.63%	NA
Cinnamon+ Ascorbic	NA	0.008%+0.63%	NA

NA: Oil-acid pairs that did not show microbial inhibition when mixed at their individual sub-inhibitory concentrations, signifying non-synergistic interactions

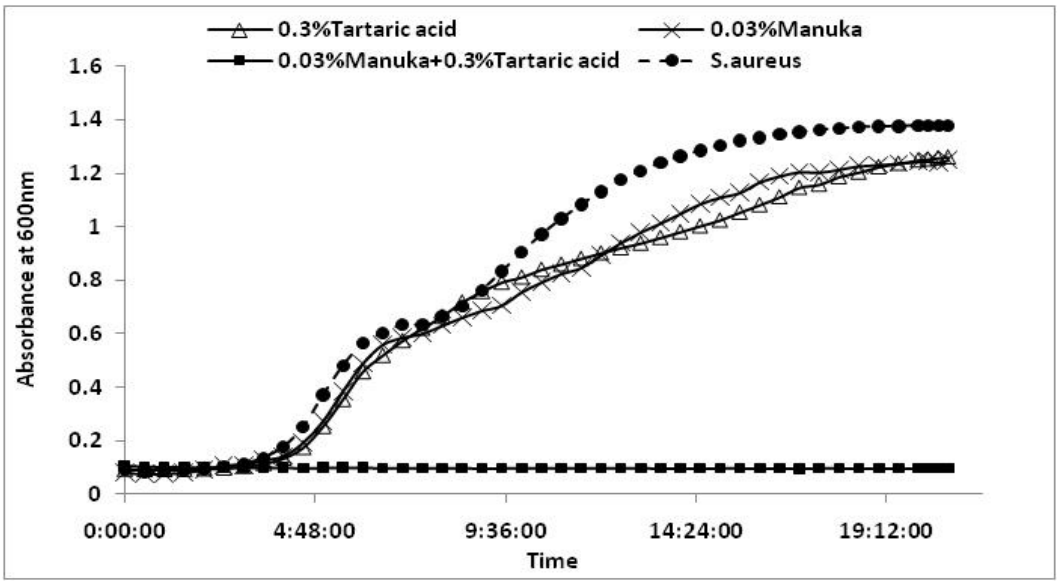

Figure 4. Antimicrobial activities of 0.03% manuka oil, 0.3% tartaric acid, and a mixture of 0.03% manuka oil and 0.3% tartaric acid against *S. aureus* ATCC25923. The cultures were grown in a micro-titer well and absorbance readings were taken using a Bioscreen for 24 h at 30 ℃. All values are means of three replicates

Some other successful EO-acid combinations gave rise to a longer lag phase as compared to their individual activities. For example, when cassia oil at 0.004% and ascorbic acid at 2.5% were used individually, they were not effective in inhibiting *C. albicans*, but when combined they were able to prevent the yeast growth for almost 17h (Fig. 5). Organic acids are known to reduce the pH and cause bleaching and discoloration to food. Among acetic, citric, hydrochloric, lactic, malic, and tartaric acids, Bal'a et al. (1998), found that malic caused the least change in the color of catfish fillets while citric caused the highest. Malic was also more efficient in reducing the cell counts of *Listeria monocytogenes* as compared to citric acid.

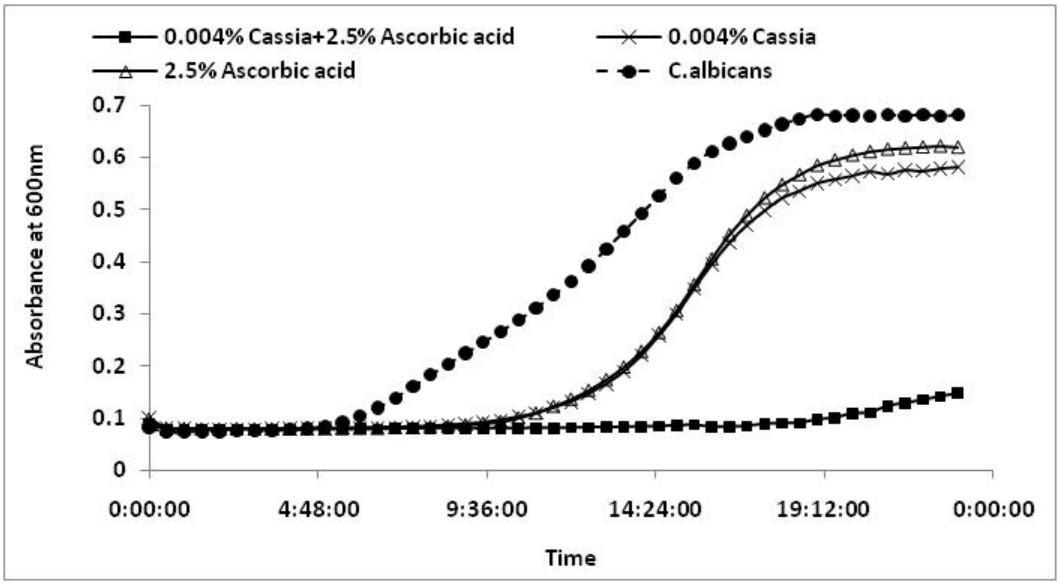

Figure 5. Antimicrobial activities of 0.004% cassia oil, 2.5% ascorbic acid, and a mixture of 0.004% cassia oil and 2.5% ascorbic acid against *C.albicans* ATCC1023. The cultures were grown in a micro-titer well and absorbance readings were taken using a Bioscreen for 24 h at 30 ℃. All values are means of three replicates

6.4.4 Plate counts

Along with the Bioscreen absorbance data, the plate count values also indicated the advantages of hurdle technology wherein the oil-acid antimicrobial mixtures caused significant reduction in the microbial cell counts as compared to the individual antimicrobial components. For example, a starting cell count of 10^5 CFU/mL of *S. aureus* increased to 10^9 CFU/mL after 24 h of incubation in the absence of antimicrobials (control) (Table 5). The presence of 0.06% mountain savory oil decreased the final cell count to 10^7 CFU/mL, while 0.02% tartaric acid reduced the final cell count to10^8 CFU/mL. When *S. aureus* was incubated with a mixture of 0.06% mountain savory and 0.02% tartaric acid, a significant reduction ($p<0.05$) in cell numbers was observed and the final cell count was 10^4 CFU/mL (Table 5).

Table5. Cell counts of *E. coli* 25922, *S. aureus* 25923, and *C. albicans* 10231, determined via dilution and plating, after a 24 h contact time between the test microbes (10^5 CFU/mL) and the individual essential oils, acids and oil-acid pairs. All values are means of three replicates

Essential oil and organic acid pairs	Microbial cell counts (CFU/mL)		
	S. aureus	*E. coli*	*C. albicans*
0.06% Lemongrass	10^8	10^8	10^8
0.03% Lemon Myrtle	10^9	10^9	10^9
0.03% Manuka	10^6	10^9	10^9
0.06% Mountain savory	10^7	10^7	10^7

0004% Cassia	10^7	10^8	10^7
0.016% Redistilled Oregano	10^6	10^6	10^6
0.008% Cinnamon	10^6	10^6	10^6
0.16% Tartaric	10^8	10^7	10^8
0.3% Malic	10^8	10^7	10^8
0.16% (0.005%)[a] Butyric	10^7	10^7	10^6
0.6% Citric	10^8	10^8	10^9
0.6% Ascorbic	10^8	10^8	10^9
0.06% Lemongrass+ 0.16% Tartaric	10^5	10^7	NG
0.03% Lemon Myrtle+ 0.16% Tartaric	10^7	10^7	NG
0.03% Manuka+ 0.16% Tartaric	NG	10^4	10^6
0.06% Mountain savory+ 0.16% Tartaric	10^4	10^3	NG
0008% Cassia+ 0.16% Tartaric	NG	10^7	10^7
0.016% Redistilled Oregano+ 0.16% Tartaric	NG	10^4	NG
0.008% Cinnamon+ 0.16% Tartaric	NG	10^3	NG
0.03% Manuka+ 0.3% Malic	NG	10^5	10^7
0.06% Mountain savory+ 0.3% Malic	10^4	NG	NG
0008% Cassia+ 0.3% Malic	NG	10^7	10^8
0.016% Redistilled Oregano+ 0.3% Malic	NG	NG	10^4
0.008% Cinnamon+ 0.3% Malic	NG	NG	10^3
0.03% Manuka+ 0.16% Butyric	NG	10^7	10^4
0.06% Mountain savory+ 0.16% Butyric	10^2	10^6	NG
0.03% Lemon Myrtle+ 0.6% Citric	10^4	10^5	NG
0.03% Manuka+ 0.6% Citric	NG	10^6	10^5
0.06% Mountain savory+ 0.6% Citric	10^2	NG	NG
0008% Cassia+ 0.6% Citric	NG	10^4	10^3
0.016% Redistilled Oregano+ 0.6%	NG	NG	10^3

Citric			
0.008% Cinnamon+ 0.6% Citric	NG	NG	10^2
0.06% Mountain savory+ 0.6% Ascorbic	10^3	10^4	NG
0008% Cassia+ 0.6% Ascorbic	NG	10^5	10^4
0.016% Redistilled Oregano+ 0.6%Ascorbic	10^6	10^5	10^7
0.008% Cinnamon+ 0.6% Ascorbic	10^6	10^7	10^5

NG: No growth (cells) was recorded

a: 0.005% butyric acid was used in case of *C. albicans*

6.4.5 Effect of pH on the MIC of EOs and Organic acids

The pH of the EOs, organic acids and EO-acid mixtures are shown in Table 6. The organic acids had different pH values and the ones with lower pH did not have the lower MIC values. This indicated that the antimicrobial activity was not solely a pH effect. Butyric acid had a pH of 4.8 and a MIC of 0.3, 0., and 0.01% against *S. aureus, E. coli & C. albicans,* respectively. While malic acid had a lower pH of 3.2 but a higher MIC of 1.25, 0.63, and 1.25% against *S. aureus, E. coli & C. albicans,* respectively. The pH values of the EOs were all around neutral while their MIC values were much lower than those of the acids. The pH of the oil-acid combinations varied between 5 and 6.3. All regulatory-approved organic acids used as antimicrobials in foods have pKa values less than 5.0, which means they are most effective in high-acid foods and vice-versa. (Davidson et al., 2005). In this study, we observed that the pH of the effective EO-acid combinations were 5.5 or higher. An example is a combination of mountain savory (0.06%) and tartaric acid (0.6%). This

system was more potent against *C. albicans* than its individual components (acid or oil) alone, while having a pH of 5.88 – much higher than the pH of 3.5 seen for tartaric acid alone at the same concentration. This definitely provides a great advantage to the safety of the more common low-acid foods, since the same or better antimicrobial efficiency can be maintained via addition of a mixture of oil and acid at much lower concentrations without substantial lowering of pH. In addition, the acids had a pH lowering effect which could result as an additional hurdle to control microbial growth in certain foods.

