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Isolation and characterization of thermophilic and hyperthermophilic microorganisms from food processing facilities

by

Ping-Shing Mak

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
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Ames, Iowa

2003

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This is to certify that the master's thesis of

Ping-Shing Mak

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

For the Major Program

To my parents, Tze-Kuen and Yin-Fong Yip Mak,

to my siblings, Jean and Joseph,

and Vivian

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ABSTRACT

Continuous and repeated-batch fermentations were performed in an attempt to isolate thermophiles and hyperthermophiles from food processing facilities. Plastic composite supports (PCS) containing polypropylene and agricultural by-products were used to promote biofilm formation for culture isolation. Rich and minimal media were used for enrichment cultivation. Two spray drier exhaust vents and sites from a corn wet milling facility were tested for potential thermophilic and hyperthermophilic microorganisms. Biological activity was monitored via CO_2 production, microscopic examination of spent culture broth, and cell count by fluorescent stain and flow cytometry. During long term fermentation a microbial consortium population consisting of Gram positive rods, Gram negative rods and Gram positive cocci able to grow at >90°C was obtained. Cells were recovered from PCS biofilm culture in the 10^6 to 10^8 cells/g PCS range. This study indicated that we could isolate potential hyperthermophiles from these environments. However the lack of reproducible results in subsequent studies suggested that the microbial population was not hyperthermophilic but stressed thermophiles.

In a subsequent study to identify the thermophilic population, continuous and repeated-batch fermentations were performed at 55-75°C for 2.5 months on a culture obtained from light corn steep liquor. PCS containing 50% (wt/wt) polypropylene, 40% (wt/wt) soyhulls, 5% (wt/wt) yeast extract and 5% (wt/wt) bovine albumin and salts was used to promote biofilm formation. LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] sodium chloride and 0.05% [wt/vol] cane molasses) was used in this study. Biological activity was monitored as previously described with the addition of plating

on 2% (wt/vol) agar using different substrates. Isolate L-10997 displayed a unique 16S rRNA sequence and was further studied. The isolate was a Gram-positive rod with an optimum growth temperature of 65°C and pH of 6-8. It was an obligately aerobic heterotroph and utilizes partially hydrolyzed animal and plant proteins. Based on its phylogenetic and biochemical characteristics a new species of thermophilic *Bacillus* was obtained and the name *Bacillus thermozeamaize* was proposed, with the type strain L-10997 (ATCC BAA-739).

GENERAL INTRODUCTION

Introduction

Hyperthermophiles are microorganisms that can thrive at temperatures of >80°C [1, 2, 66]. They can be found both natural and man-made hot environments, such as surroundings of volcanoes, hydrothermal deep-sea vents, and geothermal power plants [66]. Most hyperthermophiles are anaerobic in nature primarily because of low dissolved oxygen concentrations [26]. Over the past twenty years these microorganisms have undergone extensive studies and scientists are amazed to find out just how different hyperthermophiles are from the other microorganisms. Enzymes from hyperthermophiles are extremely valuable because of their ability to function at elevated temperatures. A few thermostable enzymes are currently available commercially, the most widely used of which is the *Taq* polymerase.

One major problem with enumeration of hyperthermophiles has been the inability to obtain sufficient cell mass [54]. At Iowa State University, a unique support for biofilm formation called plastic composite supports (PCS) has been developed. PCS are made of 50-70% (wt/wt) plastic and 30-50% (wt/wt) agricultural products and microbial nutrients [52]. PCS blends can be customized for individual bacterial strains, and have been proven to increase lactic acid production rates, reduce lag phase of microorganisms, reduce requirements of micronutrients, and increase cell density [28, 52, 72]. We believe PCS could solve the insufficient biomass problem by providing a platform for attachment of hyperthermophiles and slow-releasing nutrients during their long-term growth studies.

High-temperature processes are often associated with the food processing industries. For example, animal and plant proteins are spray-dried for use as animal feed, sugar syrup is produced by starch liquefaction at an elevated temperature, and corn is processed at a high temperature during corn starch production. The food materials and equipment are constantly being exposed to high temperature, which resembles a natural hot vent. The only difference is that these places are aerobic and nutrient-rich. Therefore hyperthermophiles could be isolated from these environments potentially.

The goal of this project is to isolate hyperthermophilic microorganisms from various food processing facilities which could produce thermostable enzymes to improve industrial wastestream processing and food processing. The first paper outlines an attempt to isolate hyperthermophilic microorganisms from various sites in food processing facilities using PCS in a biofilm reactor. The second paper outlines an attempt to isolate and characterize thermophilic microorganisms in corn steep liquor via a PCS biofilm reactor at 55-75°C.

Thesis Organization

This thesis follows an alternative format and is divided into two papers. Each paper contains an abstract, introduction, materials and methods, results and discussion, acknowledgment, and references with tables and figures included in the text. The papers are written following the specifications of Applied Microbiology and Biotechnology and the International Journal of Systematic and Evolutionary Microbiology. A general introduction including a literature review and general conclusion are included. All experiments, data collection, and data analysis were performed by the candidate.

Literature Review

Extremophiles

"Extremophiles" are microorganisms that are adapted to survive and grow in extreme environments such as extremes of temperature, pH, high salt concentrations, and high pressures [1, 2]. Extensive studies of ecology, physiology and molecular biology over the past two decades have demonstrated just how different extremophiles are from other microorganisms. Extremophiles can be classified according to their tolerance to extreme environmental factors. Acidophiles and alkaliphiles grow at acidic (pH <3) and alkaline (pH >9) conditions, respectively. Halophiles can tolerate a salt concentration in excess of 3.0%. Psychrophiles and hyperthermophiles thrive at temperatures of <5°C and >80°C, respectively. This thesis will focus on hyperthermophiles and a more in-depth introduction to this group of microorganisms will be presented.

Hyperthermophiles

It has been almost 15 years since the establishment of *Archaea* as a new domain of life [75]. Almost all of the known hyperthermophiles belong to this category [3, 73]. Although many species have been discovered in recent years, terms such as thermophilic, extremely thermophilic, and hyperthermophilic have yet to be clearly defined. However, it is probably appropriate to classify an organism as a hyperthermophile if it can grow ≥80°C [1, 67]. As a rule, hyperthermophiles are unable to grow <60°C [64, 65, 66].

The currently known thermophilic *Archaea* can be divided into three groups. The majority of them are sulfur-dependent chemolithoautotrophs, which means they grow

primarily by the metabolism of CO_2 as carbon source and sulfur as inorganic electron donors and acceptors [66]. There are also sulfate-reducing *Archaeoglobus* and thermophilic methanogens. Most of these anaerobic heterotrophs utilize only complex peptide mixtures such as yeast and meat extracts as carbon and nitrogen sources. Only a few of these organisms metabolize carbohydrates, including starch, glycogen and maltose, but they also require peptides as a nitrogen source.

At temperatures >100°C, even low molecular weight compounds such as ATP and NAD hydrolyze quite rapidly and some thermolabile amino acids (e.g. cysteine and glutamic acid) are decomposed [51, 64]. The survivial and growth of hyperthermophiles rely on the re-synthesis of these critical compounds. In considering the effects of temperature on growth physiology, it is apparent that at the present upper temperature limit of life, the predominant metabolism is strictly anaerobic with heterotrophic S° reduction. In addition, almost all of the hyperthermophilic species require complex organic mixtures as carbon and nitrogen sources. The chemolithoautotrophic archaeon *Pyrolobus fumarii* is able to grow at 113°C and represents the current upper temperature limit of life [68].

Hyperthermophiles have been reported in almost all shapes and forms. The majority of them are Gram-negative, and can be cocci, lobed cocci, plated-shaped, or rod-shaped [67, 68]. The coccoid and plate-shaped cells can be highly variable in size even within the same culture [64]. A few of them develop terminal spherical bodies which resemble "golf clubs" during the exponential growth phase [34, 65, 74]. Some coccoid and rod-shaped isolates are motile by means of flagella [23, 33, 34, 35, 67]. A complex envelope consisting of murein was observed and the GC content of the DNA ranges from 31 to 67% [27, 68].

Hyperthermophiles have already revolutionized the fundamental way in which life forms are classified and have extended the limits of the conditions under which life can thrive. They harbor unusual metabolic pathways containing some new enzymes and have new versions of known enzymes but with unprecedented thermal stability. This thesis will focus on the isolation of thermophiles and hyperthermophiles obtained from high-temperature food processing facilities.

Previous Work and Isolation Protocols for Hyperhermophilic Microorganisms

More than 30 years ago T.D. Brock and his colleagues were among the first to attempt to grow microorganisms at temperatures which were considered too high to support microbial life [12]. In 1982 K.O. Stetter expanded the known thermal limits of life by isolating the first microorganism from a submarine solfataric field that could grow at the normal boiling point of water [62, 63]. Since then the number of species known to be "heat-loving" have continued to expand, to more than 70 species by the end of 1999 [73]. The majority of hyperthermophiles were isolated from terrestrial geothermal areas such as the surroundings of volcanoes [32, 21, 62] and in subterranean hydrothermal sea floors [35, 76], sometimes up to thousands of meters below sea level [8, 40, 64]. Man-made extreme environments such as the boiling outflows of geothermal power plants [34], a uranium mine [23] and smoldering coal refuse piles [16, 49] have also been reported to contain thermophiles and hyperthermophiles.

On land, volcanic exhalations from deep magma chambers heat up soils and surface waters, forming sulfur-harboring acidic solfataric fields and neutral to slightly alkaline hot springs [68]. The salinity of such terrestrial hydrothermal systems is usually low (<2%).

More than 20 species [67, 73] were isolated from these sites. For example, representatives of the genera *Sulfolobus*, *Acidianus* and *Pyrobaculum* were isolated from a great number of these habitats around the world, including Iceland [32, 34], Italy [34, 63], Indonesia [31], New Zealand [10], the Azores [34], and the United States [12, 59].

Submarine hydrothermal systems, which can range in depth from a few meters to thousands of meters, harbor many unique hyperthermophiles. These environments generally contain a high salt concentration (>3%) and exhibit a slightly acidic to alkaline pH (5-8.5) [67]. For example, *Thermotoga maritima* was isolated from shallow sea floors in Italy and Iceland [33], *Aquifex pyrophilus* was isolated from hot marine sediments in Iceland at a depth of 106 m [35], and *Staphylothermus marinus* was isolated from a hydrothermal vent 2600 m below sea level at the East Pacific Rise [21].

Deep subterranean non-volcanic geothermally heated biotopes were discovered in 1993 about 3500 meters below the bottom of the North Sea and below the Alaskan north slope permafrost soil, where *in situ* temperatures are approximately 100°C [65 ,68]. The production fluids from oil-drilling activities yielded up to 10^4 cells/ml at the Thistle platform (North Sea) and 10^7 cells/ml at North Slope (Alaska) production wells, respectively [65, 67].

Artificial biotopes, such as smouldering coal refuse piles and hot outflows from geothermal power plants, have also been reported to contain hyperthermophiles. For example, *Metallosphaera prunae* was isolated from a uranium mine in Germany [23], and *Sulfolobus* and *Thermoplasma acidophila* (both thermophiles) were isolated from coal refuse piles in the UK and USA, respectively [16, 49].

Because of the low concentration of dissolved oxygen and the presence of hydrogen sulfide, the predominant microorganisms are anaerobic in most of these hot environments

[26]. One major limitation of the study of hyperthermophiles has been the small amount of biomass that can be generated [54]. Problems with culture stability have also been encountered, as cultures will abruptly stop growing after a few transfers [27].

The enrichment methods for thermophilic and extremely thermophilic microorganisms are based on the protocols developed for mesophilic microorganisms [41]. Modifications are made to accommodate the requirement of maintaining high temperature and other extreme conditions such as pressure and pH [27]. Most hyperthermophiles are sulfur-metabolizing, and therefore considerations have to be taken concerning the materials of construction; for example, glass should be used instead of stainless steel in the construction of the reactor. When isolating hyperthermophiles, the nutrients present at these unique sites will need to be incorporated into the culture medium. For example, in solfataric fields and hot springs around volcanoes, the environment is usually high in sulfur and low in salt, while deep sea vents are usually high in salinity and acidic [68].

Anaerobic culture techniques, such as that developed by Hungate [37] and modified by Balch and Wolfe [7], are often used. Oxygen is reduced by the addition of Na_2S [21, 33, 34, 74]. In addition, deep-sea microbes may be able to use chemical reactions running only under high pressure by shifting their equilibrium towards an energy-yielding direction. For example, the hyperthermophilic methanogens grow chemolithautotrophically in seawater in the presence of H_2 and CO_2 [67]. Aerobic cultures may be stirred (150 rpm) or slightly gassed with compressed air (25 ml/min) [30]. When a microaerobic culture condition is needed, the medium can be gassed with nitrogen for 20 minutes after autoclaving [35].

Novel cloning methods for hyperthermophiles have also been developed. In 1995, Huber et al. [36] successfully applied whole cell hybridization, a computer-controlled

microscope equipped with a strongly focused infrared laser, and a cell separation unit, to isolate a hyperthermophilic archaeum from a mixed culture in a hot pool at Yellowstone National Park. These microorganisms are impossible to isolate by conventional plating and serial dilution methods because of their inability to form colonies and due to their low numbers they are often dominated by other microorganisms within the mixed culture [36]. The isolation of hyperthermophiles is particularly challenging because, in addition to gaining access to their natural environments, the use and maintenance of high temperature, high hydrostatic pressures, anaerobic conditions, and highly acidic or highly reducing media are all factors that may have to be considered.

Some Commercial Products Derived from Hyperthermophiles

Cultivation of extreme thermophiles on a scale sufficient to provide biomass for enzyme purification presents many challenges [41, 53]. However, some successes have been achieved in recent years to isolate and develop thermostable enzymes for industrial and commercial use.

DNA Polymerases

The emergence of polymerase chain reaction (PCR) to amplify DNA fragments [57] has led to research regarding isolation and characterization of the DNA polymerases from thermophiles and hyperthermophiles. Their high temperature optima offer unique advantages over their mesophilic (e.g. *Escherichia coli*) counterparts. To date, more than 50 DNA polymerases have been cloned, sequenced, and characterized from thermophilic and hyperthermophilic eubacteria and archaea [29].

The first thermostable DNA polymerase developed for PCR was obtained from *Thermus aquaticus* (*Taq*) [46, 57]. DNA polymerases from various *Thermus* species, for example, *T. thermophilus* (*Tth*) [60], *T. flavus* (*Tfl*) [4], *T. brockianus* (*Tbr*) [29] and *Thermotoga maritima* (*Tma*) [29] have also been developed for commercial use. A number of DNA polymerases from hyperthermophilic archaea have also been isolated and characterized (e.g. *Pyrococcus furiosus* (*Pfu*)) [48, 70]. *Taq* polymerase is the enzyme of choice for sequencing and detection procedures because of its high processing rates [73]. However, the proofreading capacity of archaeal DNA polymerases has led to their preference over *Taq* DNA polymerase in high-fidelity PCR applications [29, 48, 73].

DNA Ligases

Thermostable DNA ligases are commercially available [73]. These enzymes represent an excellent addition to PCR technology with an optimum temperature range of 45 to 80°C. They are perfect for ligating adjacent oligonucleotides that are hybridized to the same target DNA. This property can be used for ligase chain reaction (a DNA amplification method) for the detection of genetically transferred diseases [47], for mutational analysis of cancer tumors by oligonucleotide ligation assay [76], and for gene synthesis by overlapping oligonucleotides [25].

Potential Applications of Hyperthermophilic Enzymes in the Food Industry

The discovery of hyperthermophiles has been beneficial to the food industry in many ways [26, 73]. If not for the thermostable DNA polymerase derived from these microorganisms, conventional and real-time PCR for the detection of foodborne pathogens would be non-existent [26, 73, 47].

The production of high-fructose corn syrup (HFCS) by the biocatalyzed conversion of glucose to fructose by glucose isomerase represents the largest existing industrial application of an immobilized enzyme [26]. Because of the thermostability limitations of the enzyme, this process is currently operated at approximately 60°C, at which temperature the equilibrium conditions limit the concentration of fructose in HFCS to a maximum of 42% [61]. A chromatographic separation step is used to remove unreacted glucose to enrich the mixture to a 55% fructose concentration, which is desirable for sweetening properties. However, because the equilibrium yield of fructose increases with temperature (55% fructose estimated at 95°C), thermostable versions of glucose isomerase that could be used at higher temperatures are desirable to minimize or eliminate the glucose removal step [26].

Pectin is a branched heteropolysaccharide abundant in plant tissues. Pectin methylesterases and depolymerases are widely used in the food industry for tissue softening [73]. In fruit juice extraction and wine making, pectinolytic enzymes increase juice yield, reduce viscosity, and improve color extraction from fruit skin. A few thermophilic pectinolytic enzymes isolated from thermophilic anaerobes show catalytic activity and stability compatible with industrial needs [73].

Animal feedstock production processes include heat treatments that inactivate potential viral and microbial contaminants. Using thermophilic enzymes (arabinofuranosidase and phytase) in feedstock production would enhance digestibility and nutrition of the feed while allowing the combination of heat treatment and feed transformation in a single step [42].

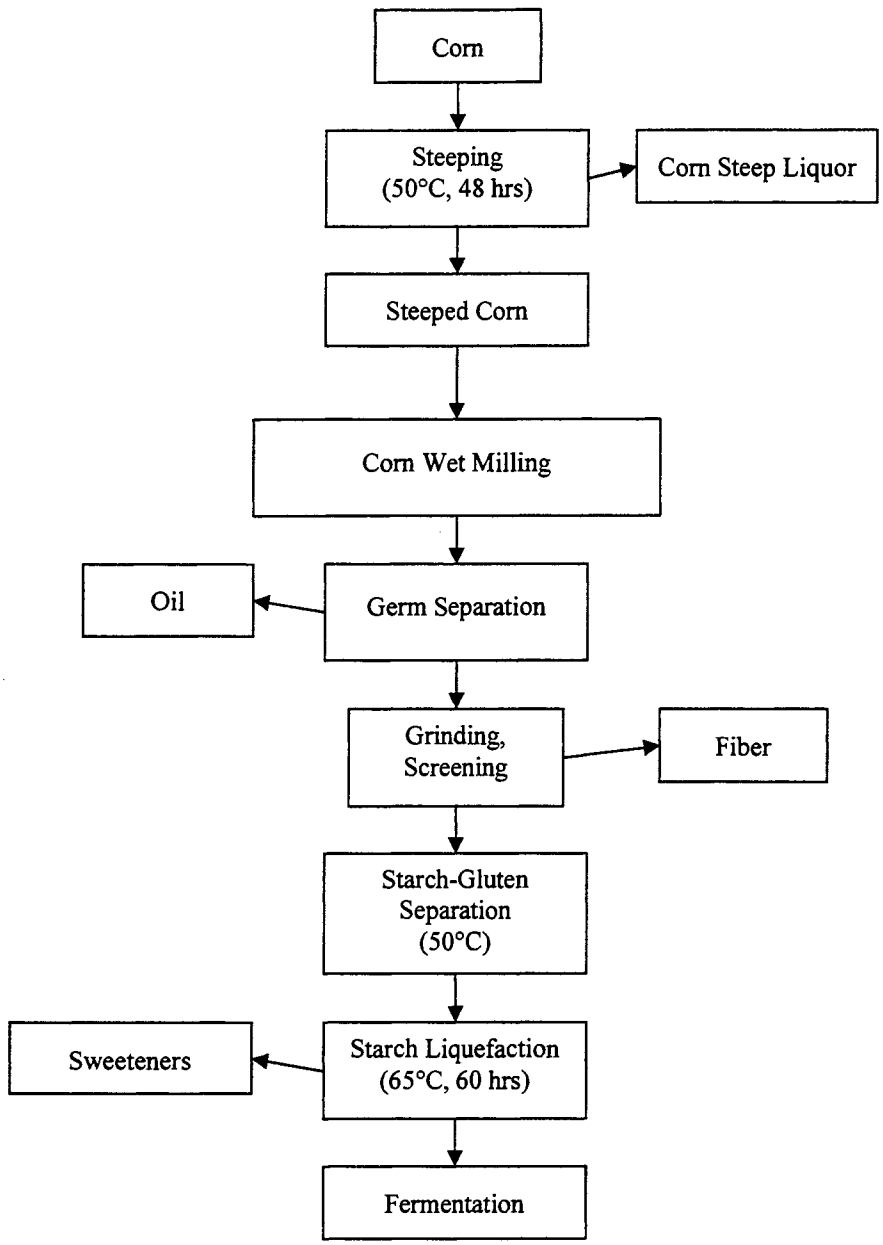
Other potential enzymes include: Keratinase to degrade poultry feathers to produce rare amino acids (serine and proline) [22]; ß-galactosidase to produce lactose-free dietary

milk products [24]; cellulase for cellulose liquefaction and cellulose alkaline pre-treatment for ethanol production [11, 13, 56]; pectinase/protease in juice clarification, food tenderizing during canning or high temperature extrusion [58].

Corn Wet Milling Facility as a Potential Source of Hyperthermophiles

Corn wet milling, in some form, has been employed in the United States since 1842 [9]. The wet milling process is designed to break down corn into its components, so that it can be further processed into products like corn syrup, ethanol, and starch. The process is largely physical involving mechanical grinding, although chemical and biological factors also play a part in steeping [9]. The wet milling process is now extremely efficient, with almost complete recovery of the constituents of corn, including starch, germ, fiber, and protein. In 2001, more than 1.4 billion bushels of corn were used by the corn refining industry, and an estimated 2 billion bushels would be used by 2010 [5]. Corn wet milling can be summarized by the following flow diagram:

Figure 1. Corn Wet Milling Process Flow Chart [6]



The objective of steeping is to prepare the corn for subsequent separation of starch and other by-products by wet milling. To achieve this, the corn is soaked in an aqueous solution containing 0.1-0.2% sulfur dioxide, which acts as an antimicrobial agent and which solubilizes and disperses the protein matrix to which starch granules are attached [15]. Proper steeping is the key to efficient starch and protein separation in the later stages of wet milling. The process is conducted at 49-53°C for 40 -50 hours.

Recently, studies have been conducted to improve the steeping process by replacing SO_2 with proteases from germinating corn [39]. Possible improvements include reduced steep time, elimination of SO_2 emissions, lower operating costs and improved yields and quality of final products. The enzymatic corn steeping process consists of two steps: 1) size reduction of corn after a brief water soaking of the kernels, and 2) controlled incubation of the coarsely ground corn slurry with enzymes [61]. Conventional wet milling steps are then performed following the enzymatic treatment. Ozone has also been studied as a potential source of SO_2 replacement [54].

Drained steepwater is called light corn steep liquor (~6% solids), which can be concentrated by flash evaporation to produce heavy corn steep liquor (30-55% solids) [9]. This high temperature treatment of corn resembles a man-made thermal hot spring, except that this vent contains nutrients and is aerobic. Corn steep liquor contains amino acids, peptides, proteins, carbohydrates, vitamins, trace metals, minerals, and several complex growth factors that will support microbial growth. It can be used as a complex nutrient for industrial microbial fermentation [50]. Thus, it is likely that thermophilic microorganisms could be isolated from this environment.

Cell Immobilization and Biofilm Formation

Microbial cells can be immobilized to improve yield in fermentation [17, 18]. The two most common types of cell immobilization are attachment and entrapment. The entrapment of cells is an artificial process in which microbial cells are trapped within a polymeric matrix [71]. The most commonly used substances for this technique involves κ-carrageenan or calcium alginate beads. The disadvantages of this technique, which limit its application on an industrial scale, include bead-swelling, cell leakage, toxicity of matrix substances, high cost of carrier and poor operational stability.

Biofilms represent a natural form of cell immobilization [45], in which microbial cells are attached to a solid surface (a substratum). Biofilms are made up of microbial cells and exopolysaccharide (EPS). The vast majority of bacterial EPS are made up of specific and nonspecific polysaccharides [14]. Specific polysaccharides are specific to individual bacterial strains and are sometimes called polysaccharide antigens [38]. A well-known example of a specific polysaccharide is xantham gum, produced by *Xanthomonas campestris*. Nonspecific polysaccharides are found in a variety of bacterial strains, are generally simplier than their specific counterparts, and many of them contain only one monomer [14].

Biofilm formation involves six stages [14]. It can be summarized as follows: Transport → Adsorption → Multiplication → Erosion → Colony Forming Unit (CFU) separation → Desorption

On a clean substratum, a conditioning film is formed by the transport and adsorption of organic molecules. It would then be possible for microbial cells to attach to the conditioned substratum via both reversible and irreversible adsorption processes. Early interactions between the bacterial cell and the substratum are reversible and consist of long

range interaction forces such as van der Waals forces, electrostatic forces and hydrophobic interactions [14]. In irreversible adhesion, polymeric fibrils produced by the bacterial cells form a bridge between the cells and the substratum. Once attached to the substratum, microbial cells can multiply to form a biofilm. As the biofilm gains in size and thickness, loosely attached daughter cells on the surface layer can be detached from the biofilm [14].

A biofilm is largely composed of water. Reported biofilm water content ranges from 87 to 99% [44]. Biofilms are generally very hydrophilic. EPS are mainly responsible for this fact, since polysaccharides, the major EPS component, contain many hydrophilic sugar residues [44].

Plastic Composite Supports

Biofilms form readily on substances such as wood chips, frittered glass particles, polyester foams, and various plastics [71]. Biofilm is vital in industrial fermentation and in waste treatment systems because of the cost savings that are associated from recycling of biomass [52]. At Iowa State University, a material called plastic composite supports (PCS) was developed using a blend of polypropylene (PP) and agricultural products by high-temperature extrusion [52]. PCS blends can be customized to enhance biofilm formation. Polypropylene gives the PCS high mechanical stability and the ability to withstand sterilization temperatures and pH extremes. Ground soybean hulls is the agricultural product of choice because of its excellent water holding capacity (up to 191% of its dry weight) [53] and its ability to puff the extruded products. This generates a porous material with high surface area [28]. The nutrients in PCS can be customized to support specific microbial requirements [28]. To protect micronutrients during high temperature extrusion, 5% (w/w)

albumin is added to all PCS blends [28]. The PCS have been shown to stimulate microbial attachment [17], serve as a vehicle for slow release of nutrients during long term lactic acid fermentation [20], provide access of nutrients for microorganisms attached on the surface of the support [52], and help prevent wash-out of biomass from the reactor vessel [52].

The PCS have also been shown to stimulate biofilm formation and enhance productivity of end products. For example, Demirci et al. [19] showed that ethanol production by *Saccharomyces cerevisiae* could be increased by up to 10 times when PCS were used during fermentation. Velazquez et al. [72] demonstrated an increase in lactic acid production by *Lactobacillus casei* in PCS-biofilm fed-repeated-batch reactors (2.45 g/l/h) compared to suspended-cell fed-repeated-batch reactors (1.75 g/l/h). Demirci et al. [17] demonstrated an increase in lactic acid production by up to five times over that of suspended culture when solid PCS were used.

The central hypothesis of the current study is that the utilization of PCS in repeated-batch and continuous bioreactor will enhance recovery of hyperthermophiles from various food processing facilities. Hyperthermophiles are extremely hard to grow and biomass concentration is often very low. Thus, the ability of PCS to release nutrients slowly will benefit long-term fermentations of hyperthermophiles by stimulating biofilm development and microbial isolation and recovery.

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ISOLATION OF HYPERTHERMOPHILIC MICROORGANISMS (>90°C) FROM FOOD PROCESSING FACILITIES

A paper to be submitted to Applied Microbiology and Biotechnology

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Abstract

Hyperthermophilic microbial isolation from a corn wet milling facility and from exhaust vents of spray driers was performed in novel 500-ml reactors in sand baths and in a customized 1-L bioreactor with external circular water baths to maintain temperature at >90°C. Different kinds of substrate-specific plastic composite supports (PCS) containing a blend of nutrients and polypropylene were used in this study to stimulate biofilm formation for recovery of hyperthermophiles. A piece of Whatman No.1 filter paper was put in each reactor as a carbon source. Cultures were obtained from various sources in food processing facilities. Repeated-batch and continuous fermentation methods employing rich and minimal media were used to isolate microorganisms able to grow at >90°C. During repeated-batch fermentations, medium was changed every 7 days. Spent culture medium was evaluated for microbial activity via pH change and cells via Gram staining. Samples showing possible growth were further examined using phase contrast microscopy and *BacLight* fluorescent stain with flow cytometry to count dead/live cells. Evolution of CO_2 was followed by pH titration of alkaline traps using an automatic titrator. A microbial consortium population consisting of Gram positive rods, Gram negative rods and Gram positive cocci able to grow at 95°C was seen. During repeated-batch and continuous fermentations $2 \cdot 10^6$ to $2 \cdot 10^8$ and

$3 \cdot 10^8$ cells/g of PCS, respectively, were obtained. This study indicated that we could isolate potential hyperthermophiles from this environment. However the lack of reproducible results in parallel studies suggested that microbial population were not hyperthermophilic but stressed thermophiles.

Key Words: Hyperthermophilic microorganisms – extremophilic microorganisms – food processing facilities – thermostable enzymes

Introduction

Microorganisms that are adapted to survive and grow in extreme environments such as extremes of temperature, pH, high salt concentrations, and high pressures are termed "extremophiles" [Adams 1993; Adams 1995]. Extensive studies of ecology, physiology, molecular biology and evolution over the past two decades have demonstrated just how different extremophiles are from other microorganisms. The most common extremophilic biotopes are of geothermal origin, for example, in submarine solfataric fields, areas surrounding volcanoes, and subterranean hydrothermal sea floors [Hicks and Kelly 1999]. Potentially, extremophiles can also be isolated from the man-made extreme environments that are often associated with the food processing industry, such as the corn steeping process, the spray drying process, and sugar syrup production. The increased interest in the study of these microorganisms is partly due to the fact that they are considered important biotechnological resources and their specific properties are expected to result in novel process applications [Vieille and Zeikus 2001]. The goal of this research is to isolate new aerobic, hyperthermophilic microorganisms which produce key thermostable hydrolytic

enzymes (cellulase, hemicellulase, protease, and xylanase) to improve industrial wastestream processing or to improve current food processing.

The goal of this research was the isolation and identification of hyperthermophilic microorganisms from food processing facilities. Studies employing repeated-batch fermentations were done, including a parallel study on selected cultures aimed at comparing growth of our cultures under rich and minimal conditions in an aerobic environment. Although microorganisms and microbial activity were observed, the isolates were not stable. However, the methodology for isolating hyperthermophiles from these hot food processing environments was demonstrated.

Materials and methods

Plastic composite supports

Table 1 summarizes the five blends of PCS discs containing polypropylene and agricultural materials. The PCS discs were designed to sink to the bottom or float on top of the reactor and were produced by high-temperature extrusion using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ). The procedures were described by Ho et al. [1997]. The PCS tubes were cut into discs (0.4 cm ID, 1.0 cm OD) with a utility knife.

Table 1 PCS blends (wt/wt)

Support	Poly-Propylene[a] (PP)	Soybean Hulls[b] (SH)	Cellulose[c] (CE)	Dried Bovine Albumin[d] (BA)	Yeast Extract[e] (YE)	Mineral Salts[f] (S)
SHYE	50	40			10	+/–
SSHBAYE+	50	40		5	5	+
FSHBAYE+	70	20		5	5	+
SCEBAYE+	50		40	5	5	+
FCEBAYE+	70		20	5	5	+

[a]PP resins; Quantum USI Division, Cincinnati, OH. Sinkers contain 50% PP and floaters contain 70% PP.
[b]Dried ground (20-mesh) soybean hulls; Cargill Soy Processing Plant, Iowa Falls, IA
[c]SigmaCell, Sigma Chemical Co., St. Louis, MO
[d]Proliant, Ames, IA
[e]Ardamine Z; Sensient Flavors, Juneau, WI
[f]Per kilogram PCS blend: 2 g of sodium acetate, 1.2 g of magnesium sulfate, 0.06 g manganese sulfate

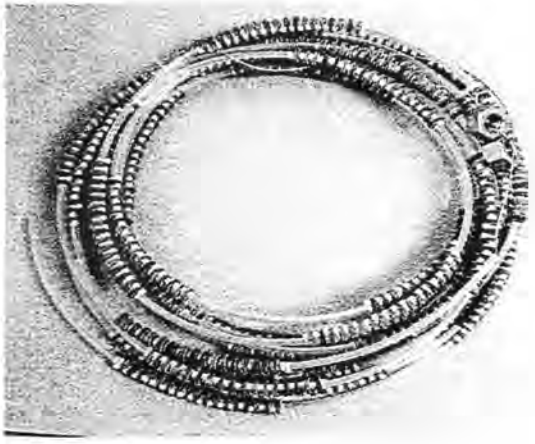
Food processing sites sampled

Spray dryer exhaust vents

Two spray drying facilities were investigated in this study. At the spray drying facilities, PCS discs were attached to a stainless steel wire inside a polypropylene tubing (3.15 m) and suspended in two spray drier exhaust vents for one year (Fig. 1). The PCS discs (0.9 cm OD, 0.4 cm ID, 0.4 cm thick) contained 50% polypropylene, 35% ground soybean hulls, 5% yeast extract, 5% soybean flour (Archer Daniels Midland, Decatur, IL), 5% bovine albumin, and mineral salts. They were positioned such that 15 PCS discs were spaced every 15 cm, with a 15 cm gap in between zones. The spray driers were located at the ISU Center for Crops Utilization Research, Ames IA and at Proliant Inc., Ames, IA. The supports were recovered after a year in the spray drier used with various products, divided into various lots based on cable depth with the PCS closest to the heating source labeled as the first lot. PCS

discs were stored at -75°C in sterile 20% glycerol in MRS medium (Difco Industries, Detroit, MI) until ready to use. To determine the temperature gradient within the exhaust vent, a temperature probe was inserted into the exhaust vent.

Fig. 1 Picture of PCS cable before use



Cultures were recovered from PCS biofilm by the stripping sand method developed by Ho et al. [1997]. Five pieces of PCS were shaken vigorously for 5 seconds in 0.1% peptone, and then transferred to a culture tube containing 9 ml of 0.1% (wt/vol) peptone and 5 g of sand. The tube was vortexed in intervals of 30 seconds for a total of 1.5 minutes. Five milliliter of the solution was used as an inoculum to the bioreactors.

Corn wet milling facility

Scrapings and samples from a corn processing facility (Penford Products Company, Cedar Rapids, IA) were obtained in May 1997 and stored at –75°C in sterile 20% glycerol in MRS medium until ready to use. The original sampling sites included 1) evaporator water; 2) hot corn steep liquor (CSL); 3) dry material on CSL pipe; 4) suet stack pipe; 5) burnt fiber

from drier; 6) evaporator tank wall; 7) biofilm from exhaust stack; and 8) scrapings from fiber drier dust window. An additional sample, 9) microbial consortium from light corn steep liquor recovered via continuous PCS biofilm culture at 55°C, was also evaluated in this study.