Table6. pH of the EOs, organic acids, and the synergistic EO-acid mixtures. All values are means of replicates of two

Essential oil/acid	pH	Oil-acid mixtures	pH
0.06% Lemongrass	7.0	0.06% Lemongrass+ 0.16% Tartaric	5.5
0.03% Lemon Myrtle	6.9	0.03% Lemon Myrtle+ 0.16% Tartaric	5.3
0.03% Manuka	7.0	0.03% Manuka+ 0.16% Tartaric	5.6
0.06% Mountain savory	7.1	0.06% Mountain savory+ 0.16% Tartaric	5.9
0.008% Cassia	7.1	0008% Cassia+ 0.16% Tartaric	5.7
0.016% Redistilled Oregano	7.1	0.016% Redistilled Oregano+ 0.16% Tartaric	6.0
0.008% Cinnamon	6.9	0.008% Cinnamon+ 0.16% Tartaric	5.7
0.016% Tartaric	3.5	0.03% Manuka+ 0.3% Malic	5.0
0.3% Malic	3.2	0.06% Mountain savory+ 0.3% Malic	5.1
0.16% Butyric	4.8	0008% Cassia+ 0.3% Malic	5.3
0.6% Citric	3.7	0.016% Redistilled Oregano+ 0.3% Malic	5.0
0.6% Ascorbic	4.3	0.008% Cinnamon+ 0.3% Malic	5.0
		0.03% Manuka+ 0.16% Butyric	6.1
		0.06% Mountain savory+ 0.16% Butyric	6.0

	0.03% Lemon Myrtle+ 0.6% Citric	5.4
	0.03% Manuka+ 0.6% Citric	5.2
	0.06% Mountain savory+ 0.6% Citric	5.1
	0008% Cassia+ 0.6% Citric	5.0
	0.016% Redistilled Oregano+ 0.6% Citric	5.2
	0.008% Cinnamon+ 0.6% Citric	5.1
	0.06% Mountain savory+ 0.6% Ascorbic	5.9
	0008% Cassia+ 0.6% Ascorbic	6.0

6.4.6 FICs

Among the fifty different combinations of oils and acids mixed at their sub-inhibitory levels, a few showed additive and a few showed synergistic behavior. Table 7 gives a summary of the interactions between the oils and the acids. The synergistic oil-acid pairs like cinnamon-citric (effective against *E. coli*), manuka-butyric (*S. aureus*) and lemon myrtle-citric (*C. albicans*) demonstrated that the addition of the respective acids allowed a significantly lower concentration of the EOs to achieve complete microbial inhibition. Similar synergistic interactions between plant oils and organic acids have also been reported by other research groups. Friedly et al. 2009 found significant synergistic antimicrobial properties when citrus EOs were combined with organic acids like citric and malic acids.

Table7. FIC_{index} for the synergistic oil-acid pairs calculated based on their MIC values against *E.coli* ATCC 25922, *S.aureus* ATCC 25923, and *C.albicans* ATCC 10231 determined using the Bioscreen. FIC_{index} near 1 indicates additivity, whereas <1 indicates synergy and >1 indicates antagonism

Essential oil and organic acid pairs	FIC_{index} values				
	Malic	Butyric	Ascorbic	Citric	Tartaric
E.coli					
Mountain savory	0.5	NA	NA	0.5	1.0
Redistilled Oregano	0.5	NA	1.0	0.3	1.0
Cinnamon	0.5	NA	1.0	0.3	NA
S.aureus					
Manuka	0.1	0.5	1.0	0.1	0.3
Cassia	0.3	NA	NA	0.5	0.5
Redistilled Oregano	0.5	NA	1.0	0.5	1.0
Cinnamon	0.5	NA	1.0	0.5	1.0
C.albicans					
Lemongrass	NA	NA	NA	NA	1.0
Lemon Myrtle	NA	NA	NA	0.5	1.0
Mountain savory	1.0	1.0	1.0	1.0	1.0
Redistilled Oregano	NA	NA	NA	NA	1.0
Cinnamon	NA	NA	NA	NA	1.0

NA: Oil-acid pairs that did not show synergistic or additive interaction

6.5 Conclusion

Results from this study demonstrate the higher antimicrobial efficiency of plant essential oil-acids combinations as compared to the individual oils or acids when tested in the MIC and MBC systems described in this study. Synergistic

interaction between the oils and acids was in general observed more against bacteria as compared to the yeast. Malic and citric acid were the most active acids showing synergistic interactions with mountain savory, RO, and cinnamon against *S. aureus* and with cassia, RO, and cinnamon against *E. coli.* The only EO-acid combination which demonstrated synergism against *C. albicans* was lemon myrtle-citric. Among the different pathogenic strains tested, the animal pathogen *R. equi* was the most resistant to the antimicrobial action of the EOs tested. Overall, our results were very promising and establish the use of acid-oil hurdle combinations as food preservatives that would help in lowering the minimum antimicrobial dosage required during preservation with least impact on product quality, or as environmental sanitizers or also as interventions useful in veterinary or clinical applications. The effectiveness of the EOs and organic acid combinations in food matrices depends on many factors such as the protein and fat contents of the food. As a further study, the successful oil-acid pairs should be tested in-vivo in a food system or any other product for their antimicrobial efficacy.

6.6 Acknowledgement

We would like to thank Van Beek Natural Science, Orange City, IA for providing the funding and essential oil samples for this work. The authors would also like to thank Dr. Hyun J. Kim and Dr. Heidi Weinkauf for their help and support.

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CHAPTER 7. GENERAL CONCLUSIONS

7.1 Summary

The research conducted in the course of this dissertation has led to the successful development of novel, low cost processes for the production of value-added, biorenewable chemicals such as nisin and microalgal/fungal-based essential fatty acids.

The *first research problem* involving the high nisin production cost was successfully addressed by demonstrating the advantages of soy whey as an alternative, high-yielding, non-dairy, agro-industrial co-product-based nisin fermentation medium. Nisin concentrations found in soy whey were 619.2 mg/L (or 24,767 IU/ml), as compared to 92.9 mg/L from cheese whey, 88.7 mg/L from cull potatoes and 672 mg/L from de Man-Rogosa-Sharpe (MRS) broth. Soy whey did not require external nutrient supplementation, indicating its high nutritive qualities and potentials for value-addition. Being a byproduct of food processing operations, the nisin produced from this feedstock is not expected to face steep regulatory hurdles for its use in foods. In the simplest example, the entire fermentate containing the soy whey, *L. lactis* and nisin could be concentrated, dried and used as a shelf life extender in foods or also as a value-added animal feed additive. Depending on the processes used, this product could potentially have both probiotic and antimicrobial properties due to the presence of viable *L. lactis* and nisin, respectively. However, soy whey-fermented nisin will face labeling issues since soy is an allergen. Selection

of the food product will also be critical since it could produce undesirable “green” or “beany” flavors.

The *second research problem* involving value-addition to biofuel co-products and development of new media for heterotrophic cultivation of oleaginous algae and fungi was successfully addressed by establishing the nutritive qualities of soy whey and thin stillage in supporting the growth of high-lipid containing cells of *Chlorella vulgaris* and *Mucor circinelloides.* The fungal study highlighted the potential of *M. circinelloides* in adsorbing/assimilating oil and nutrients when cultivated on thin stillage (TS), and producing lipid and protein-rich fungal biomass. A biomass yield of 20 g/L (db), with a lipid content of 46% (dwb) was obtained from TS while the synthetic medium, YM broth gave 5 g/L (db) of biomass containing 20% (dwb) lipids. The polyunsaturated fatty acids (PUFAs) in TS-derived fungal oil were 52% of total lipids and comprised of both omega 3 (C 18:3) and omega 6 (C 18:2) essential fatty acids. This indicated dual application of the fungal oil as either a biodiesel feedstock or a source of neutraceuticals (PUFAs). Thus, fungal cultivation on TS produced a high-protein animal feed and high-value fungal oil, thus improving corn-ethanol process economics.

High density microalgal cell growth was observed in both TS and SW with the highest biomass and lipid yields obtained from cells grown on TS (9.8, 4.2 g/L). The microalgal oil samples differed in their fatty acid composition based on the growth substrate composition. Most importantly, the PUFA content in TS (56%) and SW-derived (38%) algal oil was higher than MBM-derived (31%). The use of ultrasonics

with toluene/methanol solvent system improved lipid extraction efficiency in terms of time and solvent use. Thus, this study demonstrated the use of inexpensive and readily available agro-industrial co-products as mixotrophic microalgal nutrient feedstock that could reduce the need for fresh water and external nutrients.

Certain trends in the research outcome were found to be similar between the fungal and the algal studies. Both fungal oil and algal oil characteristics (fatty acid composition) were found to vary dependant on the growth media properties. When the microbes were grown on synthetic media, the saturated and monounsaturated fatty acid contents (C16:0, C18:0, C18:1 in Chlorella-MBM_{oil} ; C13:0, C14:0, C16:0, C16:1, C18:0, C18:1 in Mucor-YM_{oil}) in their cellular lipids were found to be higher than PUFAs (C18:2, C18:3); whereas, when they were grown on the high-organic co-product streams (TS and SW) the microbial lipids had a higher content of PUFAs. In addition, Mucor-TS_{oil} contained notably higher free fatty acids (38% of total lipids) as opposed to 4% in Mucor-YM_{oil}; while the triacylglycerol (TAG) content in Mucor-YM_{oil} (34%) was more than double than in Mucor-TS_{oil} (15%). All these observations indicated that the characteristics of TS/SW-derived algal oil and TS-derived fungal oil are better suited as raw material for high-value essential fatty acids production rather than as a biodiesel feedstock.

Economic considerations

SW-derived nisin: The daily volume of soybean whey production in a typical soy processing plant is ~ 4 MGD (Personal communication with Kerry Biosciences). The average nisin yield obtained from lab-based soy whey was 0.62g/L. Therefore,

assuming similar yields from commercial-grade soy whey, the total nisin production would be ~ 9 t/day. Based on a bulk supply rate from a Chinese producer, the cost of nisin is $50 per kg. At this rate, the revenue earned per day would be ~ $450,000, which would lead to an annual revenue generation of $164 million.