Medium preparation

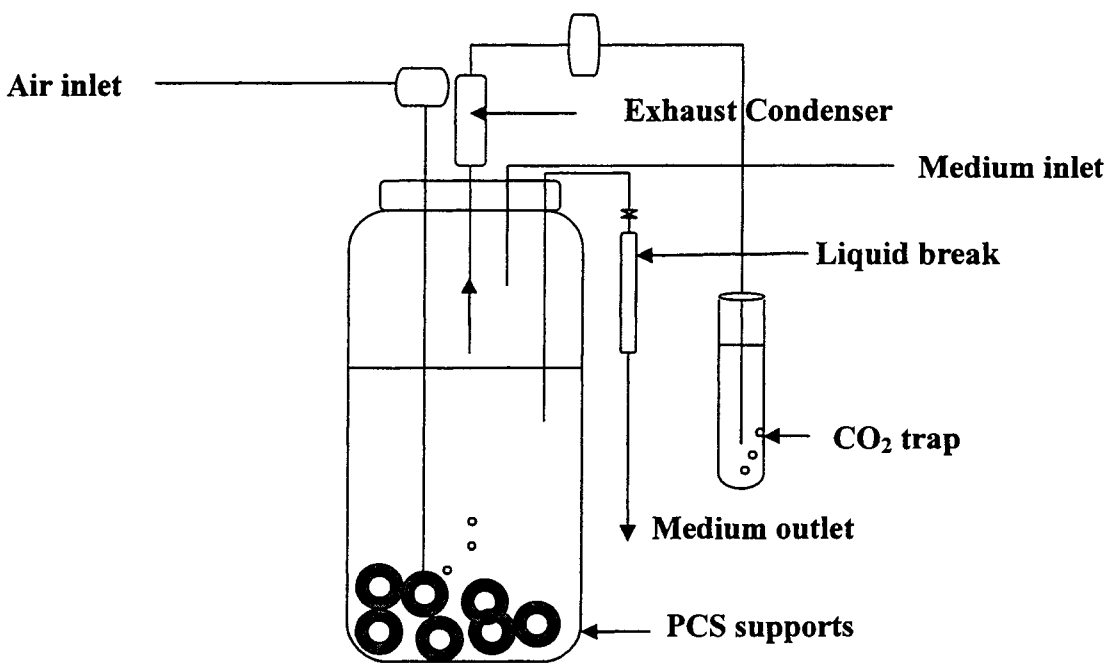
LB medium contained 10 g/L of tryptone peptone (Difco Industries, Detroit, MI), 5 g/L of yeast extract (Ardamine Z, Sensient Flavors, Juneau, WI), 5 g/L of sodium chloride, and 0.5 g/L of cane molasses (Diamond V Mills, Cedar Rapids, IA). The pH was adjusted to 7.0 with 1 N NaOH prior to autoclaving. Light corn steep liquor was obtained from Penford Products Company. Mineral salts solution contained 3.0 g/L of $(NH_4)_2SO_4$, 5.03 g/L of Na_2HPO_4, 1.98 g/L of KH_2PO_4, 0.20 g/L of $MgSO_4 \cdot 7H_2O$, 0.20 g/L of NaCl, 0.05 g/L of $CaCl_2 \cdot 2H_2O$, and 1 ml/L of trace element solution [Pridham and Gottlieb 1948].

Initial repeated-batch fermentations for enrichment

Customized 500-ml reactors using glass wide-mouthed bottles (48 mm screw cap size) fitted with silicone stoppers were designed for small-scale repeated-batch fermentations (Fig. 2). A sand bath was used to maintain temperature at >90°C. Each reactor was continuously aerated with filter-sterilized CO_2-free humidified air [Pometto et al. 1998]. The CO_2 in the exit gas was trapped by 10 ml of 2 N NaOH. Sterile fresh medium was replaced every 7 to 14 days and spent medium pH was recorded. For some fermentations, a piece of Whatman No.1 filter paper (7 cm diameter) was placed at the bottom of the reactor. Fifty ml (vol/vol) of different PCS blends were placed in the reactors to promote biofilm formation

and to provide slow release of nutrients. The reactor was autoclaved with 10 ml of deionized water at 121°C for 30 minutes. Each reactor contained 100 ml of medium. Three sets of experiments were performed: 1) an initial enrichment study on all sites sampled, 2) an enrichment study of selected sites using a rich medium, and 3) a confirmation study employing rich and minimal media on selected potentially positive samples obtained from the enrichment study.

Fig. 2 Reactor design for isolation of hyperthermophiles at 95°C



Continuous and repeated-batch biofilm fermentations of selected samples from corn wet milling facility

A B-Braun BioStat-M reactor (Allentown, PA) was used in this phase of the research. Using the bioreactor design of Velazquez et al. [2001] a customized stainless steel basket was placed around the agitator shaft to separate the PCS discs from the agitator. The vessel was loaded with 350 ml (vol/vol) of each SSHBAYES and FSHBAYES PCS blends. Three pieces of Whatman No.1 filter paper (11 cm diameter) were placed at the bottom of the reactor. The reactor working volume was 880 ml. The reactor was sterilized with 50 ml of deionized water for 90 minutes at 121°C. After sterilization, the water was aseptically drained and sterile LB medium was added. During the temperature screening experiment, the reactor temperature was set at 55, 65, 75, 80 and >90°C with an external circulator water bath set at 57, 68, 80, 85 and 97°C. Agitation speed was 150 rpm. The pH was automatically controlled at pH 6.0 with sterile 3 N HCl or 1 N NaOH. Filter sterilized air was continuously sparged through the medium (51 ml/min). The exit gas was scrubbed to monitor CO_2 production via four culture tubes in series, each containing 10 ml of 4 N NaOH.

Sample analysis

CO_2 evolution was measured every 7 days for 500-ml customized reactors and every 1-2 days for B. Braun reactor by pH titration using a Mettler DL-12 automatic titrator (Metler-Toledo, Hightown, NJ) [Pometto et al. 1998]. Suspended cell biomass was measured indirectly by absorbance at 620 nm using a Spectronic® 20 Genesys™ (Spectronic Instruments, Rochester, NY). Gram staining was performed on cell pellets obtained from centrifugation at 8816 x *g* for 15 minutes at 4°C of spent culture medium and of sand

stripped cells from PCS biofilm. At the end of the run supports and suspended cells were recovered and stored separately at -75°C in sterile 20% glycerol in MRS medium.

Quantitative microbial population was determined by *BacLight* Live/Dead bacterial viability test kit (Molecular Probes, Eugene, OR) in conjunction with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL) [Demirci et al. 1999].

The presence of extracellular hydrolytic enzymes in spent culture medium was tested. Spent culture medium was concentrated by 6 kD dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) with polyethylene glycol (Sigma Chemical Company, St. Louis, MO) for 24 hours at 4°C. The dialysate was resuspended in 10 ml of 0.06 M sodium acetate buffer and stored at 4°C until ready to use. The hydrolytic enzymes assayed in buffer were cellulase, hemicellulase, and xylanase with commercial cellulose (SigmaCell; Sigma Chemical Company), carboxymethylcellulose (Sigma Chemical Company), defatted ground soyhulls, and xylan (Sigma Chemical Company). For all enzymes the assays were carried out at 65°C by the Somogyi-Nelson analysis of reducing sugars [Wood and Bhat 1988].

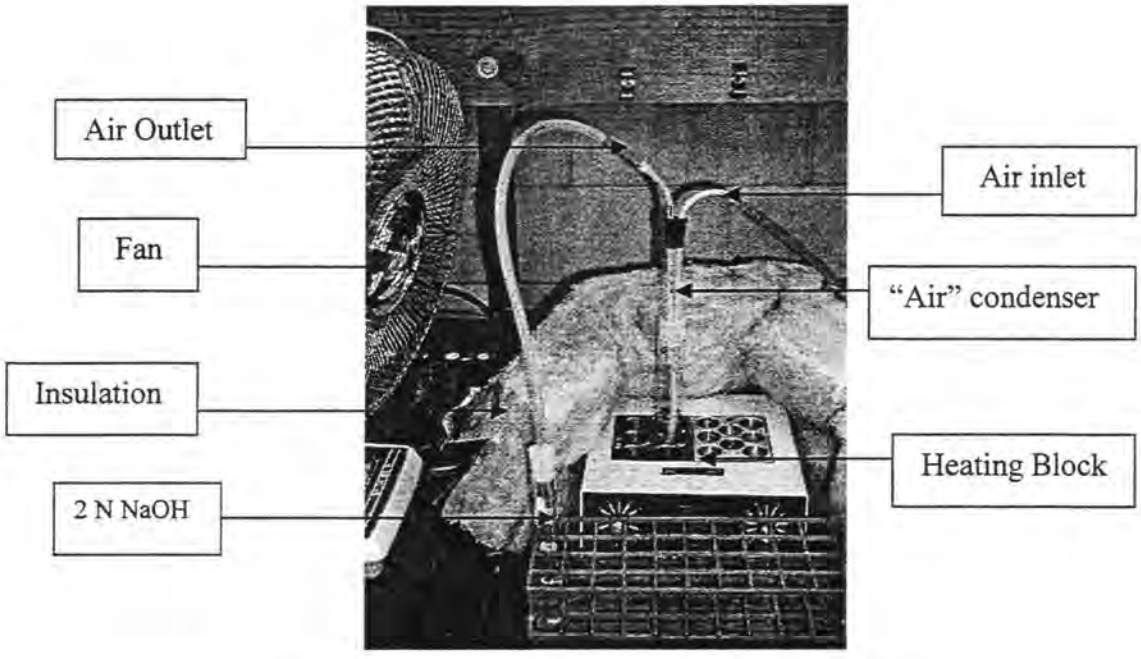
A plate assay employing the Congo Red stain was performed for xylanase and carboxymethylcellulase. Each plate contained 125 mg agarose, 12.5 mg CMC or xylan, and 23 ml of sodium acetate buffer (0.06 M, pH 6.0). Wells of 4 mm were cut using a sterile cork borer (no. 1) and the gel removed by aseptic suction. The wells were inoculated with concentrated spent culture medium and incubated at 65°C for 2 to 114 hours. Zones of hydrolysis were made visible by the addition of 20 ml of Congo Red solution (1 mg/ml dH_2O) for 15 minutes, then washed with water and stained by 1 M sodium chloride solution for 10 minutes. Positive enzyme activity was indicated by a clear zone around the sample well. For positive controls commercial cellulases and xylanases from *Trichoderma viridae*

(Sigma Chemical Company) were used. An estimate of quantitative enzyme production relative to diffusion zones was determined by methods of Wood and Bhat [1988].

Substrate-specific bubbler culture-tube study

A novel bubbler culture-tube setup was utilized to evaluate isolates from repeated-batch fermentors for hydrolytic enzymes (Fig. 3). Cellulase, hemicellulase, and cellobiase activity was evaluated based on CO_2 production of cultures grown in the tubes. Bubbler culture-tube setup consisted of a 25 x 150 mm culture tube with a 15 x 120 mm glass extension tube as an air condenser, cotton plugged air inlet and outlet tubes, a submerged air inlet tube with reduced orifice for delivery of small air bubbles, and a CO_2 trap consisting of 10 ml of 4 N NaOH. Temperature was controlled at 65, 75, 85 and >90°C via a heating block (Model #11-718-2 and #11-718-4; Fisher Scientific, Pittsburgh, PA). CO_2-free air was sparged slowly into the bubbler tubes for aeration (<15 ml/min). The unit was wrapped in fiberglass insulation to reduce heat loss. Tubes contained 20 ml of LB medium (pH 7.0) or 20 ml of mineral salts solution with nitrogen (pH ~7.2) supplemented with 0.5% yeast extract. Substrates were evaluated at 2% (wt/vol). The substrates evaluated were cellulose, defatted ground soyhulls, carboxymethylcellulose and cellobiose (Sigma Chemical Co.). Controls were set up without inoculation. CO_2 evolution traps contained 10 ml of 2 N NaOH. All treatments were performed in triplicate. CO_2 evolution was determined by pH titration with standardized 0.4 N HCl every 7 days by an automatic titrator.

Fig. 3 Novel bubbler tube setup at 95°C



Results

Spray drier cable repeated-batch fermentations

No temperature gradient was observed throughout the exhaust of the spray drier at the CCUR site and all PCS were exposed to a temperature of ~73°C during spray drying. Cumulative CO_2 production, pH of spent medium, cell count results and Gram staining observations are summarized in Tables 2 and 3. In all cases CO_2 production was higher for cultures grown in LB medium than in mineral salts solution with nitrogen. All samples from Proliant spray drier in LB medium demonstrated higher CO_2 production than in the uninoculated control, thus indicating biological activity. Also, samples PROL225 and PROL285 exhibited the highest CO_2 production, and Gram negative small rods were observed (Fig. 4). Cell count results, however, were contradictory, as cell counts from both

Table 2 Cultures recovered from Proliant Inc. (PROL) spray drier cable, repeated-batch at 95°C for 26 weeks in LB and mineral salts solution with $(NH_4)_2SO_4$

Sample[a]	Average CO_2 production (mg CO_2/week)		Cell count on stripped PCS (cells/g PCS)[b]		Gram staining observations		pH of spent medium	
	LB[c]	MS[c]	LB	MS	LB	MS	LB	MS
PROL045	**15.0**	8.1	5.3×10^7	1.7×10^8	No visible cells	No visible cells	4.7-6.3	4.8-6.7
PROL105	**15.8**	6.9	9.3×10^7	2.7×10^8	G+ cocci	No visible cells	4.7-6.2	5.0-6.5
PROL165	**16.0**	8.5	Not determined	2.2×10^8	No visible cells	No visible cells	4.7-5.3	4.8-6.3
PROL225	**13.5**	**9.3**	5.3×10^7	2.1×10^8	No visible cells	No visible cells	4.9-6.1	4.6-5.2
PROL285	**15.9**	**12.3**	6.6×10^7	6.4×10^7	G- small rods	No visible cells	4.9-6.3	4.5-6.4
Control[d]	11.0	9.0	1.0×10^8	2.3×10^8	No visible cells	No visible cells	5.3-6.4	4.8-6.2

a) Sample code indicates distance of supports inside the exhaust; PROL045 indicates supports were 45 cm down the exhaust on average, PROL285 indicates supports were 285 cm deep in exhaust on average

b) Determined by *BacLight* fluorescent stain and flow cytometry from PCS harvested at the end of experiment by the stripping sand method [Ho et al. 1997]

c) Medium used: LB: LB medium; MS: Mineral salts solution with $(NH_4)_2SO_4$. Refer to Materials and Methods for list of components

d) Controls contain uninoculated culture bottles with sterile medium and same treatment

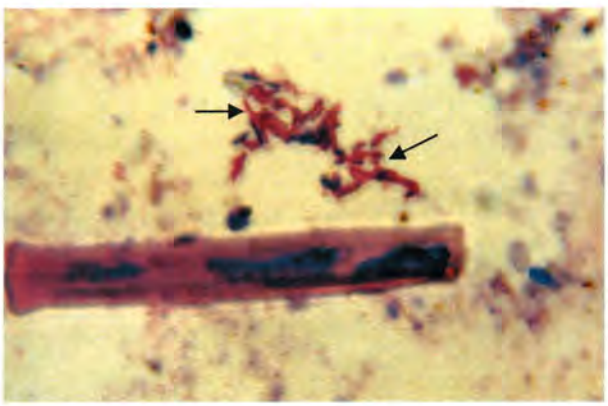
Table 3 Cultures recovered from Center for Crops Utilization Research (CCUR) spray drier cable, repeated-batch at 95°C for 26 weeks in LB and mineral salts solution with $(NH_4)_2SO_4$

Sample[a]	Average CO_2 production (mg CO_2/week)		Cell count on stripped PCS (cells/g PCS)[b]		Gram staining observations		pH of spent medium	
	LB[c]	MS[c]	LB	MS	LB	MS	LB	MS
CCUR045	11.8	8.6[d]	2.6×10^5	Not determined	No visible cells	No visible cells	4.7-6.4	4.7-6.9
CCUR105	**13.2**[e]	5.4[d]	2.1×10^5	Not determined	No visible cells	No visible cells	5.2-6.4	5.1-6.8
CCUR165	**12.5**	**10.4**	1.8×10^6	2.0×10^5	G- rods	No visible cells	4.7-6.2	4.4-6.7
CCUR225	10.6	7.4	1.8×10^5	2.8×10^5	No visible cells	No visible cells	4.7-6.2	4.8-6.7
CCUR285	10.7	8.8	3.2×10^5	1.8×10^6	No visible cells	G+ cocci	4.8-6.2	4.8-6.7
Control[f]	11.4	9.5	6.9×10^5	3.0×10^5	No visible cells	No visible cells	4.8-6.1	4.9-6.6

a) Sample code indicates distance of supports inside the exhaust; CCUR045 indicates supports were 45 cm down the exhaust on average, CCUR285 indicates supports were 285 cm deep in exhaust on average
b) Determined by *BacLight* fluorescent stain and flow cytometry from PCS harvested at the end of experiment by the stripping sand method [Ho et al. 1997]
c) Medium used, LB: LB medium; MS: Mineral salts solution with $(NH_4)_2SO_4$. Refer to Materials and Methods for list of components
d) Final cell count could not be determined because of equipment failure
e) Sample CCUR105 in LB medium went for 21 weeks instead of 26 weeks
f) Controls contain uninoculated culture bottles with sterile medium and same treatment

samples were smaller than those of the controls. For example, PROL165 released the most CO_2 during the 26-week period (average 16 mg/week) but no cells were visible under the light microscope. This may be due to high concentration of background material. Gram positive cocci and a high CO_2 production were observed in PROL105, but again cell counts were lower than the control. No correlation between spent culture medium pH and biological activities was observed; the range was very similar for the samples and the controls.