Fungal-based biodiesel: A 110 MGY corn-ethanol plant would produce around 330 MGY of thin stillage based on 6 gal TS/gal ethanol and a 50% backset (Rasmussen et al., in press). Laboratory test results indicate a Mucor biomass yield of 20 g (db) and an oil yield of 9.2 g/L of TS. This would lead to a production of 25,080 t/yr of Mucor biomass and 11,537 t/yr of fungal oil. On a volume basis, oil production would be ~ 3.1×10^6 gal/yr (assuming 890 kg/m^3 density). In a base catalyzed transesterification process of oil conversion to biodiesel, 98% conversion efficiency is usually observed (National Biodiesel Board, 2006). Therefore, assuming a cost of $2/gal of biodiesel (www.biodiesel.org), fungal-based biodiesel should generate a potential income of ~6.6 million. The left-over fungal biomass could also be sold as animal feed after the required toxicity testing. Assuming 10 g/L of residual biomass is available for feed applications, total production per year would be ~ 12, 540 t/yr. This could generate additional revenue of ~$ 2.8 million (assuming $225/ton based on Rasmussen, 2009).

Algal-based Biodiesel: Laboratory research data indicate algal biomass yields of 9.8 g/L of TS and 6.3 g/L of SW; and algal oil yields of 4.2 g of oil/L of TS and 0.7 g/L of SW. Based on above mentioned TS and SW generation rates, algal oil production could be estimated to be 5267 t/yr from TS and 3873 t/yr from SW. Or,

1.4 $\times 10^6$ ga/yr from TS and 1.1×10^6 ga/yr from SW. Assuming the same oil to biodiesel conversion efficiencies and biodiesel price, as considered above, an annual potential revenue generation of ~ $ 2.7 million and $2.2 million can be estimated from the sale of TS-based and SW-based algal biodiesel, respectively. Residual algal biomass can also be sold as animal feed similar to fungal biomass or used for further energy production via thermo-chemical pathways.

Fungal and algal-based polyunsaturated fatty acids (PUFAs): Both fungal oil and algal oil samples were found to contain significant quantities of PUFAs which have high economical value when purified and sold as neutraceuticals. Therefore, a further study aimed at optimization of PUFA production and detailed chemical analysis of their classes and types is required.

And finally, the *third research problem* involving limited application of plant essential oils as antimicrobials in food and feed due to alterations in end-product quality was addressed by successful demonstrations of higher antimicrobial efficiency by plant essential oil-organic acid combinations, as compared to the individual oils or acids tested in the MIC and MBC systems. Among the 35 different EO-acid pairs tested, synergism was found between 16 pairs, while the rest were additive or antagonistic. Interestingly, synergistic interaction between the oils and acids was in general observed more against bacteria, specifically Gram positive, as compared to the yeast. Gram negative bacteria were more resistant due to their outer lipopolysaccharide membrane which is responsible for their higher

pathogenicity. In case of the yeast, *C. albicans,* the only inhibitory organic acid was citric which showed synergistic behavior with lemon myrtle oil. The effectiveness of citric acid against all three pathogens could be due to its chelating properties, which may have enhanced the EO antimicrobial activity by helping it to permeabilize the cell membranes and lyse the cells. Overall, our results were very promising and successful in establishing several synergistic acid-oil pairs which could be used in hurdle combinations in food or feed, lowering the required antimicrobial dosage and rendering the least impact on product quality.

7.2 Recommendations for Future Research

Based on the promising results of this study, future work should be performed, focusing on the use of commercial soy whey and on scaled-up production of soy-fermented nisin to industrially relevant levels. Additional monitoring of *L. lactis* gene expression (*nisP*) during growth on soy whey could also enable us to more fully characterize this substrate as a medium for nisin production. In addition, the entire soy whey fermentate containing nisin could be added to a suitable food product preferably soy-based and a sensory analysis as well as shelf life study performed.

It has been established beyond doubt that in order to decrease production costs of nisin and expand its application for improved food safety, finding an inexpensive yet nutritionally rich, non-dairy and non-allergen-based medium is the key. In the quest to identify new substrates, the primary concerns are toxicity,

reliability of supply and consistency in quality of such feedstocks. Harder economic times and stricter environmental norms have compelled the food processing and agri-based companies to find new ways to add value to their byproducts and effluents before disposal. Using them as substrates for nisin fermentation definitely holds promise and therefore further search towards finding such suitable alternative media and exploration of their commercialization potentials should be encouraged.

TS-derived algal and fungal biomass was found rich in lipids and also contained significant amounts of protein and fiber, thus holding promise as a nutritious animal feed or feed supplement. Hence as a future study, toxicity testing of the biomass after lipid extraction should be performed along with feeding trials with the residual biomass. The algal/fungal-derived fatty acids were found to vary in composition dependant on the growth media characteristics. Therefore, another future study which holds potential would involve media optimization for enhanced production of omega-3 and omega-6 fatty acids specifically due to their increasing demands as high-value neutraceuticals.

Finally, the effectiveness of the EOs and organic acid combinations in food matrices depends on many factors such as the protein and fat contents of the food. As a further study, the successful oil-acid pairs should be tested in-vivo in a food system or any other product for their antimicrobial efficacy.

APPENDIX A

ANTIMICROBIAL ACTIVITY OF AQUEOUS CATIONIC POLYURETHANE DISPERSIONS (PUDs) AGAINST *SALMONELLA TYPHIMURIUM* AND *LISTERIA MONOCYTOGENES*

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A paper to be submitted to *Journal of Progress in Organic Coatings*

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Abstract: Environmentally-friendly, vegetable oil-based, cationic PUDs with excellent coating properties were successfully synthesized from various vegetable oil-based polyols. In this study, we tested the potentials of these PUD samples for their antimicrobial properties, both in the liquid dispersion as well as film forms, against two very difficult to control food-borne pathogens, *Listeria monocytogenes* 2045 and *Salmonella typhimurium* 13311. PUD 5 showed wide spectrum activity and was potent against both Gram positive and Gram negative bacteria; while PUD 7

was group-specific in its action being most active against *L. monocytogenes* and having negligible activity against *S. typhimurium*. The presence of acrylic acid in PUD 7 and acetic acid in PUD 5 could have influenced the antimicrobial nature of the two PUDs. Higher inhibitory activity by PUDs 4 and 5 as compared to PUDs 1, 2, and 3 could be a result of increased relative amounts of aminium ions (substituted N cations). The varying numbers of equivalents of acid used (1.5 to 5) to neutralize the PUD samples was another factor that could have contributed to the varying antimicrobial efficiencies observed in the PUD samples. PUDs 5 and 7 also proved cidal in action. They were able to completely kill *S. typhimurium* and *L. monocytogenes* cell suspensions at an initial cell count of 10^6 CFU/mL, in 48 and 12 h respectively. The antimicrobial activity of the PUDs was not found to be a surface phenomenon. The active components were able to diffuse into the surrounding medium (TSB in this case) and cause growth inhibition. PUD 8, which was the anionic PUD sample and used in this study as a control was not found to be antimicrobial in nature.

Keywords: Cationic polyurethane dispersions, antimicrobial activity, *Salmonella typhimurium, Listeria monocytogenes*

1.0 Introduction

The polymers which have a principle chain structure made of rigid hard and flexible soft segments are known as polyurethanes (Szycher, 1999). Due to their unique and favorable properties, they are widely used as adhesives, coatings, paints

etc. (Howard, 2002). However, conventional PU products are solvent-based and also contain free isocyanate monomers which are both toxic and environmentally-unfriendly (Kim et al., 2005; Lu et al., 2004). Due to increasing restrictions on the emission of volatile organic compounds and hazardous air pollutants into the atmosphere, the PUs are seeing decreasing popularity and being replaced by aqueous PUDs. PUDs are a binary colloidal system made of PU particles dispersed in water (Ahn et al., 207).

The past few years have seen considerable research being done to investigate different agricultural commodities from the agro/food industries as raw material for the synthesis of PUs (Lu and Larock, 2010a; Campos et al., 2011). Vegetable oil is a commonly used renewable raw material with environment friendly properties and other advantages like biodegradability, low toxicity, and comparatively low prices (Lu and Larock, 2009). Anionic PUDs have been successfully prepared in the past using 50-70% vegetable oil polyols (Lu and Larock, 2008). Cationic PUDs based on vegetable oils, have been found to exhibit excellent adhesion properties (Sundar et al., 2006; Lu and Larock, 2010a and b). Thermo-chemical property analyses of these PUDs have demonstrated the potential of these coatings as possible replacements for petroleum-based latexes, thus leading to waste minimization, pollution control and the conservation of our dwindling petroleum reserves. Unlike the anionic PUDs, coatings prepared from cationic PUDs have also demonstrated some antimicrobial properties due to the inclusion of antiseptic chain extenders. Their inherent positive charge has presumably enabled them to interact with and permeabilize bacterial cell membranes (Chen et al., 1998).

Environmentally-friendly, vegetable oil-based, cationic PUDs with excellent coating properties were successfully synthesized from various vegetable oil-based polyols by a team led by Dr. Richard C. Larock of the Chemistry department at ISU. In this study, our aim is to test the potentials of these PUD samples for their antimicrobial properties, both in the liquid dispersion as well as film forms, against two very difficult to control food-borne pathogens, *Listeria monocytogenes* and *Salmonella typhimurium.* These bacterial strains are among the most common causative agents of food-borne illnesses (Friedly et al., 2010; Zhang et al., 2011). Their potential antimicrobial properties (if any) could widen their application base and be used as value-added food packaging material.

2.0 Materials and Methods

2.1 Microbial strains and media

The test cultures, *Salmonella typhimurium* ATCC13311 and *Listeria monocytogenes* NADC 2045 were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). *S. typhimurium* and *L. monocytogenes* were maintained as frozen stocks at −75 ℃ in TSB (BD Diagnostics Systems, Sparks, MD) containing 20% (v/v) glycerol. Working cultures of *S. typhimurium* and *L. monocytogenes* were maintained as slants on TSA.

2.2 Inoculum preparation

Salmonella typhimurium and *Listeria monocytogenes* were grown in TSB for 18h at 35℃. The cell density was adjusted to 0.1 absorbance at 600nm (Abs600) which

corresponded to a cell count of 10^8 CFU/mL. The suspension was diluted to 10^6 CFU/mL using TSB.

2.3 PUD synthesis

The PUD samples were prepared by the Larock laboratory in the department of chemistry, ISU. Soybean oil was purchased at the local supermarket and used directly. Methoxylated soybean polyols (MSOL) with hydroxyl numbers of 155 and 140 mg KOH/g were synthesized as previously reported (Lu and Larock, 2008). Acrylic acid, dimethylolpropionic acid (DMPA), *N*-methyl diethanol amine (NMDA), and isophorone diisocyanate (IPDI) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Glacial acetic acid and methyl ethyl ketone (MEK) were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). All materials were used as received.

The IPDI, MSOL, and NMDA were added to a three-necked flask equipped with a mechanical stirrer, condenser, and thermometer. The molar ratio of NCO groups from IPDI was varied from 2.0 to 2.75. The molar ratio of OH groups from MSOL was kept constant at 1.0, while the molar ratio of OH groups from NMDA varied from 0.95 to 1.7 (corresponding to the NCO molar ratio of IPDI). The reaction was first carried out at 80 ℃ for 10 minutes and then MEK (50 wt% based on the reactant) was added to reduce the viscosity. After 2 h reaction, the reactants were then cooled to room temperature and neutralized by the addition of acid (1.5 to 5 equivalents of acetic acid or 5 equivalents acrylic acid, respectively), followed by dispersing at high speed with distilled water to produce the cationic PUD with a solid

content of about 12.5 wt. % after removal of the MEK under vacuum. The corresponding PU films were obtained by drying the resulting dispersions at room temperature in polystyrene petri dishes. As a control, a vegetable oil-based anionic dispersion was prepared using DMPA as previously reported (Lu and Larock, 2008). The molar ratios for the anionic PUD control were 2.0 NCO from IPDI, 1.0 OH from MSOL, and 0.95 OH from DMPA. The nomenclature used for the various PUD samples and their composition is enlisted in Table 1. Figure 1 shows the PUD samples 5 and 7.

Table 1 Nomenclature of the PUD samples and their composition

PUD sample	Composition	% Total solids
1	2.0 IPDI NCO:1.0 MSOL-155 OH : 0.95 NMDA OH 1:1 MEK, 5x AcOH	12.9
2	2.0 IPDI NCO:1.0 MSOL-155 OH : 0.95 NMDA OH 10g MSOL – 155, 1:1 MEK,3x AcOH	13.9
3	2.0 IPDI NCO:1.0 MSOL-140 OH : 0.95 NMDA OH 1:1 MEK, 1.5x AcOH	14.7
4	2.47 IPDI NCO:1.0 MSOL-155 OH:1:45 NMDA OH 10g MSOL-155, 1:1 MEK, 2x AcOH	13.2
5	2.75 IPDI NCO:1.0 MSOL-155 OH : 1.7 NMDA OH 7.5g MSOL-155, 1:1.25 (PU: MEK), 1.5x AcOH	12.2
6	2.0 IPDI NCO:1.0 MSOL-155 OH : 0.95 NMDA OH 10g MSOL-155,1:1 MEK, 1.5x AcOH	14.2

7	2.0 IPDI NCO:1.0 MSOL-155 OH : 0.95 NMDA OH 1:1 MEK, 5x Acrylic acid	12.4
8 (Control)	2.0 IPDI NCO:1.0 MSOL-140 OH : 0.95 DMPA OH 1:1 MEK, 1.5x TEA	11.0

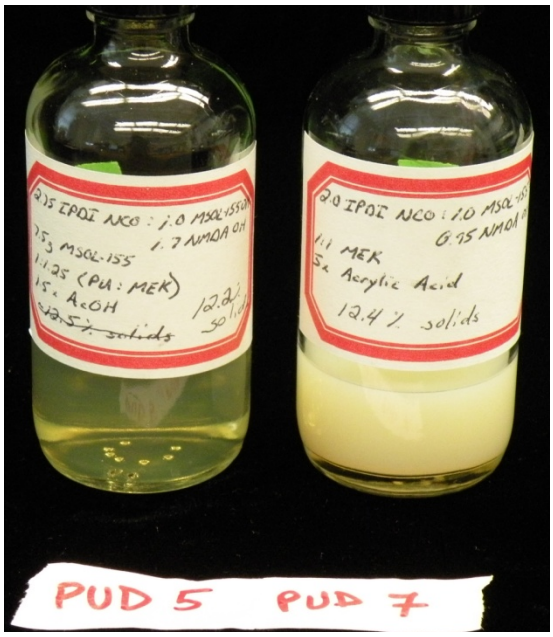

Figure1. Polyurethane dispersion samples No.5 and 7

2.4 Antimicrobial testing of the PUDs

2.4.1 Agar Disk Diffusion assay

The base agar plates with TSA were prepared by pouring 15 mL of sterile molten TSA onto the petri-plates and allowing them to solidify at room temperature. To 9 mL of tempered TSA overlay agar (0.7% agar) in polypropylene tubes, 1 mL of the test bacterial suspension (10^6 CFU/mL) was added, vortexed and poured over the base plates. The final bacterial cell count on the plates was 10^5 CFU/mL. After the overlay was allowed to harden, 20 µL of the PUD suspensions was added to

paper discs and placed on the overlay agar. As a control to check for pH-based zone of inhibition (ZOI) formation, a 2% solution of citric acid at pH 5 was added to one disc. This particular pH was chosen to mimic the pH of the PUD samples which were all at a pH ~5. The second control was PUD 8. The plates were kept at 4 ℃ for 2 hours to allow diffusion of the PUDs through the agar, and then incubated at 35 ℃ for 24 h to allow growth of the test cultures. The ZOI formed around the disc due to the inhibitory action of the PUDs against the test cultures, was calculated by measuring the diameter (in mm).

2.4.2 Agar Film Diffusion assay

Films were prepared from PUD 5, 7 and 8 by pouring 20 mL of the dispersions on 150 mm glass agar plates and air-drying them in a bio-safety cabinet for 12 h. The dried films were peeled off and divided into smaller pieces each weighing about 0.2-0.23 g. The base agar plates with TSA were prepared by pouring 15 mL of sterile molten TSA onto the petri-plates and allowing them to solidify at room temperature. To 9 mL of tempered TSA overlay agar (0.7% agar) in polypropylene tubes, 1 mL of the test bacterial suspension (10^6 CFU/mL) was added, vortexed and poured over the base plates. The final bacterial cell count on the plates was 10^5 CFU/mL. After the overlay was allowed to harden, the film pieces were placed on top of the overlay agar. PUD 8 film was used as the control. Plates were kept at 4℃ for 2 hours to allow diffusion of the PUDs through the agar, and then incubated at 35℃ for 24h to allow growth of th e test cultures.

2.4.3 Determination of surface-active vs. diffusible antimicrobial activity of the PUD films

The active components of the PUDs could be present only on the film surface giving rise to an otherwise inert film with only surface-active antimicrobial activity. Furthermore, these active components could be diffusible in nature, leach out into the surrounding medium and exert its inhibitory action at the suspension level. In order to test the antimicrobial mode of action of these films, 200 µL each of PUDs 5, 7 and 8 were added to 5 mL sterile glass tubes and allowed to dry overnight in order to form a film on the base. To the coated and dried tubes, 3 mL of TSB was added. Equal volume of TSB was also added to the control tubes which had no coating. All tubes were kept in a 35℃ incubator for 3 days to allo w any diffusible components of the film to disperse into the TSB medium. The treated and untreated TSB samples from the tubes with and without the coatings, respectively, were then used as growth media and tested in the Bioscreen. The TSB samples in the Bioscreen plate were inoculated with the test cultures, *L. monocytogenes* and *S. typhimurium* (10^5 CFU/mL). The micro-titer plates were incubated for 24 h at 35 ℃ in the Bioscreen and absorbance readings were recorded every 15 min. As a second control, normal TSB without the time-temperature treatment was also tested in parallel in the Bioscreen.

2.4.4 Time course plating

In order to determine the potency of the PUD samples, a time-kill experiment was done by measuring the cell counts of the test microbes in the presence of PUDs

5, 7 and 8 by time-course plating. *S. typhimurium* and *L. monocytogenes* inocula were prepared as mentioned before to get an initial cell density of 10^6 CFU/mL. Six hundred microliters of each of the PUDs 5, 7 & 8 were added to separate sterile glass tubes and allowed to air dry in the hood. The inactive PUD 8 was the control. To the coated and dried tubes with PUDs 5 and 8, 3 mL of *S. typhimurium* suspension (10^8 CFU/mL) was added, and to tubes with PUDs 7 and 8, 3 mL of *L. monocytogenes* suspension (10^8 CFU/mL) was added. The second set of control tubes did not have any coating and contained the test cell suspensions only. All the tubes were incubated at 35℃. Samples were taken at d ifferent time intervals and cell counts were measured by the spread-plate technique.

2.4.5 Anti-biofilm activity of PUD 4 against L. monocytogenes

A distinct area on the slide was marked and 20 µL of PUD 4 was added to the marked area and air-dried in the bio-safety cabinet. The control slide did not have any coating. The CoverWell perfusion chambers (model PC1R-2.0; Grace Bio-Labs, Bend, OR) were placed on the test slide enclosing the coated area and on the control slide. *L. monocytogenes* inoculum was prepared as mentioned before having a cell count of 10^8 CFU/mL. Using a sterile, glass Pasteur pipette and a rubber bulb, 300 µL of *L. monocytogenes* cell suspension were added to the chamber. Perfusion chamber inlet ports were sealed using transparent adhesive tape. The flexible silicone base of the perfusion chamber formed a water-tight cell-suspension containing compartment. The slides with the chambers were placed in petri-plates and incubated at 37℃ for 3 days to allow biofilm for mation. After 3 days, the

suspension was pipetted out and the slides were washed with phosphate buffered saline (PBS) and dried. The cells were stained with crystal violet, mounted with a cover-slip and examined using a Leitz LaborLux S microscope equipped with a Canon PowerShot A640 consumer-grade digital camera controlled by Axiovision software (v. 4.6; Carl Zeiss Microimaging, Inc.,Thornwood, NY). Biofilm formation/prevention was noted and photographed under 45×10 x magnification).

3.0 Results and Discussions

3.1 Agar Disk Diffusion assay

All the PUDs were found to have inhibitory activity against both *S. typhimurium* and *L. monocytogenes.* The largest zones of inhibition against *S. typhimurium* were 11.9 and 11.5 mm shown by PUD 4 and 5, respectively. While, *L. monocytogenes* was most susceptible to PUDs 4 and 7 with zones of inhibition of 13.4 and 15.7 mm, respectively. PUD 8 was not active against either *S. typhimurium* or *L. monocytogenes* and showed no visible inhibition zone. Citric acid, the second control, was also inactive (no ZOI) against both *S. typhimurium* and *L. monocytogenes.* The antimicrobial activity of the PUDs against the two test cultures, represented by the size of the zones of inhibition is shown in Table 2, and figures 2 and 3. Figure 4 demonstrates a comparison in inhibitory activity between PUDs 5 and 8 and PUDs 7 and 8 when tested against *S. typhimurium* and *L. monocytogenes,* respectively. Figure 5 demonstrates a comparison in inhibitory activity between PUD 5 and citric acid and between PUD 7 and citric acid against *S. typhimurium* and *L. monocytogenes,* respectively. The antimicrobial activity of a few

PUDs was group-specific. PUD 7 was the most active dispersion against Gram positive *L. monocytogenes* while it showed least activity among the PUDs against Gram negative *S. typhimurium* (Fig. 6). On the other hand, PUD 5 was equally active against both the groups of test cultures (Fig. 7).