Fig. 4 Gram positive rods observed on sand-stripped PCS of PROL285. Magnification: 1000x



Samples from the CCUR spray drier cable showed less uniformity, as only three samples in LB (CCUR045, 105, 165, grown in LB) showed a higher CO_2 production than the uninoculated control. However, CCUR 165 did demonstrate a higher CO_2 production than the control when grown in both rich and minimal media. Cell count results indicated that CCUR165 grown in LB was higher than the control, and Gram negative rods were observed. Again no correlation between spent medium pH and biological activity was observed.

The two sets of controls, undergoing the same treatment, showed significant differences in their total cell count via flow cytometry (10^5 range for CCUR cable versus 10^8 range for Proliant cable). This discrepancy in cell counts between the two sets of controls may be due to background fluorescence from soybean hull fragments released into the culture medium. A study involving autoclaving PCS sinkers and floaters in LB for 72 hours followed by *BacLight* fluorescent stain and flow cytometry confirmed this finding with it reporting 10^7 to 10^8 cells/g of PCS. Gram negative rods and Gram positive cocci were observed in four samples (Tables 2 and 3). However, we were not able to obtain sufficient biomass in subsequent scale-up experiments at 80°C. Thus we conclude that some thermophiles may have survived but they were not stable at >90°C.

Isolation of hyperthermophiles from a corn wet milling facility

Nine enrichment studies were performed via customized 500-ml repeated-batch reactors (Table 4). Repeated-batch fermentations were carried out at >90°C as outlined in materials and methods. Cultures from sampling sites 1 (evaporator water), 4 (suet stack pipe), 5 (burnt fiber from drier) and 6 (evaporator tank wall) did not show positive enrichment cultures and were not studied further. Samples 2 (hot corn steep liquor), 3 (dry material on CSL pipe), 7 (biofilm from exhaust stack), 8 (scrapings from fiber drier dust window) and 9 (microbial consortium from light corn steep liquor recovered via continuous PCS biofilm culture at 55°C) were used for additional enrichment studies using LB with cane molasses medium. Supernatant and PCS biofilm consortia showed Gram positive cocci, Gram positive rods and Gram negative rods (Table 5). PCS biofilm culture demonstrated 10^7 to 10^8 cells/g of PCS as determined by *BacLight* fluorescent stain and flow cytometry. Highest CO_2

production came from cultures PPC1.b1 (light corn steep liquor consortium) grown for 4 months in LB medium with cane molasses at pH 7.

Table 4 Sampling sites from corn processing facility

Site	Description	Give Positive Enrichment Cultures?
1	Evaporator Water	No
2	Hot Corn Steep Liquor	Yes
3	Dry Material on CSL Pipe	Yes
4	Suet Stack Pipe	No
5	Burnt Fiber from Drier	No
6	Evaporator Tank Wall	No
7	Biofilm from Exhaust Stack	Yes
8	Scrapings from Fiber Drier Dust Window	Yes
9	Microbial Consortium from LCSL	Yes

Table 5 Culture collection of hyperthermophiles from a corn processing facility, isolated at >90°C

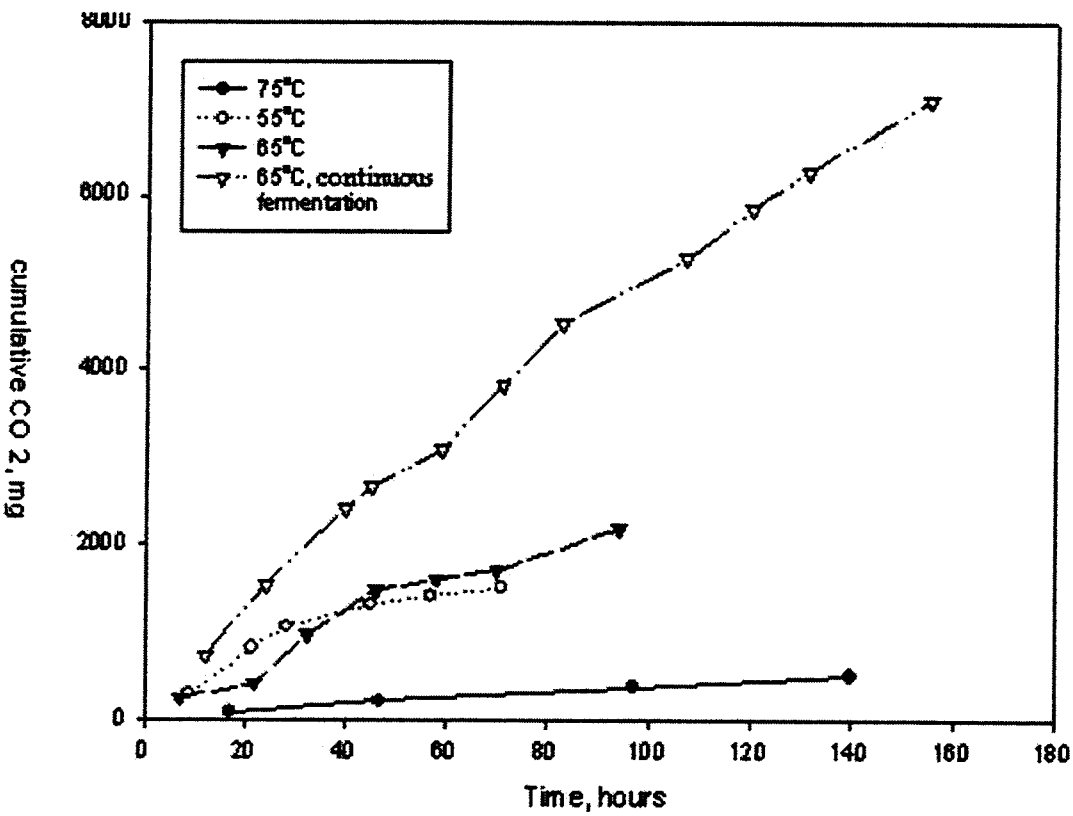
Source	Short Name	Average CO_2 production, (mg/week)[a]	Cell Count on PCS (cells/g PCS)	Gram Staining of PCS Biofilm	Run Length	PCS in Reactor[b]
Light CSL (Penford Products, Cedar Rapids, Iowa)[c]	PPC1	52.5	2.4×10^8	G+ rods, G- cocci	7 months	SSHBAYE+
Light CSL for 7 months at >90°C with SHYE[d]	PPC1.a	2.8	1.2×10^8	G+ rods, G+ cocci	9 months	SCEBAYE+
Light CSL for 7 months at >90°C with SHYE[d]	PPC1.b	5.6	5.3×10^7	G+ rods	9 months	SSHBAYE+ WF
Light CSL at >90°C for 7 months with SSHBAYE+ and cellulose paper[d]	PPC1.b1	490	2.4×10^7	G+ cocci	4 months	50% v/v SCEBAYE+ 50% v/v FCEBAYE+ WF
Light CSL for 7 months at >90°C with SHYE[e]	PPC1.c	7.0	8.5×10^7	G+ rods	2.5 months	FCEBAYE+ WF
Light CSL from 55°C continuous run with SHYE[d]	PPC1.1	12.6	1.3×10^8	G- rods	9 months	SSHBAYE+
Hot CSL (Penford Products, Cedar Rapids, Iowa)[d]	PPCS2	5.6	1.3×10^8	G+ cocci, G+ rods	9 months	SHYE+
Dry material on CSL pipe (Penford Products, Cedar Rapids, Iowa)[d]	PPCS3	19.6	2.0×10^8	G+/- rods, G+ cocci	9 months	SHYE+
Biofilm from stack (Penford Products, Cedar Rapids, Iowa)[d]	PPCS7	4.6	3.6×10^8	G+/- rods	9 months	SHYE
Scrapings from drier window (Penford Products, Cedar Rapids, Iowa)[d]	PPCS8	3.5	2.8×10^8	G+ cocci, G+ rods	9 months	SHYE

[a]Respective controls (sterile medium with same treatment) were subtracted from reported numbers
[b]PCS abbreviations: refer to Table 1; wf indicates a piece of Whatman No. 1 filter paper at bottom of reactor
[c]medium contains non-sterile light corn steep liquor
[d]medium contains per liter distilled water: 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl, 0.025% v/v cane molasses, pH 7.0
[e]medium contains per liter distilled water: 10g Tryptone, 5 g Yeast Extract, 5 g NaCl, 0.025% v/v cane molasses, 0.5% cellobiose (Sigma Chemical Company, St. Louis, MO), pH 7.0

Repeated-batch and continuous fermentations of isolate PPC1 (Light CSL) from corn processing facility

Isolate PPC1 was chosen to study at a lower temperature because of its ability to form a visible biofilm at 55°C. A 1-L fermentor was employed. The sample was most active at 65°C compared to 55 and 75°C based on CO_2 production (Fig. 5). Gram positive rods were observed and biochemical tests done by Silliker (South Holland, IL) suggested the culture was most probably *Bacillus stearothermophilus*.

Fig. 5 CO_2 production of PPC1 at different temperatures in 1-L repeated batch fermentations.



Repeated-batch Fermentations of Selected Cultures from Corn Processing Facility

From our culture collection, cultures were selected based on their CO_2 production, and a parallel study was performed using complex and minimal media at 95°C (Table 6). Cultures showed Gram positive cocci and Gram negative rods, which were also observed in the preliminary experiment. PCS biofilm ranged from 10^7 to 10^8 cells/g of PCS, which was consistent with previously described results. Highest CO_2 production came from culture PPC1.1 (light corn steep liquor) with 16.89 and 14.25 mg/week in LB and MS medium, respectively. However only a few Gram negative rods were observed under light microscope, because of the presence of high background material (Fig.6).

Fig. 6 Gram negative rods observed on sand-stripped PCS of PPC1.1. Magnification: 1000x

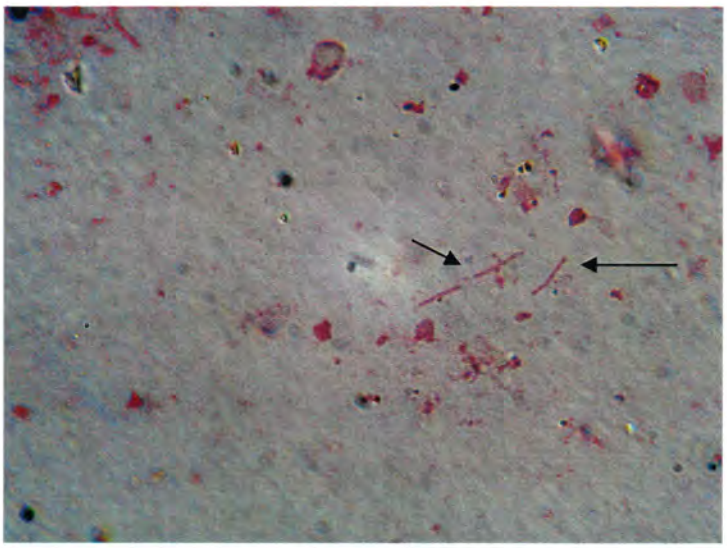


Table 6 Parallel study of selected cultures from a corn processing facility, 6 months at >90°C

Sample[a]	Average CO_2 production (mg/week)		Cell Count on PCS (cells/g PCS)[b]		Gram Staining of PCS	
	LB[c]	MS[c]	LB	MS	LB	MS
PPC1.b1	12.87	8.02	8.8×10^7	2.2×10^8	G+ cocci/G- rods	Few G- rods
PPCS3	11.33	6.20	6.9×10^7	5.0×10^8	Few G- rods	No visible cells
PPC1.1	16.89	14.25	1.0×10^8	8.6×10^7	No visible cells	Few G- rods
PPCS2	10.80	7.67	6.8×10^7	2.2×10^8	No visible cells	No visible cells
PPCS7	13.00	5.65	9.8×10^7	1.0×10^8	No visible cells	No visible cells
PPCS8	12.51	6.28	5.9×10^7	9.9×10^7	No visible cells	No visible cells
Control[d]	12.80	8.26	Not available	Not available	Not available	Not available

[a]refer to Table 4 for sample short name; reactors contained 25 ml (vol/vol) SSHBAYE+ and 25 ml (vol/vol) FSHBAYE+
[b]Determined by *BacLight* fluorescent stain and flow cytometry from PCS harvested at the end of experiment by the stripping sand method [Ho et al. 1997]
[c]Medium used, LB: LB medium; MS: Mineral salts solution with $(NH_4)_2SO_4$. Refer to Materials and Methods for list of components
[d]Controls CO_2 values are average of previous runs; used as a comparison

Tests for the Presence of Extracellular Hydrolytic Enzymes

Enzymatic activity was not detected at 65°C for all cultures grown at >90°C. Culture PPC1 grown at 55-75°C demonstrated positive activities on LB plates supplemented with xylan. Zones of hydrolysis were observed upon Congo Red staining (Fig. 7). A maximum xylanase activity of 1.6 μg of xylose/hr/mg protein was detected (Table 7).

Fig. 7 Congo Red staining on plates with agarose and xylan for continuous culture of PPC1 at 65°C

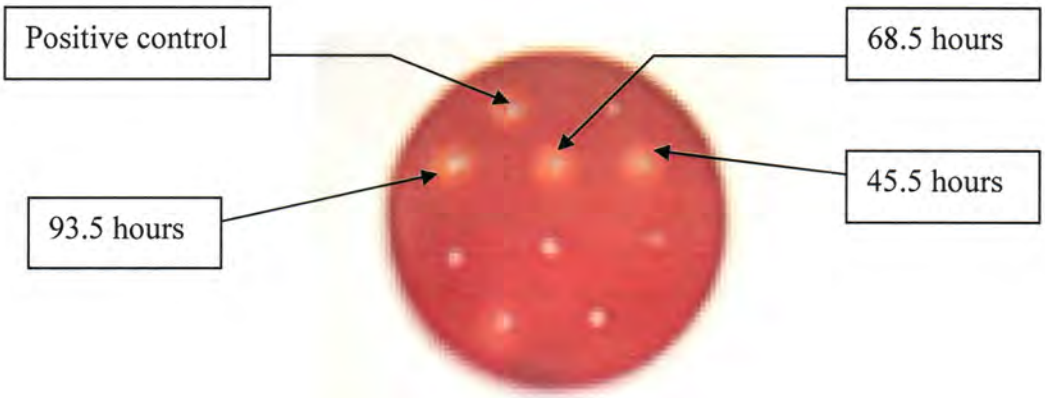


Table 7 Culture conditions and specific xylanase activity from repeated batch and continuous fermentation of sample PPC1. Continuous fermentation with 0.2 h^{-1} starts at 72 hours. Protein concentration was determined by Lowry assay. Specific activity was determined by Somogyi-Nelson analysis of reducing sugars at 65°C.

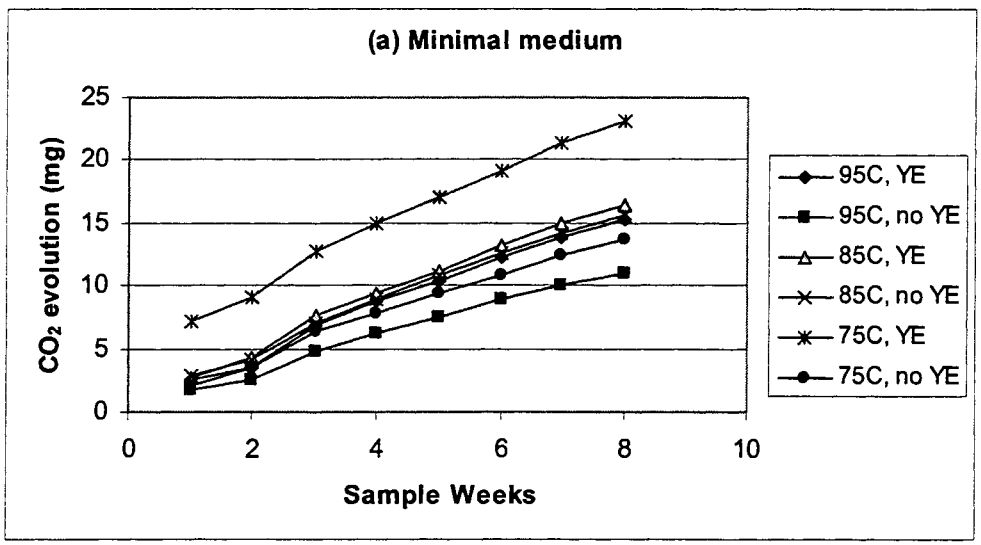
Fermentation Time (hrs)	Culture pH	Culture Temperature (°C)	Protein (mg/ml)	Specific Activity (μg xylose/h/mg protein)
2	6.6	65	0.45	0
17.5	7.4	65	0.9	0
45.5	7.8	64.2	Not determined	Not determined
68.5	7.4	63.8	1.7	1.36
93.5	7.2	62.4	2.6	1.6
113.5	7.1	60	0.8	0

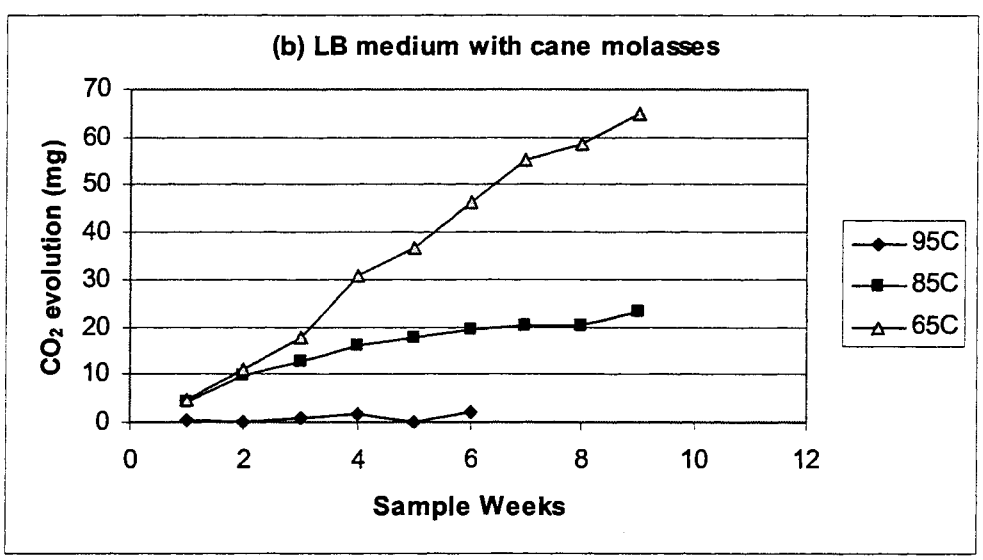
Substrate-specific Bubbler Culture-tube Study

Only a few tubes showed positive activity. Sample PPC1.b (Light CSL) showed a positive reaction based on CO_2 production in bubbler tubes containing cellulose (Figure 8a). Gram negative rods were observed under the light microscope at termination in tubes with yeast extract incubated at 75 and 85°C. Highest CO_2 production (23 mg over 8 weeks) was observed when the culture was grown at 75°C with 0.5% yeast extract in mineral salts with nitrogen. Thus, CO_2 production decreases as temperature goes up, and CO_2 production is increased when culture was supplemented with yeast extract than when it was omitted from the medium. This indicated that the optimum temperature for the microorganism was closer to 75°C than 95°C, and a requirement of yeast extract for growth.