The main difference in the synthesis of PUDs 5 and 7 was the presence of acrylic acid in 7 and acetic acid in 5 which could have influenced the antimicrobial nature of the two PUDs. Higher inhibitory activity by PUDs 4 and 5 as compared to PUDs 1, 2, and 3 could be a result of increased relative amounts of aminium ions (substituted N cations). The varying numbers of equivalents of acid used (1.5 to 5) to neutralize the PUD samples was another factor that could have contributed to the varying antimicrobial efficiencies observed in the PUD samples.

Table 2 Antimicrobial activity of the PUDs against *L. monocytogenes* 2045 and *S. typhimurium* 13311 represented by the zones of inhibition (in mm) and measured using the agar disc assay. All data are expressed as means ± SD for three replicates

Polyurethane dispersions (PUDs)	*S. typhimurium* 13311 ZOI (diameter in mm)	*L. monocytogenes* 2045 ZOI (diameter in mm)
PUD 1	9.6 ± 0.3	10.4 ± 0.3
PUD 2	9.8 ± 0.4	11.2 ± 0.2

PUD 3	10.6 ± 0.3	11.8 ± 0.4
PUD 4	11.9 ± 0.4	13.4 ± 0.3
PUD 5	11.5 ± 0.4	12.2 ± 0.3
PUD 6	9.8 ± 0.2	10.8 ± 0.2
PUD 7	9.3 ± 0.2	15.7 ± 0.3
PUD 8	No zone	No zone
2% Citric acid	No zone	No zone

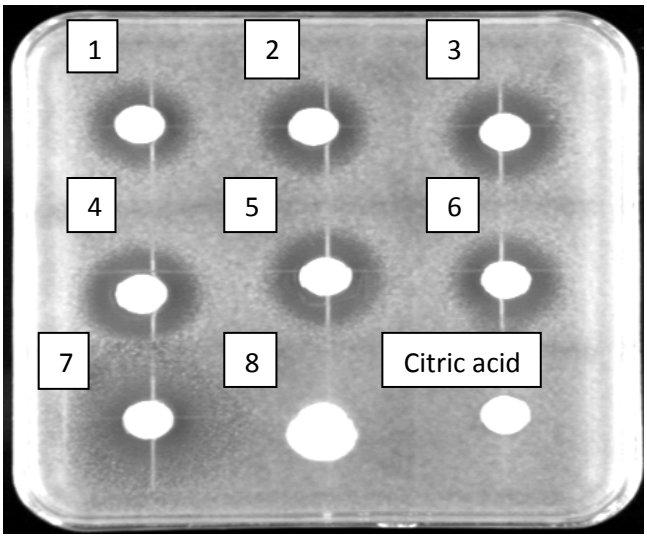

Fig. 2 Antimicrobial activity of PUDs 1-8 against *L. monocytogenes* 2045 (10^5 CFU/mL), represented by the zones of inhibition around the paper discs impregnated with 20 µL of the polyurethane dispersions

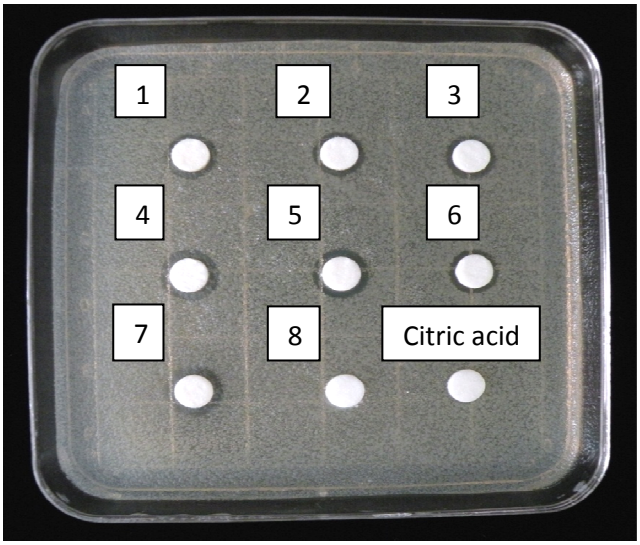

Fig. 3 Antimicrobial activity of PUDs 1-8 against *S. typhimurium* 13311 (10^5 CFU/mL), represented by the zones of inhibition around the paper discs impregnated with 20 µL of the polyurethane dispersions

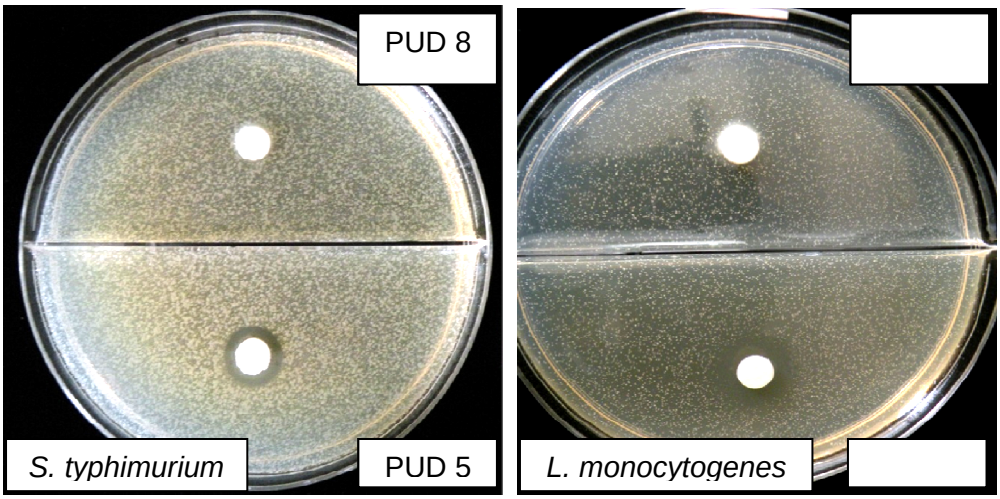

Fig. 4 Inhibitory activity of PUDs 5 and 8 and PUDs 7 and 8, against *S. typhimurium* 13311 and *L. monocytogenes* 2045 (10^5 CFU/mL) as tested by the agar disc assay. The paper discs were impregnated with 20 µL of the polyurethane dispersions

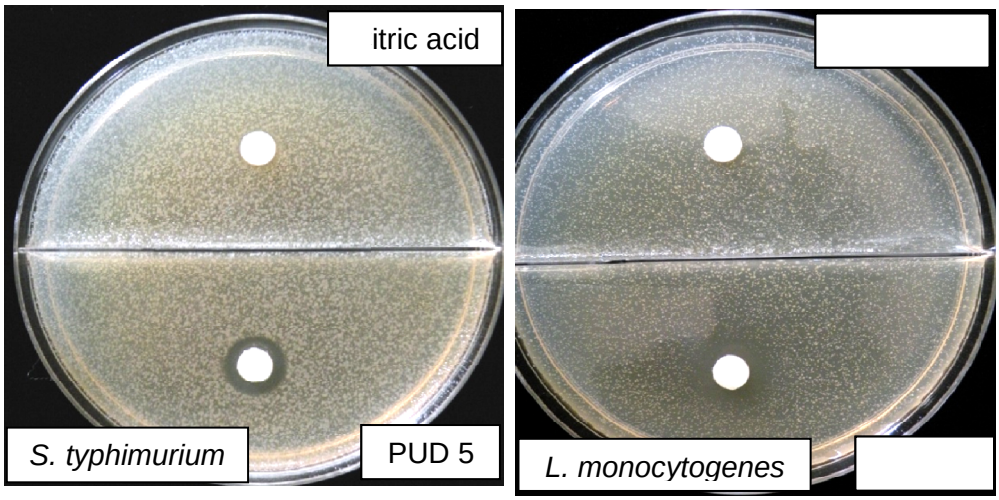

Fig. 5 Inhibitory activity of PUD 5 and citric acid and PUD 7 and citric acid against *S. typhimurium* 13311 and *L. monocytogenes* 2045 (10^5 CFU/mL) as tested by the agar disc assay. The paper discs were impregnated with 20 µL of the polyurethane dispersions

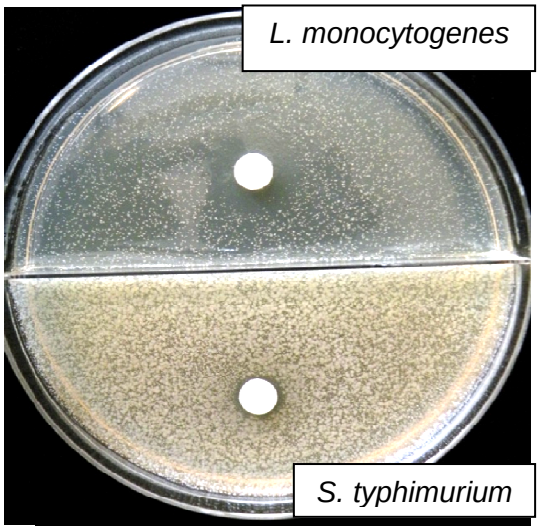

Fig. 6 Inhibitory activity of PUD 7 against *S. typhimurium* 13311 and *L. monocytogenes* 2045 (10^5 CFU/mL) as tested by the agar disc assay. The paper discs were impregnated with 20 µL of the polyurethane dispersions

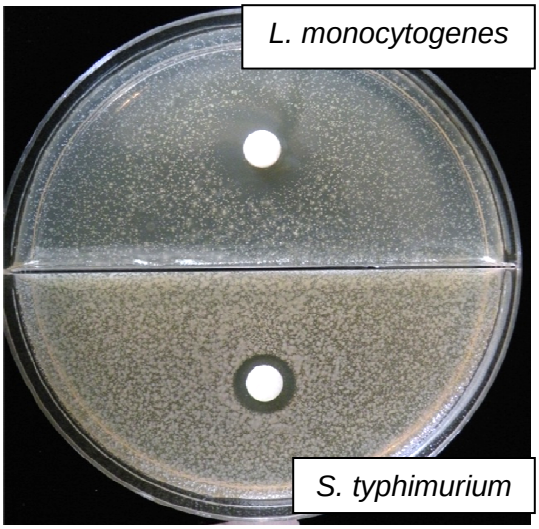

Fig. 7 Inhibitory activity of PUD 5 against *S. typhimurium* 13311 and *L. monocytogenes* 2045 (10^5 CFU/mL) as tested by the agar disc assay. The paper discs were impregnated with 20 µL of the polyurethane dispersions

3.2 Agar Film Diffusion assay

Similar antimicrobial activity results were obtained with the film forms of the PUDs against the two test cultures, as observed with the liquid state of the dispersions. PUD 7 was more active against *L. monocytogenes* while PUD 5 was reactive against both *L. monocytogenes* and *S. typhimurium.* In order to make the right comparison, the ZOI was calculated by subtracting the diameter of the film from the outer diameter including the zone. The zones of inhibition formed by PUD 5 and 7 against *L. monocytogenes* were 8 and 22 mm, respectively. While zones formed by PUD 5 and 7 against *S. typhimurium* were 19 and 6 mm, respectively (Fig. 8).