The observations were very similar when sample PPC1.b was grown under rich medium (LB with cane molasses, pH 7.0) (Figure 8b). CO_2 production increases with a decrease in temperature, and reached the highest at 65°C (65 mg over 9 weeks, control subtracted). The tube became visibly turbid indicating very vigorous microbial growth (Gram negative rods under light microscope). Again this indicated sample PPC1.b having a lower temperature optimum. Results from other substrates were inconsistent among the triplicates.

Fig. 8 Cumulative CO_2 production for PPC1.b grown in (a) mineral salts solution with $(NH_4)_2SO_4$, supplemented with 0.5% yeast extract and 2% sigmacellulose, and (b) LB with cane molasses at pH 7 in a bubbler tube, at different temperatures





Discussion

The two sets of spray-drier experiments indicated no hyperthermophilic microorganisms, since only a few cells were observed at >90°C. Initial cell count on PCS cable had ~5 x 10^7 cells/g of PCS. Another possible source of microbial collection is the spray drier dust bags. Furthermore, enrichment at the exhaust temperature of 73°C might yield some isolates.

Biological activity was observed in cultures obtained from a corn processing facility. Corn steep liquor has been reported to contain a variety of lactic acid bacteria [Blanchard 1992], and some strains might be able to survive at >90°C. Hence >90°C can be used as a screening tool for isolating thermophilic microorganisms that possibly grow at a lower temperature, e.g. 70-80°C.

The very low activity observed in the substrate-specific bubbler tube cultures indicated that none utilized these polysaccharides very well at >90°C. Under temperature stress cells might shut down their production of these hydrolytic enzymes, which they otherwise would use at a lower temperature (e.g. xylanase production at 65°C).

Our ability to look for cells under the light microscope was significantly hindered because of the high background material on spent medium and from PCS biofilm sand-stripped supports. These background material most likely came from soyhulls in the PCS, pieces of filter paper in reactors from prolonged heat treatment, and sand from sand stripping of PCS biofilm. This and the low cell mass made observing cells under the light microscope nearly impossible. Attempts to improve the staining process were carried out, including filtering the cell solution with a Whatman No. 1 filter paper prior to Gram staining, and also an alternative staining method (DAPI), which binds to double-stranded DNA and gives

fluorescence under a blue filter. Both methods were unsatisfactory. A successful method to filter out background material remains a major task in future research.

Biological activity was determined by CO_2 production, *BacLight* live/dead fluorescent stain and flow cytometry, and Gram staining during most initial isolations. However, attempts to confirm microbial growth in sequential studies proved negative at >90°C but positive at thermophilic temperatures (65-70°C). This suggests that stressed thermophiles were being isolated and not hyperthermophiles. Thus, future work on isolation of thermophilic microorganisms would be more productive. Finally, protocols were developed for isolating thermophilic and hyperthermophilic microorganisms from these high-temperature food processing environments.

Acknowledgements

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CHARACTERIZATION OF A NEW ISOLATE FROM LIGHT CORN STEEP LIQUOR, *BACILLUS THERMOZEAMAIZE* SP. NOV.

A paper to be submitted to the International Journal of Systematic and Evolutionary Microbiology

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Abstract

Continuous and repeated-batch fermentations using a culture obtained from light corn steep liquor were performed in a customized bioreactor. Plastic composite supports (PCS) containing polypropylene and agricultural by-products were used to promote biofilm formation for culture isolation. The bioreactor was operated for 2.5 months at pH 6.0 and temperature between 55 and 75°C. LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] sodium chloride and 0.05% [wt/vol] cane molasses) was used in the study. Biological activity was monitored via CO_2 production, microscopic examination of spent culture broth, cell count by fluorescent stain and flow cytometry, and plating on 2% (wt/vol) agar using different substrates. Hydrolytic enzyme production from isolate L-10997 was tested by plate assay and by inoculating colonies in bubbler tubes containing 1% (wt/vol) tryptone and 0.5% (wt/vol) yeast extract and a different carbon source. Results indicated that isolate L-10997 is a motile, aerobic, gram-positive bacilli that grows in the temperature range 45-73°C. It produces subterminal, ellipsoidal spores and forms smooth, opaque colonies with a shiny appearance and smooth edges. Optimum pH for growth is 6.0-8.0. Doubling time is 56 hours at 63°C under aerobic conditions. This isolate was further characterized on the basis of biochemical and nutritional properties. Sequencing of 500 bp and 1500 bp of bacterial 16S

rRNA gene indicated that isolate L-10997 was related to *Bacillus thermoglucosidasius* and *Geobacillus thermocatenulatus* (formerly *Bacillus thermocatenulatus*). Based on the phylogenetic and physiological differences this isolate represents a new species of aerobic thermophilic *Bacillus* with an optimal temperature of around 65°C, which we proposed the name *Bacillus thermozeamaize*. The type strain of this species is strain L-10997 (ATCC BAA-739).

Key words: *Bacillus thermozeamaize* – Obligate thermophile – corn steep liquor

INTRODUCTION

"Extremophiles" are microorganisms that are adapted to survive and grow in extreme environments such as extremes of temperature, pH, high salt concentrations, and high pressures [Adams, 1993; Adams, 1995]. Extensive studies of ecology, physiology, and molecular biology over the past two decades have demonstrated just how different extremophiles are from other microorganisms. The most common extremophilic biotopes are of geothermal origin, for example, in submarine solfataric fields, areas surrounding volcanoes, and subterranean hydrothermal sea floors. Potentially, extremophiles can also be isolated from the man-made extreme environments that are often associated with the food processing industry, such as corn wet milling, spray drying, and syrup production. The increased interest in these microorganisms is due to their biotechnological resources that are expected to result in novel process applications. The goal of this research was to isolate new thermophilic microorganisms from light corn steep liquor (LCSL) which produce key

thermostable hydrolytic enzymes to improve industrial wastestream processing or to improve food processing.

A preliminary characterization of these bacteria based on cell shape, spore formation, and several phenotypic features indicated that they are thermophilic organisms that belong to the genus *Bacillus*. These isolates were further investigated by partial sequencing of their 16S rRNA. Isolate L-10997 exhibited a unique rRNA sequence and was chosen for further analysis. The results of 16S rRNA sequencing indicated that isolate L-10997 was related to *Bacillus thermoglucosidasius* and *Geobacillus thermocatenulatus* (formerly *Bacillus thermocatenulatus*). Isolate L-10997 was compared with *B. thermoglucosidasius* (ATCC 43732), *G. thermocatenulatus* (DSM 730), and *B. stearothermophilus* (ATCC 7953) for their physiological differences. Based on these differences, we confirmed strain L-10997 as a new thermophilic species of the genus *Bacillus*.

METHODS

Organisms. *Geobacillus thermocatenulatus* (DSM 730) was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. *Bacillus stearothermophilus* (ATCC 7953) was obtained from American Type Culture Collection (Manassas, VA).

Plastic composite supports (PCS). PCS are customized solid supports developed at Iowa State University to stimulate biofilm formation [Pometto *et al.*, 1997] and for this study to enhance isolation of thermophilic microorganisms from light corn steep liquor. PCS discs were produced that would sink or float in culture medium. PCS blends that sink contained

50% (wt/wt) polypropylene (Quantum USI Division, Cincinnati, OH), 40% (wt/wt) dried ground soybean hulls (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (wt/wt) dried bovine albumin (Proliant, Ames, IA), 5% (wt/wt) yeast extract (Ardamine Z; Sensient Flavors, Juneau, WI) and mineral salts [Ho *et al.*, 1997], abbreviated SSHBAYES, whereas PCS blends that float contained 70% (wt/wt) polypropylene, 20% (wt/wt) dried ground soybean hulls, 5% (wt/wt) dried bovine albumin, 5% (wt/wt) yeast extract and mineral salts, abbreviated FSHBAYES. All PCS blends were produced by high-temperature extrusion using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W.Brabender Instruments, Inc., South Hackensack, NJ) by using the procedures described by Ho *et al.* [1997]. PCS tubes were cut manually into discs (0.4 cm ID, 1.0 cm OD) with a utility knife.

Microbial consortia recovery from light corn steep liquor (LCSL). Light corn steep liquor is the product from corn steeping, which is the initial step in the corn wet milling process to produce corn starch, corn oil, corn syrup, etc [Blanchard, 1992]. A continuous four-bioreactor train was constructed to recover microorganisms from LCSL. Reactor one was a 15-L microferm fermentor (New Brunswick Scientific, Edison, NJ) with pH 5.0 and 45°C temperature control and a 10-L working volume. This was followed by three 1-L customized bioreactors without PCS [Ho *et al.*, 1997] with pH monitoring and temperature controlled via a 45-48°C water bath. The last reactor was a packed bed biofilm reactor filled with 190 g/L PCS disc composed of 50% (wt/vol) polypropylene, 35% (wt/vol) ground soybean hulls, 5% (wt/vol) yeast extract, 5% (wt/vol) bovine albumin, 5% (wt/vol) soybean flour, and mineral salts [Ho *et al.*, 1997]. The bioreactor train was fed LCSL from Penford Products Company (Cedar Rapids, IA) with an overall dilution rate for the train of 0.05 h^{-1}

and a biofilm reactor dilution rate of 0.75 h^{-1}. Reactor train was operated for 7 days. For culture preservation PCS biofilm cultures were aseptically placed into sterile capped tubes, filled with 20% glycerol in Difco MRS Lactobacilli medium (Difco Industries, Detroit, MI), then stored at -75°C. Fermentation inoculum was prepared by the stripping sand method described by Ho *et al.* [1997]. Five pieces PCS discs containing LCSL PCS biofilm consortia were shaken vigorously for 5 sec in 0.1% (wt/vol) peptone, then transferred to a culture tube containing 9 ml of 0.1% (wt/vol) peptone and 5 g of sand. The tube was vortexed in intervals of 30 seconds for a total of 1.5 min. Five milliliter of the solution was used to inoculate the bioreactor.

Medium preparation. LB medium was prepared by sterilizing 10 g/L tryptone peptone (Difco Industries), 5 g/L Ardamine Z yeast extract, 5 g/L sodium chloride, and 0.5 g/L cane molasses (Diamond V Mills, Cedar Rapids, IA) in 90 L of deionized water in a 100-L sterilizable-in-place fermentor (IF 150 Fermentor, New Brunswick Scientific, Edison, NJ) with continuous agitation for 24 minutes at 121°C. The pH was adjusted to 6.0 using sterile 3 N HCl. The 90 L of LB medium was aseptically transferred to two sterilized (121°C for 3 h) 50-L carboys. The carboys were equipped with a medium transfer line with stopcock, a medium delivery line with a liquid break, and two 0.45μm air filters.

Continuous and repeated-batch fermentations. A B-Braun BioStat-M reactor (Allentown, PA) was used in this study. Based on the bioreactor design of Velazquez *et al.* [2001] a customized stainless steel basket was placed around the agitator shaft to separate the PCS discs from the agitator. The vessel was loaded with 350 ml (vol/vol) of PCS blends

SSHBAYES and FSHBAYES. Three pieces of Whatman 1 filter paper (11 cm diameter) were placed at the bottom of the reactor. The reactor working volume was 880 ml. The reactor was sterilized with 50 ml of deionized water for 90 minutes at 121°C. After sterilization, the water was aseptically drained and sterile LB medium was added. The reactor temperature was set initially at 55°C with an external circulator water bath. Agitation speed was initially at 100 rpm, and then increased to 150 rpm. A pH of 6.0±0.1 was maintained automatically with sterile 3 N HCl or 1 N NaOH. Filter-sterilized air was continuously sparged through the medium (51 ml/min). The exit gas was trapped to monitor CO_2 production via a series of four tubes with 10 ml of 4 N NaOH. The reactor was inoculated with 5 ml of culture from LCSL PCS biofilm consortia. The bioreactor was operated as a batch culture until biofilm became visible, then changed to continuous culture at 0.1 h^{-1} dilution rate for one day, then 0.2 h^{-1} dilution rate for 6 days. At the end of the period the reactor was returned to batch and temperature was increased to 65°C. This process was repeated for 70 and 75°C with similar pattern of run time and dilution rate. The goal was to slowly change the LCSL biofilm consortia microbial population to all thermophiles. The PCS were recovered aseptically and placed in sterile 20% glycerol in MRS medium and stored at -75°C.

Fermentation sample analysis. Production of CO_2 was measured by pH titration via automatic titrator (Mettler DL-12; Mettler-Toledo, Hightown, NJ) every 1-2 days. Suspended cells samples were collected every day (30 ml). Suspended cell biomass was measured indirectly by absorbance at 620 nm. Gram staining was performed on suspended cell pellets obtained from centrifugation at 16110 x *g* for 3 minutes at room temperature. To confirm cell

viability, to determine isolate optimum temperature, and to screen for hydrolytic enzyme activity, culture broth samples were plated on 2% (wt/vol) agar (Sigma Chemical Co., St. Louis, MO) with various substrates and incubated at room temperature, 37, 45, 50, 55, 65 and 70°C. For spent culture medium, quantitative microbial population was determined by *BacLight* Live/Dead bacterial viability test kit employing the SYTO 9 and propidium iodide stains (Molecular Probes, Eugene, OR) in conjunction with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL) [Demirci *et al.*, 1999]. At the end of each temperature run suspended cells were recovered, centrifuged at 8816 x *g* at 4°C, and stored separately at -75°C in sterile 20% glycerol in MRS medium. At the end of the experiment PCS biofilm culture was recovered aseptically and stored at -75°C in sterile 20% glycerol in MRS medium. Pure culture was obtained by serial dilution and streak plating of suspended cells and stripped PCS biofilm culture on LB-cane molasses plates (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 0.05% [wt/vol] cane molasses and 2% [wt/vol] Sigma agar).

Isolate electron microscopy. For transmission electron microscopy (TEM) a modification of method as described by Zillig *et al.* [1990] was used. Cells were pelleted from culture broth and fixed with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in deionized H_2O for 48 hours at 4°C. Samples were rinsed 2 times in deionized H_2O (this and all subsequent procedures except for polymerization were carried out at room temperature). Samples were pelleted after each step in a microcentrifuge. The samples were post-fixed in 1% osmium tetroxide in dH_2O for 1 hour, followed by a 5-minute wash in dH_2O. The samples were then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and

embedded using a modified EPON epoxy resin (Embed 812; Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 70°C. Thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Ultrathin sections were collected onto copper grids and counter-stained with 5% uranyl acetate in dH_2O for 3 min followed by Sato's lead stain for 1.5 min in a microwave processor. Images were captured using a JEOL 1200EX scanning and transmission electron microscope (Japan Electron Optic Laboratories, Akishima, Japan).

Isolate biochemical tests. Biochemical tests were performed by Silliker Inc (South Holland, IL) and our lab on strain L-10997, *B. thermocatenulatus* and *B. stearothermophilus*. Data for *B. thermoglucosidasius* were obtained from studies by Nazina *et al.* [2001] and Suzuki *et al.* [1983]. A doubling time estimation of strain L-10997 was performed by direct measurement of dry cell mass in a shaker flask culture running at 63°C and 150 rpm. Cell density was followed by absorbance at 620 nm by using a Spectronic® 20 Genesys™ (Spectronic Instruments, Rochester, NY), then converted to dry weight biomass via standard curve [Demirci *et al.*, 1999].

Isolates 16S rRNA sequence analysis. For preliminary culture identification 16S rRNA gene sequencing was performed by Accugenix (Newark, DE). Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal RNA, and purification of PCR products were carried out as described previously [Rainey *et al.*, 1992; Rainey *et al.*, 1993]. Purified PCR products were sequenced by using Ampli*Taq* FS DNA polymerase and *d*Rhodamine dye terminators (Applied Biosystems, Foster City, CA) as directed by the manufacturer.