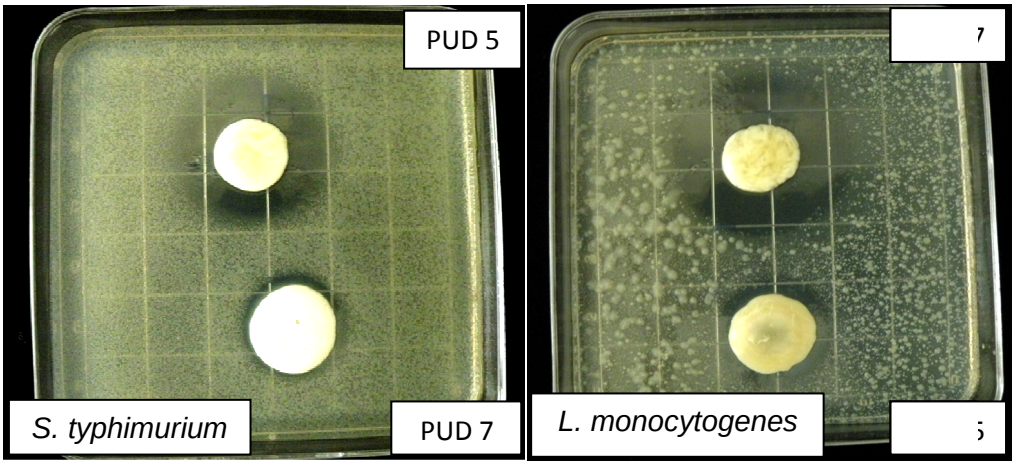

Fig. 8 Inhibitory activity of PUD films 5 and 7 against both *S. typhimurium* 13311 and *L. monocytogenes* 2045 (10^5 CFU/mL) as tested by the agar film assay. The films were made by pouring 20 mL of the dispersions onto 150 mm glass petri plates, air-drying and cutting pieces of films weighing about 0.2 - 0.23 g

3.3 Surface-active vs. diffusible properties of antimicrobial activity of the PUD films

Figure 9 shows a glass tube coated on the base with PUD 5 and filled with TSB. When TSB was kept in contact with PUDs 5 and 7 and used as growth medium for the test cultures in the Bioscreen, it was unable to support the growth of the test pathogens as demonstrated in Figures 10a and b. Normal, untreated TSB used as a control was able to support excellent growth of both the test strains. The other control was TSB treated with the un-reactive PUD 8, and normal growth of the cultures was found in this case too (Fig.10c). These observations proved that the active components of the potent PUDs 5 and 7 were able to diffuse into the TSB when sufficient contact time was allowed and were able to inhibit microbial growth. Time-temperature treated TSB was found to undergo certain degree of nutrient

denaturation represented by the lower final cell numbers in case of *L. monocytogenes* and a longer lag period in case of *S. typhimurium* (Figs. 10a and b).

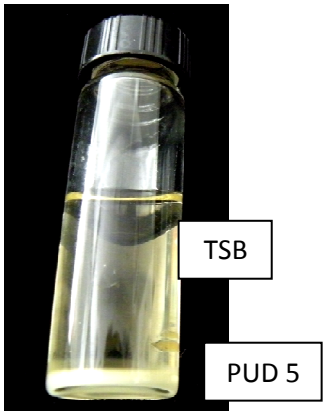

Fig. 9 Sterile glass bottle with PUD 5 coating on the base and 3 mL TSB which was kept in contact with the PUD coating for 5 days and later tested in the Bioscreen as a growth medium for *S. typhimurium* 13311

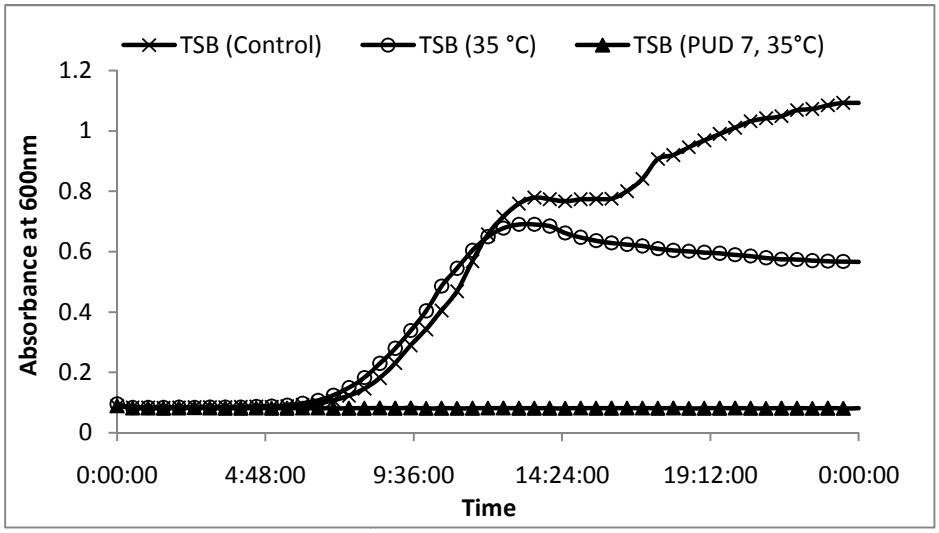

Fig. 10a Growth of *L. monocytogenes* 2045 in untreated TSB broth, time and temperature treated (35 ℃) TSB broth, and PUD 7 tr eated TSB broth, measured in terms of absorbance at 600 nm in a Bioscreen

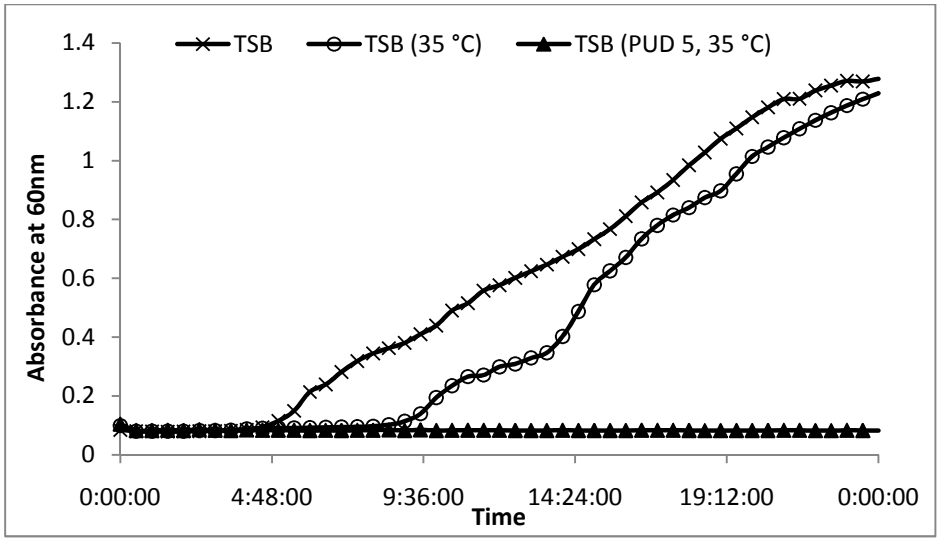

Fig. 10b Growth of *S. typhimurium* 13311 in untreated TSB broth, time and temperature treated (35 ℃) TSB broth, and PUD 5 tr eated TSB broth, measured in terms of absorbance at 600 nm in a Bioscreen

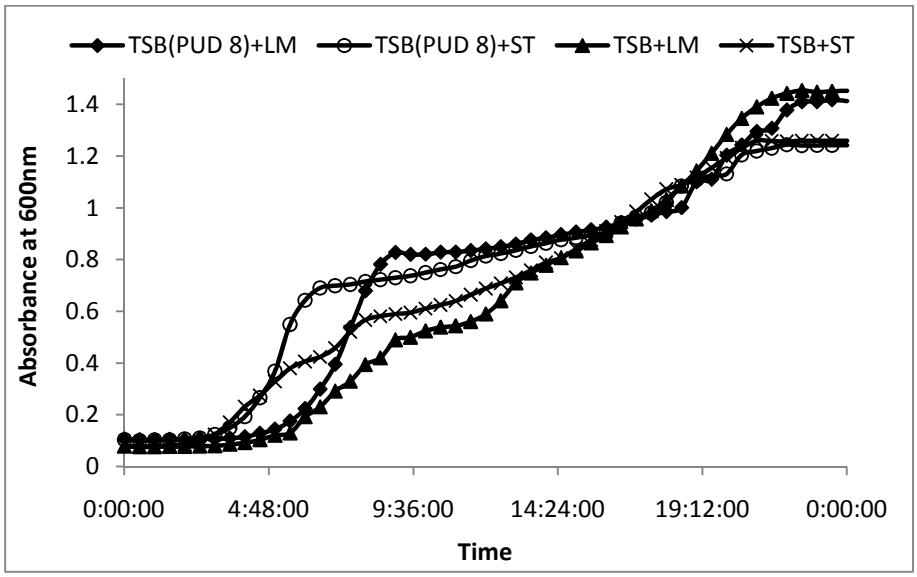

Fig. 10c Growth of *S. typhimurium* 13311 (ST) and *L. monocytogenes* 2045 (LM) in untreated TSB broth, and PUD 8 treated TSB broth, measured in terms of absorbance at 600 nm in a Bioscreen

3.4 Time course plating

PUDs 5 and 7 were able to completely kill *S. typhimurium* and *L. monocytogenes* cell suspensions in 48 and 12 h respectively (Figs. 11a and b). In presence of PUD 5, the initial cell count of 10^6 CFU/mL of *S. typhimurium* was reduced by three logs (10^3) in 12 h and by six logs (10^6) in 48 h. While in presence of PUD 7, the initial cell count of 10^6 CFU/mL of *L. monocytogenes* was reduced by three logs (10^3) in 7 h and by six logs (10^6) in 12 h (Table 2). The control tubes (without any coating) as well as tubes with PUD 8 showed normal logarithmic increase in bacterial cell counts over time with the initial count of 10^6 CFU/mL increasing to 10^9 CFU/mL in 48 h (Fig. 11c).