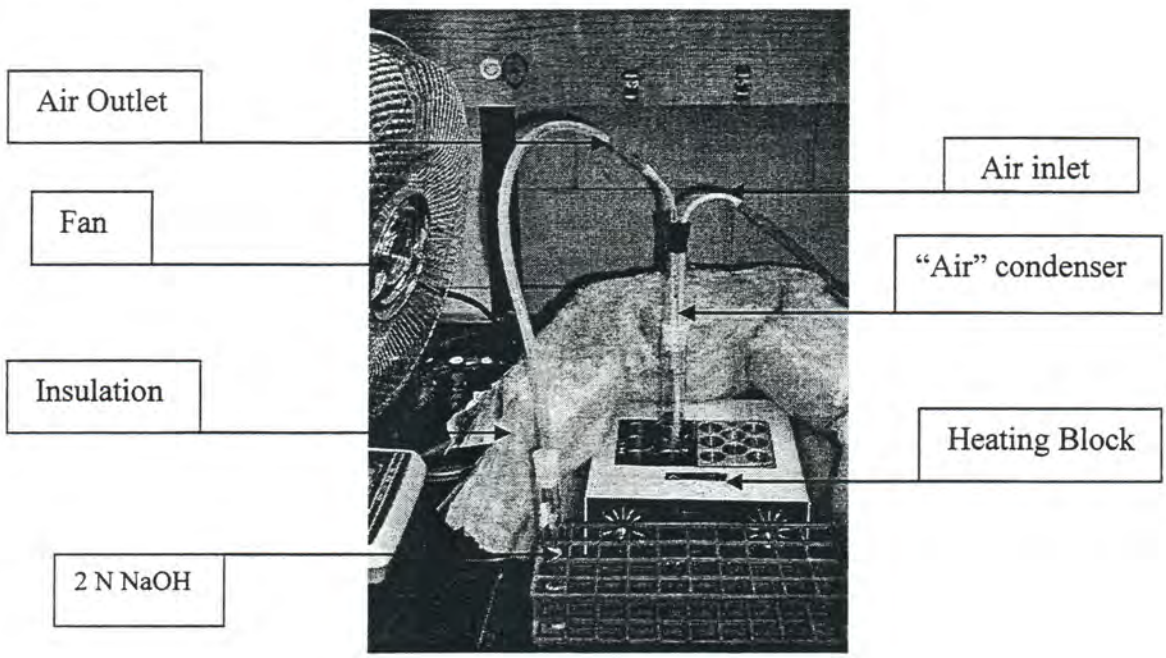
Excess dye-labeled terminators were removed from the extension products using Montage Seq96 filter plates (Millipore, Bedford, MA). Extension products were electrophoresed using an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). The 16S rRNA sequences were aligned and analyzed using Applied Biosystems MicroSeq® Microbial Analysis software.

Isolate cellular fatty acid compositions. Cellular fatty acid compositions were determined by Accugenix. Biomass grown in trypticase soy broth (Becton Dickinson, Sparks, MD) was treated with sodium hydroxide and methanol for 30 minutes at 100°C to saponify the lipid material. Sodium salts of the free cellular fatty acids were converted to their methyl esters by heating the preparations for 10 minutes at 80°C with methanol and hydrochloric acid and were then extracted with n-hexane and tert-butylethylether. The extracts were analyzed by gas-liquid chromatography with a Sherlock™ System (Applied Biosystems) by using phenylmethyl silicone as the stationary phase, and the components were identified by flame ionization detection.

Substrate-specific bubbler culture tube study. A novel bubbler culture tube setup was utilized to evaluate isolate L-10997 for hydrolytic enzymes (Fig. 1). Bubbler culture-tube setup consisted of a 25 x 150 mm culture tube with a 15 x 120 mm glass extension tube as an air condenser, cotton-plugged air inlet and outlet tubes, a submerged air inlet tube with reduced orifice for delivery of small air bubbles, and a CO_2 trap consisting of 10 ml of 4 N NaOH. Temperature was controlled at 65°C via a heating block (Model #11-718-2 and #11-718-4; Fisher Scientific, Pittsburgh, PA). Carbon dioxide-free air was sparged slowly into the

bubbler tubes for aeration (<15 ml/min). The unit was wrapped in fiberglass insulation to reduce heat loss. Tubes contained 20 ml of 1% (wt/vol) tryptone in deionized water (pH ~7.1). The substrates evaluated were cellulose (Sigmacell, Sigma Chemical Co.), defatted ground soyhulls (Penford Products Co.), carboxymethylcellulose (Sigma Chemical Co.) and cellobiose (Sigma Chemical Co.). Uninoculated controls containing the same medium were set up to monitor growth. To stimulate growth sterile 0.5% (wt/vol) yeast extract (Ardamine Z) was added to all tubes after the first week of incubation. All treatments were performed in replicates of three. Trapped CO_2 was determined by pH titration with standardized 0.4 N HCl every 7 days by an automatic titrator.

Fig. 1. Novel Bubbler Tube Setup at 95°C



A series of plate assays employing the Congo Red stain was done for carboxymethylcellulose (CMC) or xylan on strain L-10997 at the end of the bubbler tube study. Each plate contained 125 mg agarose, 12.5 mg CMC or xylan, and 23 ml of sodium acetate buffer (0.06 M, pH 6.0). Wells of 4 mm were cut using a sterile cork borer (no. 1) and the gel removed by aseptic suction. The wells were inoculated with cultures grown in bubbler tubes supplemented with CMC and incubated at 65°C for 7 days. Every day a sample was taken and zones of hydrolysis were made visible by the addition of 20 ml of Congo Red solution (1 mg/ml dH_2O) for 15 minutes, then washed with water and stained by 1 M sodium chloride solution for 10 minutes. Positive enzyme activity was indicated by a clear zone around the sample well. For positive controls commercial cellulase and xylanase from *Trichoderma viridae* (Sigma Chemical Company) were used.

RESULTS

Culture isolation and pre-characterization from PCS bioreactor. In the PCS biofilm reactor a visible biofilm developed on PCS at 55°C by day 2. Bioreactor agitation speed of 100 rpm did not generate a consistent suspended cell absorbance; therefore the speed was increased to 150 rpm. Figures 2 to 4 illustrate CO_2 evolution over the course of each temperature run. Average CO_2 production at 65, 70 and 75°C were 129, 124 and 63 mg/day, respectively. Quantitative cell densities in spent culture medium and on PCS biofilm as determined by *BacLight* fluorescent stain flow cytometry are summarized in Table 1. Four isolates, designated L-10994, L-10995, L-10996 and L-10997, were obtained by serial dilution and subsequent streak plating on LB with cane molasses agar at 65 and 70°C.

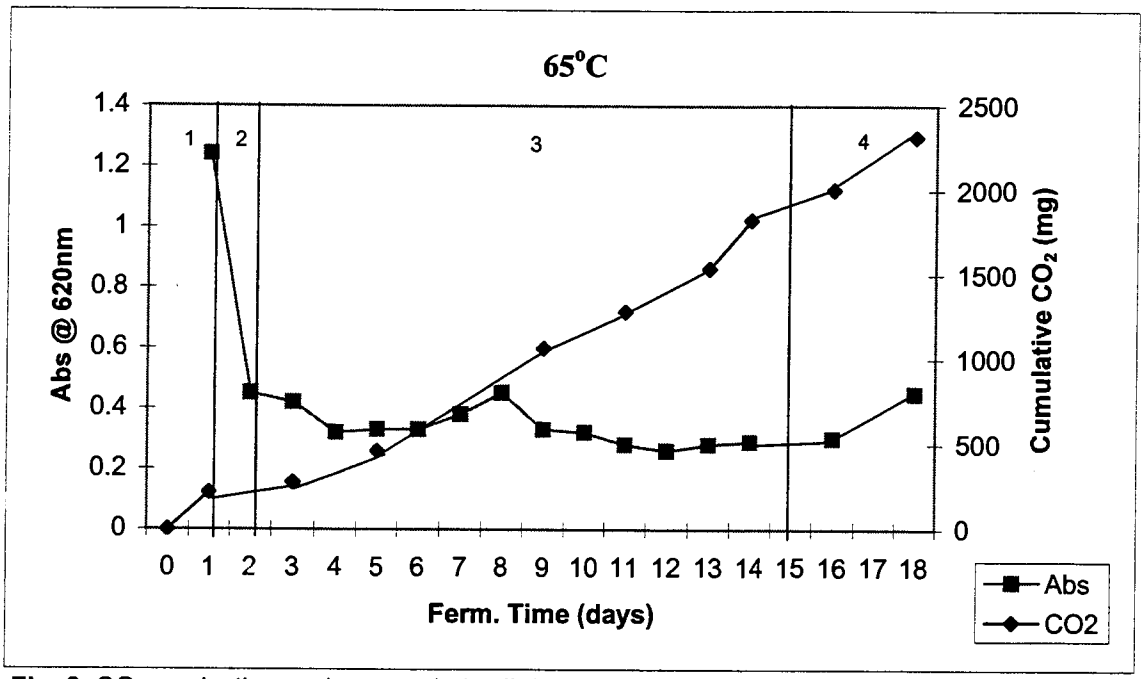


Fig. 2. CO_2 production and suspended cell density determined by absorbance reading at 620 nm for LCSL biofilm consortia at 65°C
1 -- Batch; 2 -- Continuous at 0.1 dilution rate; 3 -- Continuous at 0.2 dilution rate; 4 -- Batch

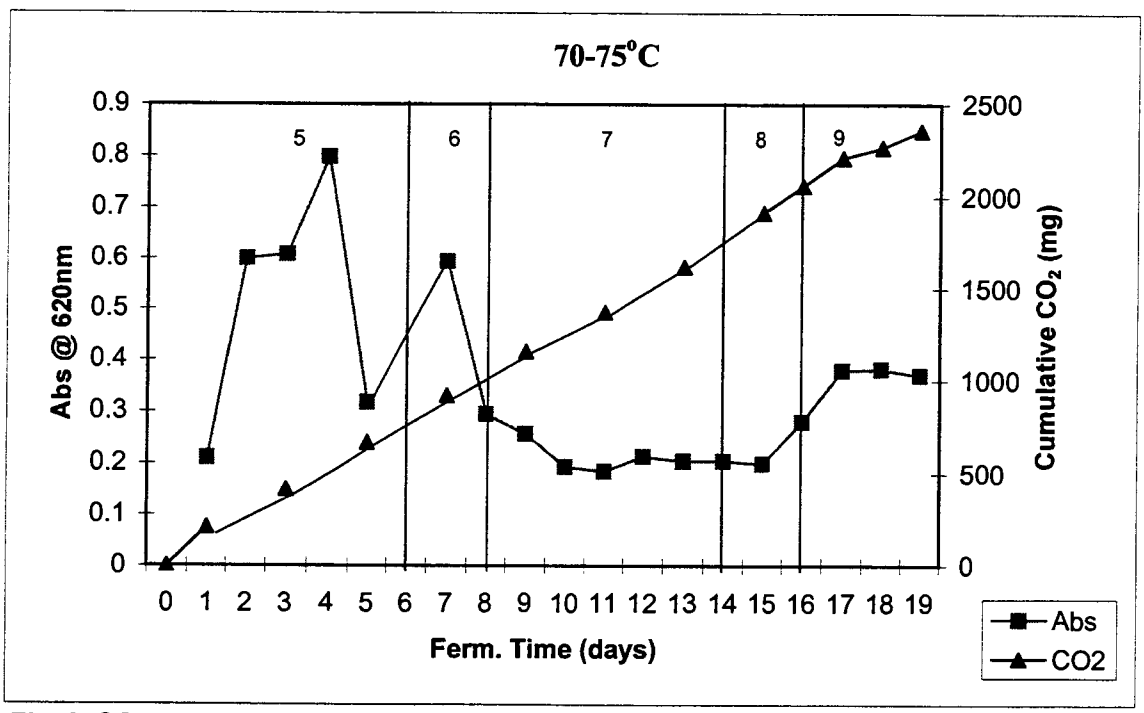


Fig. 3. CO_2 production and suspended cell density determined by absorbance reading at 620 nm for LCSL biofilm consortia at 70-75°C
5 -- Batch at 70°C; 6 -- Continuous at 0.1 dilution rate at 70°C;
7 -- Continuous at 0.2 dilution rate; 8 -- Batch at 70°C; 9 -- Batch at 75°C

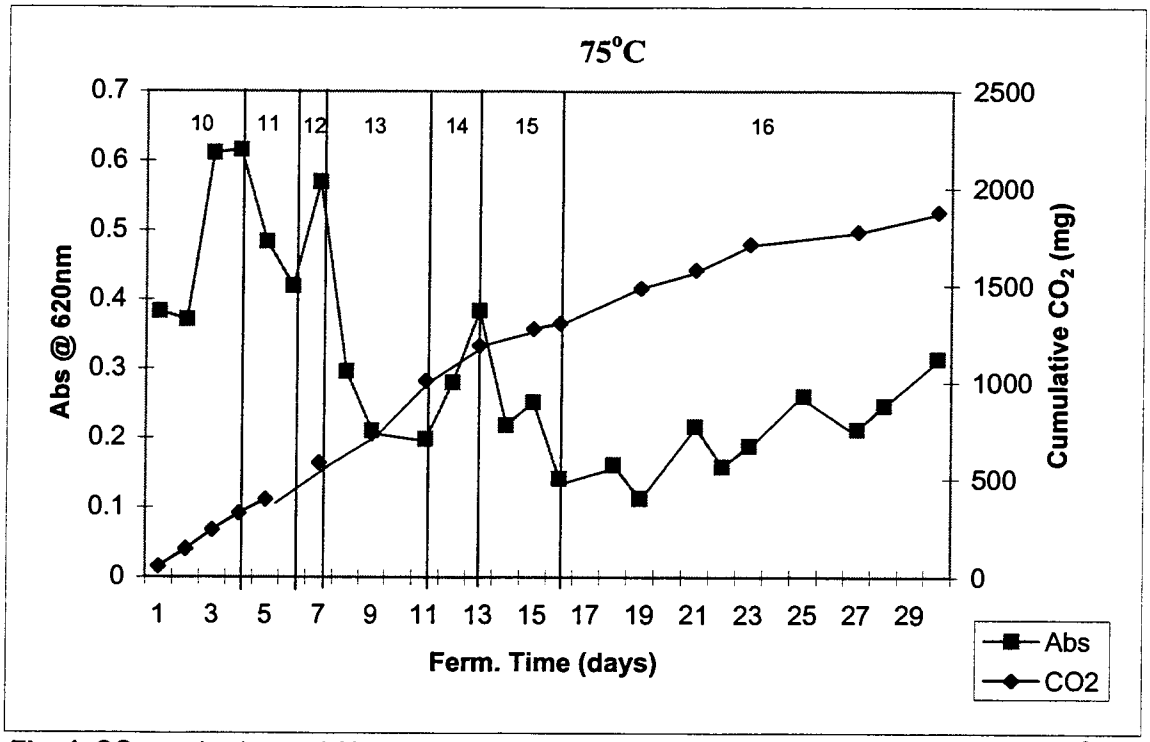


Fig. 4. CO_2 production and Absorbance reading at 620 nm for LCSL biofilm consortia at 75°
10 -- Batch; 11 -- Continuous at 0.1 dilution rate; 12 -- Batch;
13 -- Continuous at 0.1 dilution rate; 14 -- Batch;
15 -- Continuous at 0.1 dilution rate; 16 -- Batch

Table 1 Quantitative cell densities as determined by *BacLight* fluorescent stain flow cytometry in spent culture medium (suspended cells) and on PCS biofilm

Temperature Run	Cell count[a]	Percentage Live / Percentage Stressed Cells[b]
End of 65°C (Day 37)	**2.39×10^7 cells/ml**	**1.31% / 0.83%**
End of 70°C (Day 56)	**5.10×10^6 cells/ml**	**0.55% / 15.3%**
End of 75°C (Day 85)	**2.50×10^6 cells/ml**	**1.02% / 1.86%**
Biofilm culture on PCS (Day 85)	**2.70×10^8 cells/g PCS**	**0.07% / 0.12%**

[a]Based on size comparison with a known amount of 6μm polystyrene beads
[b]Percentage live cells was defined as % of cells which were dyed green; % stressed cells was defined as % of cells that picked up both red and green dyes

Colony and cell morphology. Suspended cells were streak-plated on LB agar and incubated at 65°C. Two types of colonies were observed which consisted of irregular-shaped colonies with extensive swarming and a dull surface (strains L-10994 and L-10996) and shiny, yellowish colonies with a diameter of about 3 mm (strain L-10995) and 1 mm (strain L-10997). Only L-10997 grew at 70°C. All isolates were Gram-positive rods at 65°C. At 70°C some cells of isolate L-10997 appeared slender, longer and Gram-negative, probably because of age and temperature stress [Prescott *et al.*, 1996] (Fig. 5). The vegetative cells of strain L-10997 were motile rods occurring in pairs or in clusters. Endospores were subterminal and ellipse-shaped.

Electron-microscopic examination showed a typical Gram-positive cell envelope profile (Fig. 6) which was confirmed by Gram-staining. Terminally non-swollen sporangia liberated ellipsoidal spores. The cytoplasmic membrane was surrounded by a thin peptidoglycan layer. Cell division was frequently asymmetric. A layer of exopolysaccharide material can be observed in some cells that were grown at 60°C.

Fig. 5. Gram stained L-10997 grown on LB agar, 65°C for 48 hours (1000x magnification; bar, 20 μm)

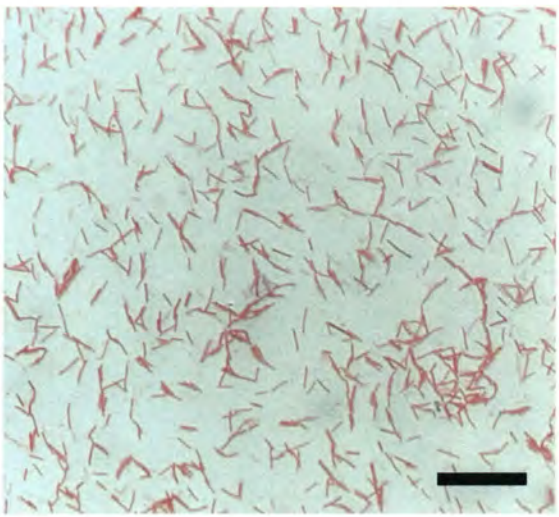
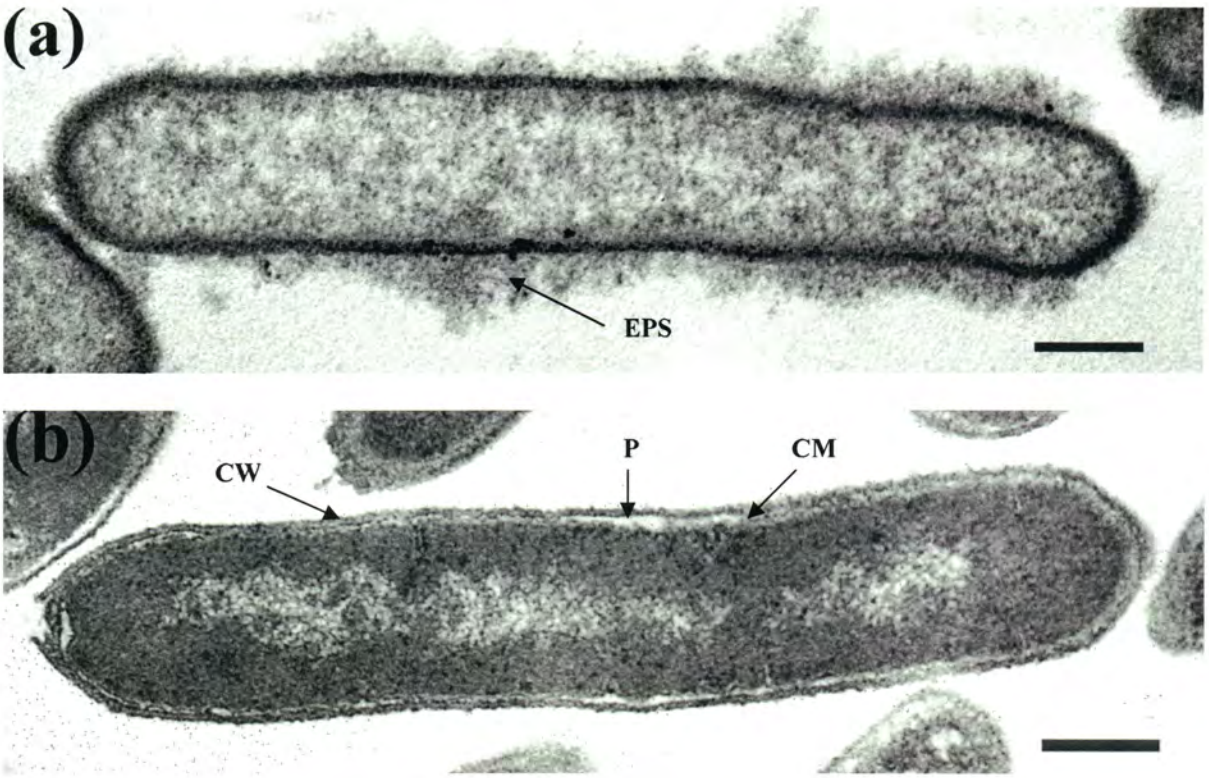


Fig. 6. Thin sections of 3-d old vegetative cells of strain L-10997, grown at (a) 60°C and (b) 70°C. CW, cell wall; CM, cytoplasmic membrane; EPS, exopolysaccharide; P, periplasmic space. Bars, 0.25 μm.



Phenotypic characterization. The isolates were aerobic heterotrophs that grew only in the presence of oxygen. They grew heterotrophically on bacto tryptone, yeast extract, and proteose peptone. Table 2 compares substrate utilization by isolate L-10997 to other thermophilic *Bacillus*. Isolate L-10997 grew only in the presence of plant-derived and partially digested polypeptides, and did not grow on plates containing only casamino acids. It did not grow at room temperature, 37 and 45°C, and grows more slowly at 55 and 70°C than it does at 65°C (Table 3). At 70°C the colonies appeared smaller than colonies at 65°C. A doubling time of 56 hours was observed by dry cell mass measurements at 63°C.

Table 2 Differentiating biochemical properties of isolate L-10997, *B.thermoglucosidasius* ATCC 43742, *B. thermocatenulatus* DSM 730 and *B. stearothermophilus* ATCC 7953

	L-10997	B. thermo-glucosidasius ATCC 43742[1]	B. thermo-catenulatus DSM 730[2]	B. stearo-thermophilus ATCC 7953[3]
Catalase	+	+	+	+
Oxidase	+	+	+	+
Voges-Proskauer reaction	–	–	+	+
Methyl red test	– (pH=5.43)	+w	+ (pH=4.60)	+w (pH=4.80)
Nitrate reduction to nitrite	–	+	+	–
Citrate utilization	–	+	+	–
Starch hydrolysis	–	+	–	–
Casein hydrolysis	+w	+	+	+w
Gelatin liquefaction	–	–	–	–
Urea decomposition	+	+	+w	–
Milk coagulation	–	–	–	–
Litmus milk reaction	Acid (pH=5.75)	Peptonization	Peptonization (pH=7.12)	Acid (pH=5.93)
Utilization of glucose:				
Production of gas	+	–	–	
Production of acid	+		–	
Production of acid anaerobically	–			
Acid from xylose	+		–	
Acid from arabinose	+		–	
Acid/Gas from sucrose	+/–		–/–	+/–
Acid/Gas from lactose	+/–		–/–	+/–
MYP- Egg Yolk	–			
MYP- Mannitol	+			
Degradation of tyrosine	–		+	
Deamination of phenylalanine	–			
2% NaCl	+		+	+
4% NaCl	+		+	–
7% NaCl	–		–	–
Lysozyme tolerance	–			
H_2S production	–		–	–
As sole carbon source:				
Casamino acids	–		+	+w
BSA	–		+	–

+, positive; +w, weakly positive; –, negative.

[1]Data obtained from Suzuki *et al.* (1983), Nazina *et al.* (2001)

[2]Data obtained from our lab; corresponded to Golovacheva *et al.* (1975), Nazina *et al.* (2001)

[3]Data obtained from our lab; corresponded to Nazina *et al.* (2001)

Table 3 Growth of organisms on nutrient agar plates at different incubation temperatures (pH 7.0)

Strain	Growth at:									
	Room Temp.	37°C	40°C	45°C	50°C	55°C	60°C	65°C	70°C	80°C
L-10997	– (7)[a]	– (7)	– (7)	– (7)	++ (2)	+++ (2)	+++ (2)	+++ (1)	++ (1)	– (3)
B. stearothermophilus ATCC 7953	– (7)	– (7)	+++ (3)	+++ (3)	+++ (2)	+++ (1)	+++ (1)	+++ (2)	++ (2)	
B. thermocatenulatus DSM 730	–	+	+	+	+	++	+++ (1)	+++ (1)	++ (1)	– (3)
B. thermoaerophilus DSM 10154[b]	– (5)	– (5)	+ (2)	++ (2)	+++ (1)	+++ (1)	+ (2)	– (5)		
B. thermoleovorans ATCC 43513[c]	–	–	–	+	+	++	++	++	+	
B. thermoglucosidasius DSM 2542[d]	–	–	–	+	+	+	++	++	–	

[a] –, no growth; + weak growth; ++ medium growth; +++ strong growth. The numbers in parentheses are the numbers of days of incubation.

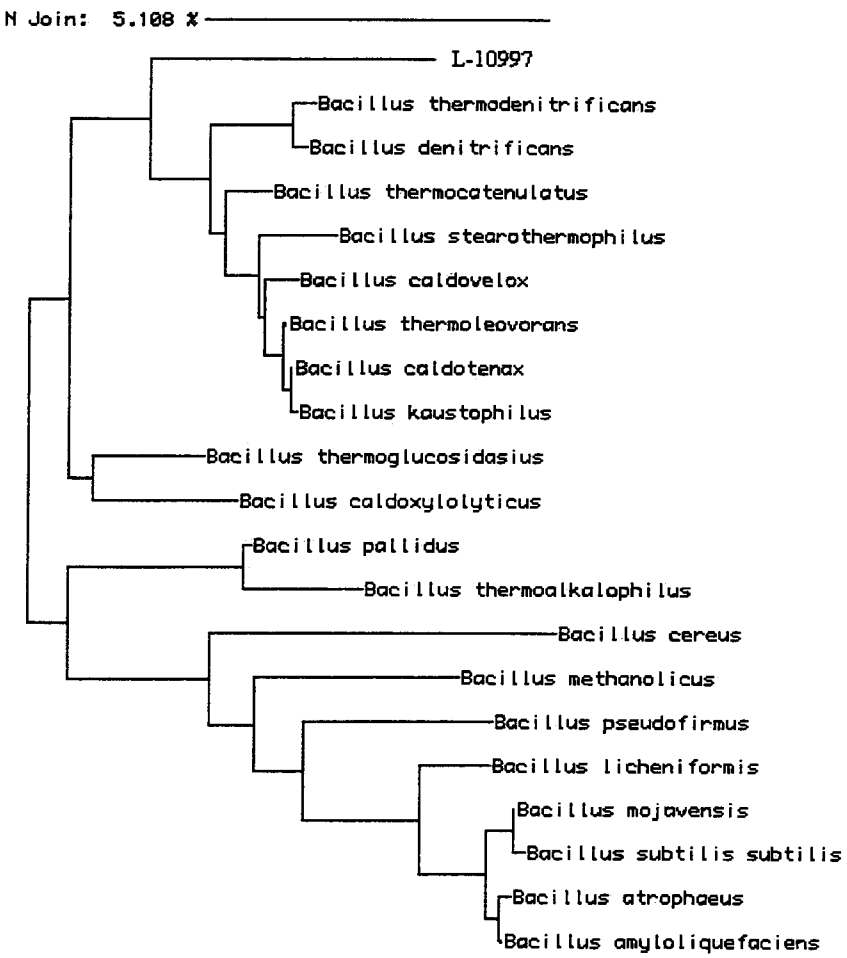
[b] Data obtained from Meier-Stauffer *et al.* (1996)

[c] Data obtained from Zarilla and Perry (1987)

[d] Data obtained from Suzuki *et al.* (1983)

Isolates 16S rRNA sequence analysis. Partial sequence of all four strains (526 nucleotides between positions 0005 and 0531 of the *Escherichia coli* nomenclature) was determined by Accugenix (Newark, DE). The sequence of strain L-10994 was identical to L-10996, and L-10995 was identical to L-10997. The closest 16S rRNA sequence match to isolates L-10994 and L-10996 was *Geobacillus thermoaerophilus* with a 0.38% difference, whereas isolates L-10995 and L-10997 were closest to *G. thermocatenulatus* with a 5.67% difference. Because a difference was seen, an almost complete 16S rRNA sequencing (more than 1500 nucleotides) was performed on isolate L-10997. Results indicated that the closest match to isolate L-10997 was *Bacillus thermoglucosidasis* with a 6.18% difference, followed by *Geobacillus thermocatenulatus* with a 6.34% difference. Thus, isolate L-10997 potentially represented a new *Bacillus* species. The unrooted phylogenetic tree (Fig. 7) shows the relationship of strain L-10997 relative to other *Bacillus* species.

Fig. 7. Phylogenetic positions of L-10997 among other members of family *Bacillacae*. The bar indicates difference between species representing ~1500 base pairs.



Cellular fatty acids of *Bacillus* L-10997. The fatty acid compositions of isolate L-10997 and selected thermophilic and mesophilic *Bacillus* are summarized in Table 4. The major fatty acid of *Bacillus* L-10997 was 16:0 (36%). Iso-16:0, 16:0 and iso-17:0 accounted for about 64% of the total fatty acids. This is in contrast to other thermophilic *Bacillus* species, in which fatty acids in the iso- form were usually the major types [Nazina *et al.*, 2001].

Table 4 Cellular fatty acid composition (% w/w) of the thermophilic isolate from light corn steep liquor and the recognized species of thermophilic and mesophilic bacilli

Fatty Acid[a]	L-10997	DSM 730	ATCC 43742	DSM 22	DSM 5366	*B. subtilis*	*B. cereus*
a13:0				5.1			0.88
14:0	2.32	0.6	0.6	1.5	1.4		3.53
i15:0	9.82	**25.5**	**22.0**	**39.8**	**22.6**	**23.27**	**36.54**
A15:0	2.27	0.6	1.6	6.4	1.3	**39.85**	3.12
15:0	5.92	1.3		0.5	2.1		0.45
I16:0	**10.52**	**31.8**	**10.4**	6.2	**21.0**	3.43	4.06
16:0	**36.37**	8.3	**11.6**	9.2	**11.2**	2.26	4.20
I17:0	**16.80**	**21.0**	**30.3**	**17.1**	**18.5**	**11.71**	6.7
A17:0	6.06	3.1	**16.6**	**13.3**	4.6	**13.27**	0.58
17:0	3.84	2.3	0.8		1.3		
I18:0	0.45	1.3			0.9		
18:0	1.94	2.2			3.4		
18:1 w9c	1.14						
18:1 w7c	0.59						
20:4 w6,9,12,15c	0.64						
Other	1.33	2.0	6.1	0.9	11.7	5.47	39.94
Total	100	100	100	100	100	100	100

Taxa are identified as: 1, strain L-10997; 2, *B. thermocatenulatus* DSM 730, 3, *B. thermoglucosidasius* ATCC 43742; 4, *B. stearothermophilus* DSM 22, 5, *B. thermoleovorans* DSM 5366; 6, *B. subtilis*; 7, *B. cereus*.
[a]Fatty acid abbreviations: a13:0, 10-methyldodecanoic acid; 14:0, tetradecanoic acid; i15:0, 13-methyltetradecanoic acid; a15:0, 12-methyltetradecanoic acid; 15:0, pentadecanoic acid; i16:0, 14-methylpentadecanoic acid; 16:0, hexadecanoic acid; i17:0, 15-methylhexadecanoic acid; a17:0, 14-methylhexadecanoic acid; 17:0, heptadecanoic acid; i18:0, 16-methylheptadecanoic acid; 18:0, octadecanoic acid; 18:1 w7c, 11-octadecenoic acid; 18:1 w9c, 9-octadecenoic acid; 20:4 w6,9,12,15c, 5,8,11,14-eicosatetraenoic acid. Data were taken from this study (column 1), Kampfer (1994) (column 3), Nazina *et al.* (2001) (columns 2,4,5), database provided by Accugenix (columns 6,7). Major components are shown in bold.

Hydrolytic enzyme production by *Bacillus* L-10997. *Bacillus* L-10997 demonstrated a higher CO_2 production when grown in bubbler tubes containing tryptone, yeast extract, and carboxymethylcellulose than in bubbler tubes containing only tryptone and yeast extract over six weeks. This indicates possible cellulase activity (Fig. 8). However, confirmation of endo-cellulase production by *Bacillus* L-10997 via Congo Red plate assay of CMC (7 days at 65°C) was negative. Utilization of other carbon sources were also inconsistent (Table 5). Bubbler culture tubes containing soyhulls demonstrated a negative effect on CO_2 production; all tubes generated lower CO_2 than the uninoculated control. At the end of 6 weeks we were able to recover the bacterium in most tubes on LB with cane molasses plates at 65°C.

Fig. 8. CO_2 production by *Bacillus* L-10997 in bubbler tubes (triplicates) containing 1% tryptone, 0.5% yeast extract and 2% carboxymethylcellulose. Average CO_2 production by cultures grown in tubes containing yeast extract and tryptone were subtracted from samples.

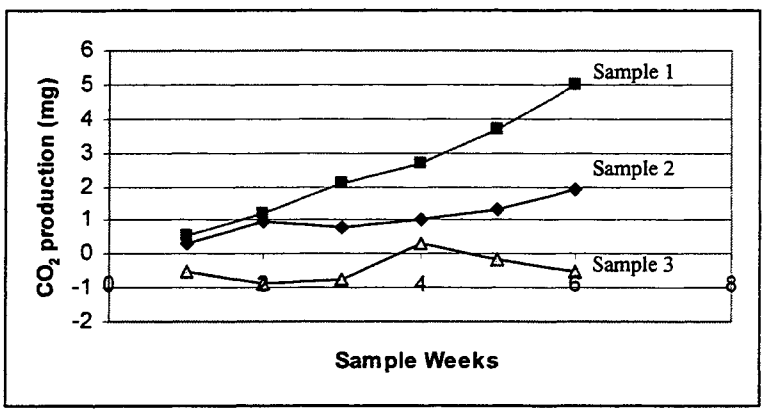


Table 5 Hydrolytic enzyme production by *Bacillus* L-10997 grown in bubbler culture tubes for 6 weeks at 65°C

Polymers[a]	No. of positives in terms of CO_2 production[b]	No. of tubes with positive growth on LB plate at 65°C at end of experiment
Cellulose	0/3	3/3
Defatted Ground Soyhulls	1/3	1/3
Cellobiose	1/3	2/3
Carboxymethylcellulose	2/3	3/3

[a]tubes also contain 1% tryptone and 0.5% yeast extract
[b]positives are defined as tubes with more CO_2 produced than culture grown in tubes containing no polymers; out of a set of 3 tubes

DISCUSSION

The high level of 16S rRNA sequence similarity (~94%) between *B. thermocatenulatus* DSM 730 and *B. thermoglucosidasius* ATCC 43742 with our isolate L-10997 indicated that these organisms belong to the same genus, *Bacillus*. Morphologically, *Bacillus* L-10997 appeared to be quite similar to *B. thermocatenulatus* and *B. thermoglucosidasius*. However, we found significant differences in the cellular fatty acid content (Table 4). Differences in biochemical reactions (e.g. gas production from glucose, acidic Litmus milk reaction, and negative methyl red test) were also observed for *Bacillus* L-10997. Hydrolytic enzyme production by bubbler tubes indicated that over the long term *Bacillus* L-10997 could produce endocellulase. Further characterization of this strain may lead to novel thermostable enzyme production which could benefit waste treatment and downstream processing. On the basis of these differences, we concluded that *Bacillus* L-10997 represented a new species in the genus *Bacillus*, which we named *Bacillus thermozeamaize*.