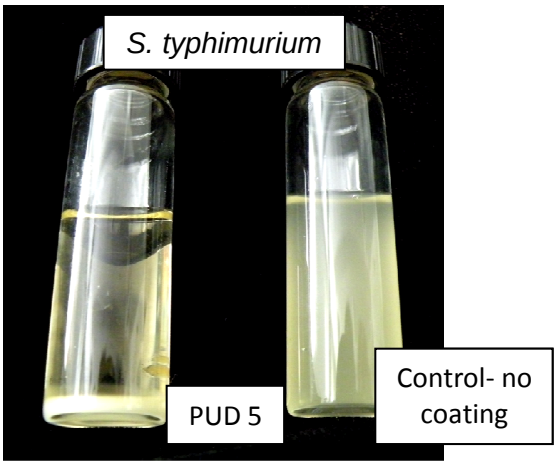

Fig. 11a Sterile glass tube with PUD 5 coating on the base (left) and control tube with no coating (right), containing 3 mL of *S. typhimurium* 13311 cell suspension (10^6 CFU/mL) and after 48 h of incubation. The coated tube showed a complete kill of the test pathogen cells noted by the transparency of the medium and plate counts, while the control tube showed a turbid cell suspension indicating growth

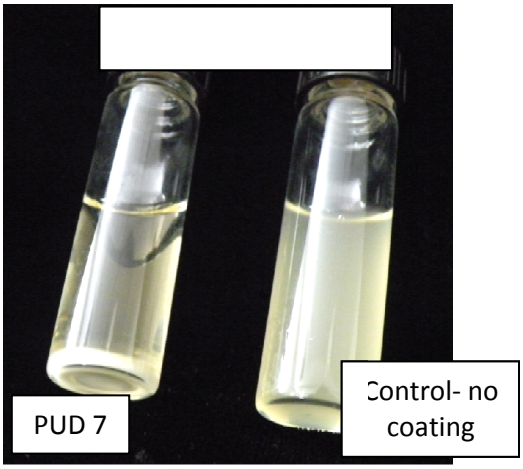

Fig. 11b Sterile glass tube with PUD 7 coating on the base (left) and control tube with no coating (right), containing 3 mL of *L. monocytogenes* 2045 cell suspension (10^6 CFU/mL) and after 48 h of incubation. The coated tube showed a complete kill of the test pathogen cells noted by the transparency of the medium and plate counts, while the control tube showed a turbid cell suspension indicating growth

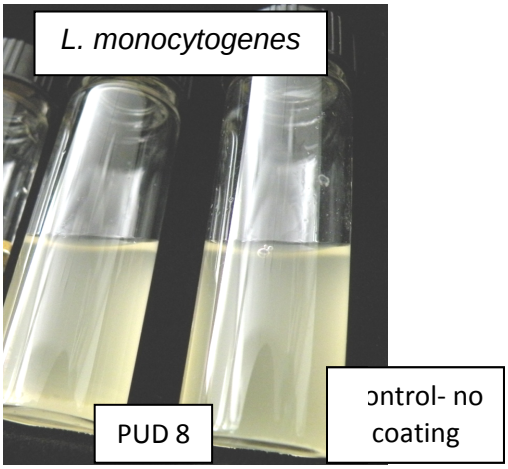

Fig. 11c Sterile glass tube with PUD 8 coating on the base (left) and control tube with no coating (right), containing 3 mL of *L. monocytogenes* 2045 cell suspension (10^6 CFU/mL) and after 48 h of incubation. The coated as well as the control tubes showed a turbid cell suspension indicating growth and demonstrating the inactive nature of PUD 8

Table 2 Cell counts of *S. typhimurium* 13311 (ST) and *L. monocytogenes* 2045 (LM) when exposed to polyurethane dispersions (PUDs) - 5, 7 and 8 at an initial cell concentration of 10^6 CFU/mL. The test cultures were kept in glass tubes coated with the PUDs at 35 ℃. Periodic sampl ing was done and cell numbers measured by dilution and spread-plate method. All data are expressed as means ± SD for two replicates

Incubation period	Cell counts (CFU/mL)					
	LM + TSB (control)	LM + PUD8	LM + PUD7	ST + TSB (control)	ST +PUD 8	ST +PUD 5
0 h	(2±1)× 10^6	(2±1)× 10^6	(2±1)× 10^6	(4±1)× 10^6	(4±1)× 10^6	(4±1)× 10^6
0.5 h	(6±3)× 10^6	(4±2)× 10^6	(5±2)× 10^5	ND	ND	ND
1 h	ND	ND	ND	(6±2)× 10^6	(8±1)× 10^6	(5±3)× 10^5
3 h	(4±2)× 10^6	(3±1)× 10^7	(6±2)× 10^4	(3±1)× 10^7	(5±2)×10^7	(7±1)× 10^4
7 h	(2±1)× 10^8	(6±3)× 10^7	(7±2)× 10^3	(7±1)× 10^7	(8±1)×10^7	(2±1)× 10^4
12 h	(3±1)× 10^9	(4±1)× 10^9	No cells	(2±1)× 10^8	(4±1)× 10^9	(6±3)× 10^3
24 h	(2±1)× 10^9	(3±2)× 10^9	No cells	(8±1)× 10^9	(5±2)× 10^9	(3±1)× 10^2
48 h	(3±1)× 10^9	(4±2)× 10^9	ND	(8±1)× 10^9	(4±2)× 10^9	No cells

ND: Cell count measurements of these test samples were not done for these time points

3.5 Anti-biofilm activity of PUD 4 against L. monocytogenes

After 3 days of contact between the PUD 4 film prepared on the glass slide and the *L. monocytogenes* cells, a very turbid cell suspension was observed in the chamber of the un-coated slide while the turbidity in the chamber on the PUD 4 coated slide was very less as seen in Figures 12a and b. When the slides were

washed, dried, stained and observed under the microscope, a distinct difference in cell morphology could be observed between the un-coated (control) and the PUD 4 coated slides. While the control slide showed a dense growth of cells in the form of a biofilm (Fig. 13), the coated slide showed a few isolated bacterial cells and a few in chains, with no indication of a biofilm (Fig. 14).

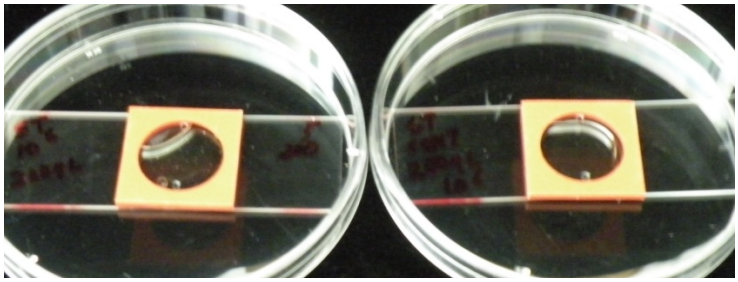

Fig. 12a Perfusion chamber experiment using sterile petri-plates, glass slides and CoverWell perfusion chambers to test anti-biofilm activity of PUD 4 against *L. monocytogenes* on PUD 4 coated (left) slides. Uncoated slides (right) were the control. All chambers contained actively growing *L. monocytogenes,* with a cell concentration of 10^8 CFU/mL

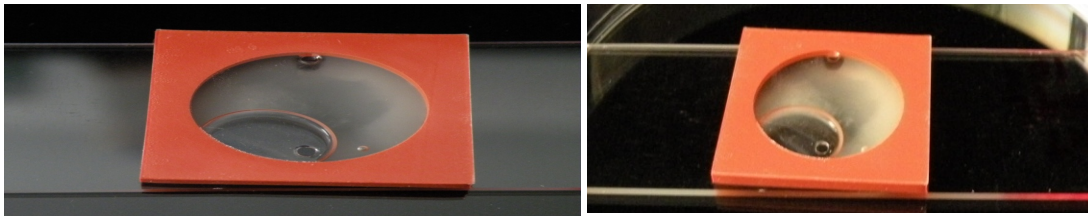

Fig. 12b Perfusion chamber experiment after 3 days of contact between *L. monocytogenes* cells and the PUD 4 coated (left) slide. Uncoated slide (right) showed dense and turbid microbial growth while PUD 4 coated slide showed much less turbidity

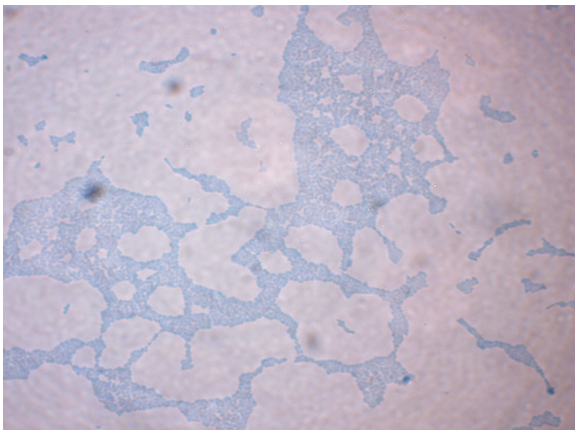

Fig. 13 Dense growth of *L. monocytogenes* biofilm formed on an uncoated slide after 3 days of incubation in a perfusion chamber. The slide was observed under 45×10x magnification using a Leitz LaborLux S microscope

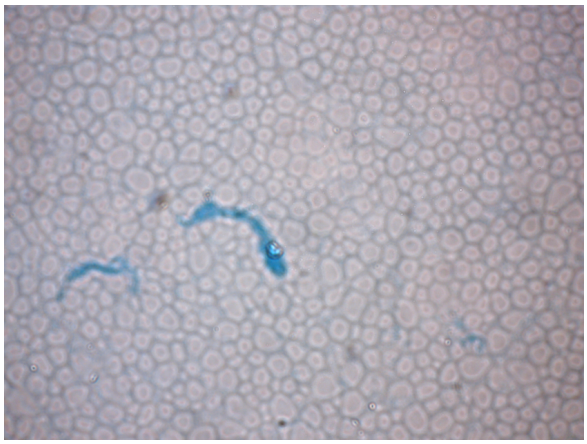

Fig. 14 Scattered and less number of cells of *L. monocytogenes* seen on the PUD 4 coated slide after 3 days of incubation in a perfusion chamber. The slide was observed under 45×10x magnification using a Leitz LaborLux S microscope. The textured background was due to the PUD coating on the slide

4.0 Conclusions

Vegetable-oil based cationic waterborne polyurethane dispersions were found to have inhibitory activity against food-borne pathogens, *Salmonella typhimurium* and *Listeria monocytogenes* in the form of liquid dispersions as well as films. PUD 5 showed wide spectrum activity and was potent against both Gram positive and Gram negative bacteria; while PUD 7 was group-specific in its action being most active against *L. monocytogenes* and having negligible activity against *S. typhimurium*. PUDs 5 and 7 also proved cidal in action. They were able to completely kill *S. typhimurium* and *L. monocytogenes* cell suspensions at an initial cell count of 10^6 CFU/mL, in 48 and 12 h respectively. The antimicrobial activity of the PUDs was not a surface phenomenon alone. The active components were able to diffuse into the surrounding medium (TSB in this case) and cause growth inhibition. PUD 8, which was the anionic PUD sample and used in this study as a control was not found to be antimicrobial in nature. Therefore to conclude, the cationic PUDs had excellent film properties along with the additional advantage of being antimicrobial in nature. Substituting fossil fuel-derived raw materials with renewable agriculture-based commodities for the synthesis of valuable polymeric materials would be very important from both a social and an environmental standpoint.