**Description of *Bacillus thermozeamaize*, sp. nov. (*ther.mo'ze.a'mays.* Gr. adj. *thermos*, hot; L. n. zea, grain, corn; Eng. adj. maize, yellow in color, *thermozeamaize*, loving heat and from corn). Cells are rod-shaped, about 4-5 μm in length, 0.4-0.5 μm in width, motile, Gram positive and produce subterminal, ellipsoidal spores. Colonies are smooth, opaque, with a shiny surface and smooth edges on nutrient agar. It is obligately aerobic and grows between 50 and 73°C, pH 5 to 9 and 0 to 4 % NaCl. It has a doubling time of 56 hours. It is obligately heterotrophic and grows on protein digests. It is positive for growth in the presence of 4% sodium chloride, hydrolysis of casein, acid production from glucose, mannitol and xylose. It is catalase and oxidase positive. It is negative for Voges-Proskauer reaction, growth in the presence of a sodium chloride concentration of 5% or higher, reduction of nitrate to nitrite, and starch hydrolysis. It does not grow in the presence of lysozyme. The major cellular fatty acids are iso-16:0, n-16:0 and iso-17:0. The 16S rRNA difference is >6% to its closest relative *Bacillus thermoglucosidasius*. It was isolated from light corn steep liquor in corn wet milling process. The type strain is L-10997 and is deposited in the American Type Culture Collection as ATCC BAA-739.

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GENERAL CONCLUSIONS

Our attempts to isolate hyperthermophilic microorganisms (>90°C) from exhaust vents of spray driers and from corn processing facility were unsuccessful. We believe the surviving microorganisms are stressed thermophiles rather than true hyperthermophiles. However, we were able to develop protocols and tests that will benefit future research for the evaluation of different potential sites.

We were able to isolate a new thermophilic Bacillus species, *Bacillus thermozeamaize*, from light corn steep liquor. It is an obligately aerobic heterotroph with a temperature optimum of ~65°C. Further studies on its enzymatic production capacity and on mechanisms of how its stress proteins work at elevated temperatures would be beneficial.

RECOMMENDATIONS FOR FUTURE RESEARCH

Microorganisms from spray drier exhaust vents

1) Instead of using a steel cable containing PCS as a trap in the spray drier exhaust, the dust bag which traps particulates during the spray drying process could be sampled. The dust bag will probably contain more trapped materials than the uncharged, dry PCS.

2) The initial enrichment temperature would be set at a temperature which the spray drier was running (~75°C) instead of 95°C to recover injured cells or spores. Once a population was established the temperature could be increased for further temperature screening.

Microorganisms from corn processing facility

1) A lower temperature closer to the processing temperature (e.g. 55°C) could be used for initial enrichment of all samples instead of 95°C. This allows injured cells and spores to rejuvenate and establish a population.

2) Isolation of other new species of thermophilic microorganisms in light corn steep liquor is possible by using selective medium (e.g. minimal medium with polysaccharides, proteins or lipids as sole carbon source).

Enzyme production by thermophilic *Bacillus* species

1) From our biochemical tests, we discovered that *Bacillus thermocatenulatus* DSM 730 was able to utilize bovine serum albumin as its sole carbon source, which was not previously reported. Currently no thermostable protease is available commercially and most of the thermostable proteases in laboratory studies come from anaerobic thermophiles or hyperthermophiles. Studies on the aerobic *Bacillus thermocatenulatus* protease could prove interesting.

2) Long-term bubbler tube study (6 weeks) indicated that *Bacillus thermozeamaize* could potentially produce endocellulase but not in short-term study (Congo Red plate assays). An inducer is probably needed to facilitate the production of this enzyme.

3) Other thermostable enzymes of interest to the food industry would be glucose isomerase, pectinases, keratinase, and more.

APPENDIX

This appendix included experiments and tests to avoid in future work on hyperthermophilic microorganisms from food processing facilities. The procedures described here either did not work well with high temperature, or was incompatible with the PCS that we were using.

Tests to avoid in future research

1) DAPI (diamidophenylindole) is a DNA-intercalating dye and normally could be used to visualize DNA material in bacteria. However, in our case the presence of background material interferes with the stain and it will also show a blue fluorescence under the microscope, thus giving false positive results.

2) One major problem we have encountered was that our controls (sterile medium, PCS, and filter paper) produced a material that interfered with the flow cytometry analysis, producing high (10^7) cell counts. We do not believe it was because of contamination since the controls contained sterile medium and were incubated at a high temperature. A study was performed by autoclaving 35 ml (vol/vol) PCS blend SSHBAYE+ (50% polypropylene, 40% soyhulls, 5% yeast extract, 5% bovine albumin, mineral salts) and 35 ml (vol/vol) PCS blend FSHBAYE+ (70% polypropylene, 20% soyhulls, 5% yeast extract, 5% bovine albumin, mineral salts) in 100 ml LB medium or mineral salts solution with $(NH_4)_2SO_4$ for 72 hours to determine background material that could interfere with cell counts. The test was performed in triplicate. Table 1 showed the amount of background fluorescence in the medium and on sand-stripped supports after 72 hours of autoclaving.

Table 1 Background counts from PCS after 72 hours autoclaving at 121°C

	LB medium			Mineral salts solution with $(NH_4)_2SO_4$		
Sample[a]	pH at end of 72 hours	Counts in medium (counts/g PCS)[b]	Counts on stripped supports (counts/g PCS)[b]	pH at end of 72 hours	Counts in medium (counts/g PCS)[b]	Counts on stripped supports (counts/g PCS)[b]
Sample 1	4.8	1.7×10^7	1.7×10^8	5.2	1.4×10^7	1.6×10^8
Sample 2	4.9	1.0×10^7	1.7×10^8	5.3	3.4×10^7	8.1×10^7
Sample 3	4.9	5.3×10^6	1.4×10^8	5.3	3.1×10^7	1.3×10^8

[a]Samples represent triplicates for each treatment
[b]Counts as determined by *BacLight* fluorescent stain and flow cytometry

Background counts after 72 hours of autoclaving yielded 10^7 to 10^8 counts/g of PCS, which we believed was from materials leaching by the PCS (soyhulls, nutrients, etc). In order to avoid interfering with flow cytometry, PCS made without soyhulls could be used, but this will reduce microbial attachment. Unless a method to filter out this background material is developed, *BacLight* fluorescent stain and flow cytometry is not recommended for hyperthermophiles with a low cell mass.

3) High background CO_2 production was also observed in bubbler tubes containing soyhulls alone. Incubation of soyhulls at high temperature (>90°C) will create turbidity in the medium and give off CO_2, thus giving false positive results. A study to determine background CO_2 production by soyhulls in LB medium was performed in bubbler tubes at 95°C. In a bubbler culture tube containing 15 ml LB medium, different amount of soyhulls were added (0.25, 0.5 and 1.0 g). The tubes were incubated at 95°C and CO_2 production was

monitored. A control containing 15 ml of LB medium was used. Figure 1 illustrates CO_2 production over 6 weeks.

Figure 1 CO_2 production for soyhulls alone at 95°C. Values shown were cumulative with background CO_2 from LB medium subtracted

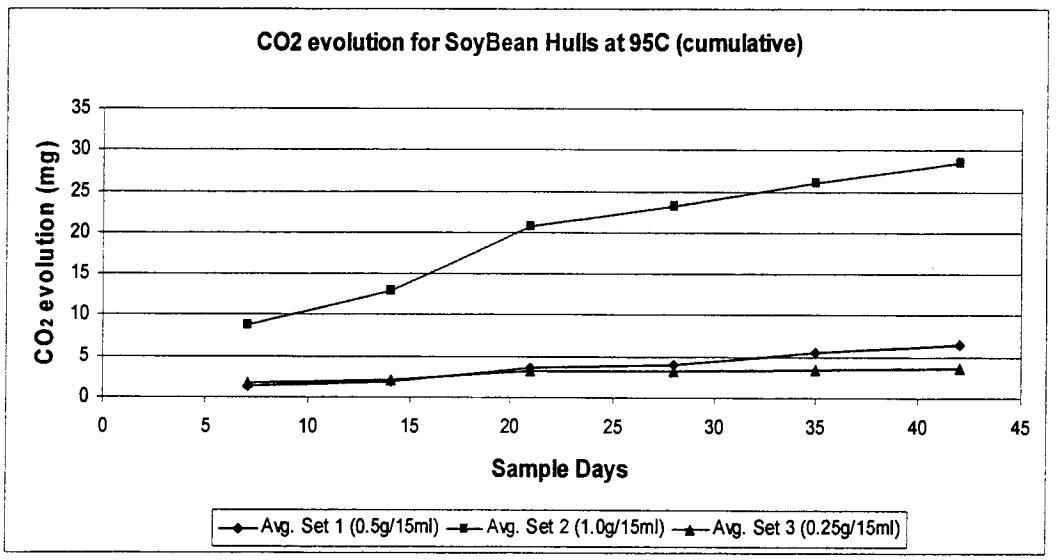
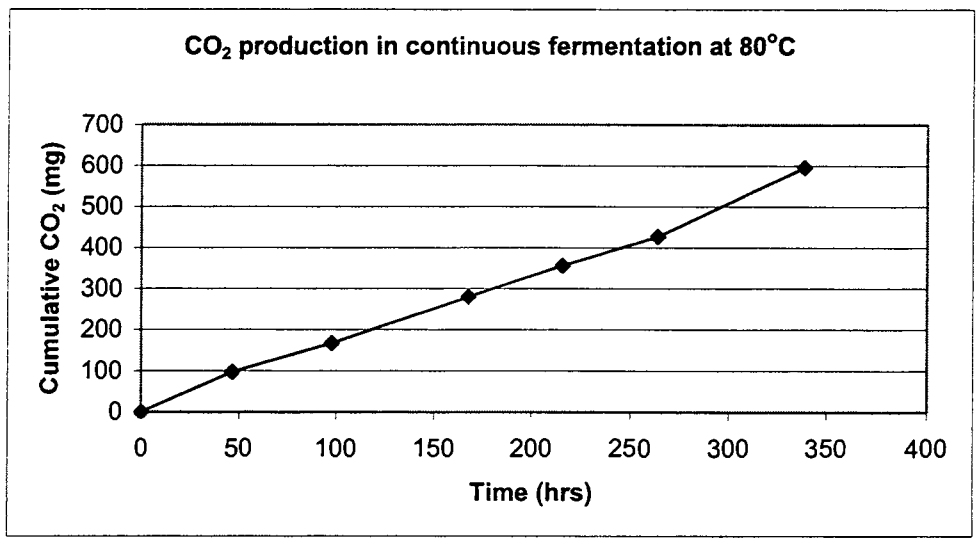


Figure 1 illustrates CO_2 production increased as more soyhulls were included in the bubbler tubes. This number corresponds to 22, 27, and 61 mg CO_2/day in a 1-L reactor containing 1.5, 3 and 6% soyhulls, respectively. This needs to be taken into consideration in the future when soyhulls are being employed.

4) An attempt to destroy the mesophilic population in culture obtained from corn steep liquor (Isolate PPC1.1) was performed. To a 1-L BioStat M reactor 350 ml (vol/vol) of each SSHBAYE+ and FSHBAYE+ were added along with 3 pieces of Whatman No. 1 filter paper at the reactor bottom. LB medium was used in this study. pH was controlled at 6.0 by addition of 3 N HCl and 1 N NaOH. A cocktail of antibiotics (final concentration for 860 ml

working volume was 50 units of Penicillin and 50 μg of Streptomycin) was added to the reactor prior to inoculation. The reactor was first run as a batch culture at 37°C for 48 hours to allow the antibiotics to kill any mesophilic microorganisms in the PPC1.1 consortia, and then the temperature was increased to 80°C which deactivates the antibiotics and the fermentation switched to continuous with a dilution rate of 0.1 to dilute any remaining antibiotics from the culture medium, thus promoting thermophiles (injured and/or spores) to grow. CO_2 production was monitored and Figure 2 shows CO_2 production over the course of the experiment.

Figure 2 CO_2 production in continuous fermentation of PPC1.1 at 80°C (with antibiotics)

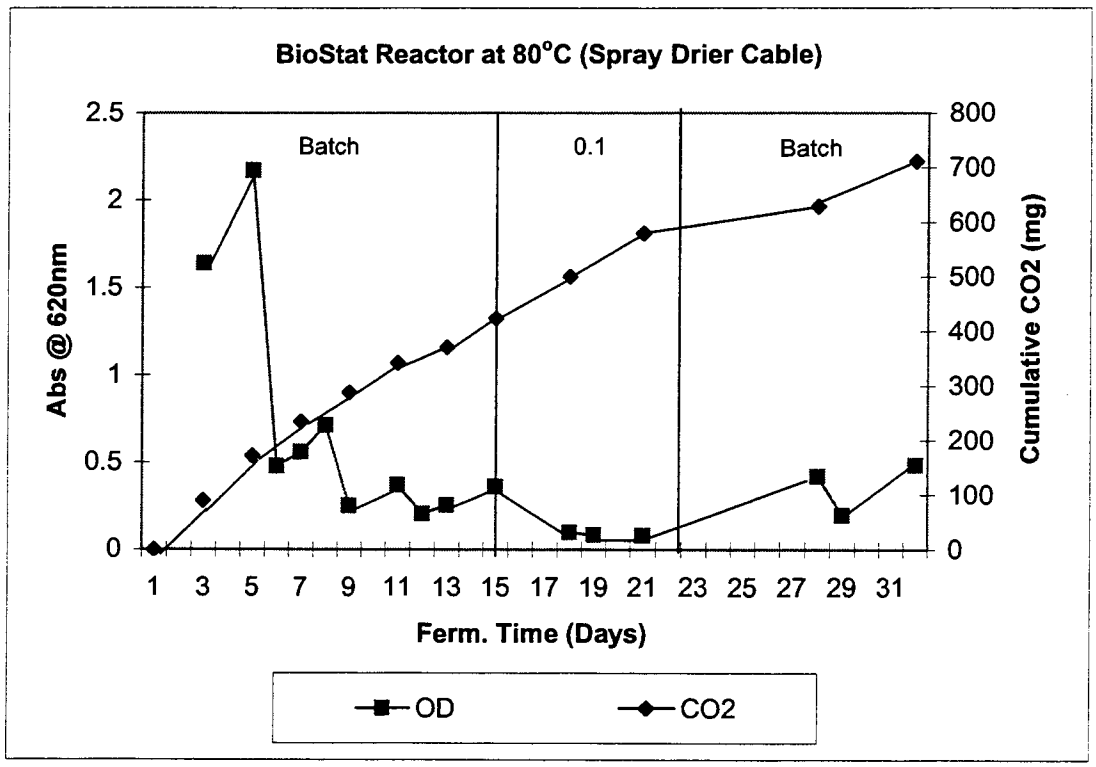


The reactor was run for 12 days. The culture medium was very clear at end of experiment indicating no microbial growth. Some CO_2 was produced, but the amount was much lower than a reactor with positive cultures (e.g. isolation of *Bacillus thermoaerophilus* and *Bacillus thermozeamaize* at 65°C produced >2000 mg CO_2 over 350 hours). Thus, the

CO_2 most likely came from background material on the PCS and also chemical reactions with high temperature treatment. At the end of experiment PCS in the reactor were sand-stripped and solution plated on LB agar. No colonies were obtained at 65°C. The antibiotics probably killed off all of the microorganisms in the reactor.

5) An experiment to obtain thermophilic cultures from spray drier cable was performed at 65 to 80°C. In a 1-L BioStat M reactor, 350 ml (vol/vol) each SSHBAYE+ and FSHBAYE+ were placed in the reactor along with 3 pieces of Whatman No. 1 filter paper at the reactor bottom. LB medium was used in this study. pH was controlled at 6.0 by 3 N HCl and 1 N NaOH. PCS from both spray driers (from zone 1 closest to exhaust) were sand-stripped and inoculated into the reactor set at 80°C. Repeated-batch fermentations were performed for the first 15 days, then changed to continuous with a 0.1 dilution rate for 7 days, and then fermentation was changed back to repeated-batch again for 7 days. At the end of the period the temperature was lowered to 65°C for 3 days. Tests for biological activity include CO_2 production, indirect cell mass measurements via absorbance at 620 nm, wet mount and Gram staining of spent culture medium, and plating on LB agar at 65°C. No growth was observed for the entire period. Figure 3 shows CO_2 and absorbance over the course of the experiment. Thus, not a lot of cells were trapped on the supports, and that some other method of trapping particulates from spray drier exhausts is needed.

Figure 3 CO_2 production and absorbance at 620 nm for BioStat M reactor at 80°C



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