Acknowledgement

The authors would like to thank The Consortium for Plant Biotechnology Research Inc. for funding this project. We would like to thank Dr. Richard C. Larock and Thomas Garrison for synthesizing and supplying us with the polyurethane

dispersion samples. We are also grateful to Dr. Hyun Jung Kim and Zongyou Zhang for their support and help.

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APPENDIX B. CURRICULUM VITAE

DEBJANI MITRA

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EDUCATION

- **Ph.D.,** Iowa State University, Ames, IA (expected graduation: Oct 2011).
  Double major in Biorenewable Resources Technology and Food Science & Technology
  Thesis Advisors: Dr. (J) Hans van Leeuwen & Dr. Byron Brehm-Stecher.
  Running thesis title: "Production and characterization of value-added biorenewable chemicals".
  Recipient of R&D100 award, 2009; Grand Prize for University Research from the American Academy of Environmental Engineers (AAEE), 2011.
- **MS,** Environmental Sciences (2005). Pune University, Pune, India.
- **BS,** Microbiology (2003). Bharati Vidyapeeth Deemed University, Pune, India.
  Ranked 2nd in college.

PROFESSIONAL EXPERIENCE

Graduate Research Assistant at Iowa State University, Ames, Iowa (2007 – present)

- Value-added nisin production via lactic acid bacterial fermentation of a non-dairy based substrate (2007-08)
- Oleaginous fungal fermentation on thin stillage for lipid production and value-addition to corn ethanol co-product (2008 – 2011)
- Heterotrophic/mixotrophic cultivation of oleaginous algae using industrial co-product steams (2009)

- Natural antimicrobials and food safety (2009 – till date)
- An Integrated fungal biodiesel production process: Mycodiesel (2008-2009)
- Fungal pilot plant for ethanol co-product beneficiation (2010-till date)
- Identification of genes conferring bacterial resistance to Sensiva SC50 (2011)
- Reduction in mucosal barrier markers with soy protein diet but not probiotic in DSS-treated mice (2010)

Internship at Kerry Fermented Ingredients Inc. (Aug-Nov 2008)

Identified, developed and implemented a new fermentation substrate for an existing product line (flavour compound). Optimized fermentation conditions of two existing product lines for higher productivity.

Achievements: Successfully established merits of a new fermentation substrate for an existing product. Fermentation optimization studies led to a significant rise in productivity.

Research Associate & summer internship at Agharkar Research Institute, Pune, India (2002, 2004, 2005-06)

- Full-time research associate - Production of secondary metabolites from extremophiles involving bacterial fermentation, antibiotic production, recovery and purification (2005-06).
- Master's thesis - Screening thermophilic actinomycetes for antimicrobial compounds against multi-drug resistant pathogenic bacteria and metallo- protease enzyme Inhibitors (2004-05).

JOURNAL PUBLICATIONS

- *Value-Added Production of Nisin from Soy Whey*. Debjani Mitra, Anthony L. Pometto III, Samir K. Khanal, Bishnu Karki, Byron F. Brehm-Stecher, J. (Hans) van Leeuwen. 2010. Appl. Biochem. Biotechnol. 162:1819-1833.

- *Reduction in mucosal barrier markers with soy protein diet but not probiotic in DSS-treated mice.* H. Jiang, J. Przybyszewski, D. Mitra, C. Becker, B. Brehm-Stecher, A. Tentinger, R. S. MacDonald. 2011. J. Nutr. 141 (7):1239-1246.

JOURNAL PUBLICATIONS IN PREPARATION / SUBMITTED

- *Low-value non-dairy co-products as fermentation feedstock for nisin production: a Review.* Debjani Mitra, Anthony L. Pometto (III), Samir K. Khanal, Aubrey F. Mendonca, J (Hans) van Leeuwen, and Byron F. Brehm-Stecher. 2011 (in preparation to be submitted to Trends in Food Science and Technology).

- *Oleaginous fungal fermentation on thin stillage for lipid production.* Debjani Mitra, Mary L. Rasmussen, Priyanka Chand, Venkat Reddy Chintareddy, Linxing Yao, David Grewell, John G. Verkade, Tong Wang, J (Hans) van Leeuwen. 2011 (Submitted to J. Bioresource Technology).
- *Heterotrophic/Mixotrophic algal cultivation on agro-industrial co-product streams for lipid production*. Debjani Mitra, J (Hans) van Leeuwen and Buddhi Lamsal. 2011 (Submitted to J. Algal Research).
- *Synergistic action of plant essential oils and organic acids and their group/species-specific antimicrobial activity against foodborne pathogens.* Debjani Mitra, J (Hans) van Leeuwen, and Byron F. Brehm-Stecher. 2011 (in preparation to be submitted to J. of Food Protection).
- *Antimicrobial activity of aqueous cationic polyurethane dispersions against Salmonella typhimurium and Listeria monocytogenes.* Thomas Garrison, Debjani Mitra, Byron F. Brehm-Stecher, Richard C Larock. 2011 (in preparation to be submitted to J. Progress in Organic Coatings).
- *Identification of genes conferring bacterial resistance to Sensiva SC50*. Hyun J. Kim, Debjani Mitra, Byron F. Brehm-Stecher, 2011 (in preparation to be submitted to J. of Food Protection).

BOOK CHAPTER

Van Leeuwen, J (H), Rasmussen, M.L., Sankaran, S., Koza, C., Erickson, D.T., Jin, B. and Mitra, D. (2012). *Fungal cultivation in thin stillage from corn-to-ethanol plants to value-added products*. Chapter 2 in *"Sustainable Bioenergy and Bioproducts".* K. Gopalakrishnan, J. (H.) van Leeuwen, and R. C. Brown editors. Springer, New York ISBN 978-1-4471-2323-1.

SELECTED CONFERENCE PAPERS / POSTERS

- Oral presentation on *"Mixotrophic cultivation of the oleaginous alga Chlorella vulgaris on industrial co-products"* at the First International Conference on Algal biomass, Biofuels and Bioproducts (**ABBB**) (St. Louis, MO, 2011).
- Poster on *"Fungal fermentation for bio-oil production from thin stillage*" presented at the Corn Utilization and Technology Conference **(CUTC**), (Atlanta, GA, 2010).
- Oral presentation on "*Fungal Fermentation for Bio-Oil Production*" at the Annual Meeting of the American Oil Chemical Society (**AOCS**), (Phoenix, AZ, 2010).
- Poster paper on "*Group- and Strain-Specific Antimicrobial Activities of Select Plant Essential Oils*", presented at the Annual Meeting of the International Association for Food Protection (**IAFP**), (Anaheim, CA, 2010).
- Poster paper on "*Utilization of a byproduct from soybean processing for the cultivation of probiotic Lactococcus lactis"* - Presented at the Annual meeting of the American Association of Cereal Chemists (**AACC**), (Baltimore, MD, 2009).
- Paper on "*Ultrasonication in Soy Processing for Enhanced Protein and Sugar Yields and Subsequent Bacterial Nisin Production*" - Presented at the Annual International Meeting of the American Society for Agricultural and Biological Engineers (**ASABE**), (Minneapolis, MN, 2007).
- Poster paper on "*Antibacterial Activity of Thermoactinomyces thalpophilus on Vancomycin resistant S. aureus and E. faecalis"* - Presented at the 47th Annual Conference of Association of Microbiologists of India (**AMI**), (Bhopal, India, 2006).

ADDITIONAL AWARDS AND HONORS

- Represented Iowa State University at 2009 EU-US exchange program at Karl Franzens University, Graz, Austria and attended intensive short-course on "Renewable Resources & Clean Technology".
- Received the Dean Klecker Global Agriculture Graduate Scholarship ($750) endowed by the Iowa Farm Bureau Federation (2010-2011).
- Received the George Washington Carver Student Scholar award ($500) in 2010 from the Bioeconomy Institute at ISU, recognising achievements by graduate students in the field of biorenewables.
- Received the College of Human Sciences International Experience Scholarship ($1000) in 2009.

CO-CURRICULAR ACTIVITIES

- Elected to the executive committee of Indian Students Association at Iowa State University for the term 2008-2009. Member of the orientation team for new graduate students at ISU in 2008.
- Member of departmental committee for selection of future faculty in fermentation
- Silver medallist in Archery at National level in India (1997), Gold medallist at State level Basketball Championship (1995), Bachelors Degree in Fine Arts (painting), Senior Diploma in Indian classical dance (Bharatnatyam).

ACKNOWLEDGEMENT

I would like to acknowledge my major professors Dr. Hans van Leeuwen and Dr. Byron Brehm-Stecher for their support and guidance. I would also like to thank my POS committee members, Dr. Anthony L. Pometto III, Dr. Aubrey Mendonca, and Dr. Buddhi Lamsal, as well as Dr. Samir Khanal, Dr. William Colonna, Dr. Stephanie Jung, Dr. Ruth McDonald, Carol Ziel and my lab mates and colleagues for their help and support. A special thanks to my husband, friends and family for being there with me through everything. And last, but not the least, I would like to dedicate my PhD dissertation to my parents without whose undying faith and encouragement this would not be possible.