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Production of aldehyde oxidase by Streptomyces species

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Production of aldehyde oxidase by *Streptomyces* species

by

Byungtae Lee

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Food Science and Human Nutrition
Major: Food Science and Technology**

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**Iowa State University
Ames, Iowa
1995**

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To my parents

and

To my wife and daughter

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ABSTRACT

Production of aldehyde oxidase (aldehyde:O₂ oxidoreductase, EC 1.2.3.1) by *Streptomyces setonii* 75Vi2 and *Streptomyces viridosporus* T7A was characterized. For *S. setonii*, aldehyde oxidase was initially induced in shake-flask culture in 0.6% (w/v) yeast extract medium. Inducer (propanal) concentration was determined to be 1.6 g/L medium and two propanal additions generated the best results. Inducer addition to cells in late-log phase was essential to enzyme induction. Dissolved oxygen and pH measurements were key parameters for determination of late-log phase in 15-L and 50-L batch fermentation. Aldehyde oxidase activity was 0.02 units (one unit was defined as 0.1 μ mole/min/mg protein) in shake-flask and CSR cultures, and 0.05, 0.1, and 0.21 units in 5-L, 15-L, and 50-L batch fermentations, respectively. However, with propanal or *trans*-cinnamaldehyde (aromatic aldehyde) as inducer, aldehyde oxidase production by *S. setonii* was inconsistent and not reproducible.

For *S. viridosporus*, vanillin (2 g/L medium) was used as inducer in 50-L fermentations in 0.6% (w/v) yeast extract and 1.0% (w/v) malt extract medium. Vanillin was a stable enzyme inducer and its oxidation to vanillic acid was monitored spectrophotometrically at 345 nm. Aldehyde oxidase activity was more stable in *S. viridosporus* than *S. setonii* and freeze-dried cell-free extract extended its shelf-life. Propanal oxidation to propionic acid by aldehyde

oxidase was confirmed by HPLC for the 30% ammonium sulfate precipitate from a cell-free extract. Oxygraphic enzyme activity measurement did not always correlate with acid production due to interference of endogenous catalase but oxygraph was beneficial for rapid aldehyde oxidase detection.

Crude cell-free extract, 45% ammonium sulfate precipitate and heat-treated (70°C) for 5 and 10 min of crude extract revealed positive aldehyde oxidase activity as shown by brown color formation on nondenaturing polyacrylamide gel electrophoresis (PAGE) zymogram with vanillin as the substrate. This same band from the nondenaturing PAGE demonstrated two broad protein bands on SDS-PAGE which corresponded to peptides of 20 and 55 kDa. These bacterial oxidases were active toward propanal, hexanal, *trans*-cinnamaldehyde, and vanillin, which suggests its possible use to reduce off-flavors in soy products.

INTRODUCTION

Soybeans (*Glycine max.*(L.)) originated in Eastern Asia. Soybeans were used as food long before the existence of written records (102), and they were introduced into the United States in the early 1800s (103). For the 1992/1993 production year, worldwide soybean production was 116 million metric tons and 60 million metric tons in the United States, which accounts for 52% of world production (104).

Soybean oil, meal, and protein are major products from soybeans. Soybean oils are widely used in cooking oils, salad oils for edible use, and in soap, printing inks, and plasticizers for non-food industrial use (104). Also, soybean meal products such as soy flour (products intended for human use), soy concentrates (containing at least 70% protein), and soy isolates (containing at least 90% protein) are used in bakery ingredients, meat products and cereals, and in adhesive, paints, and plywood (104). Today, the major industrial market for soy protein is in paper coatings (54).

Proximate chemical composition of soybeans varies depending on the variety and the growing conditions. On an average, soybeans are 40% protein, 20% lipid, 35% carbohydrates, and 5% ash on a dry weight basis (103).

Human consumption of this protein-rich food is limited due to some off-flavors, which are associated with lipid oxidation by lipoxygenase activity.

Some volatile components of soybeans, flours, concentrates, isolates, and textured soy proteins have been identified (73). Hexanal, a product of lipid oxidation is one of the compounds that demonstrates very low threshold to sensory evaluation (30). Investigation of hexanal and soy protein interactions suggested that structural changes in protein might occur due to hexanal binding to soy proteins, which might prevent this off-flavor removal (104).

Several methods were reported to inactivate lipoxygenase (3, 11, 105, 114). However, soy protein functionality alteration could be another problem. Enzymatic removal by alcohol dehydrogenase (71), aldehyde dehydrogenase (21), and bovine aldehyde oxidase (115) were attempted and none was practically successful. Bacterial source of aromatic aldehyde oxidase was reported by Deobald and Crawford (29). No researchers to date, however, have attempted production of bacterial aldehyde oxidase for possible use in off-flavor removal in soy products.

Research Objectives

The main objective of this research was to optimize aldehyde oxidase production by *Streptomyces* species in 50-L batch fermentations for removal of off-flavors from soy products. The specific objectives were: 1) to determine enzyme induction protocol for consistent aldehyde oxidase production (i.e., culture growth phase for enzyme induction, identify the best inducer, number

of inducer additions, the concentration of inducer added, the need for pH and dissolved oxygen control by the fermentor, and the inoculum type used [spore vs. vegetative cells]); 2) to scale-up enzyme production to 50-L fermentations (i.e., cell harvest, cell disruption, and recovery of cell free extracts); 3) to develop reliable enzyme assays for aldehyde oxidase detection (i.e., oxygraph, spectrophotometric, and/or chromatographic methods); and 4) to partially purify by ammonium sulfate and characterize the aldehyde oxidase produced by two *Streptomyces* species (i.e., ammonium sulfate precipitation, extract shelf-life, native- and SDS-polyacrylamide gel electrophoresis).

Literature Review

Soybeans processing

This soybean processing section is summarized from Chapter 3 (p74-p144) by Snyder and Kwon (103). Soybean processing involves all the steps necessary to make whole beans to final products.

Preparation. Soybeans are cleaned, dried and cracked to separate hulls. These dehulled soybeans are processed to produce soybean meat, which is conditioned by heating before flaking.

Flaking. The conditioned soybean meat is processed through smooth rollers, thus it facilitates the extraction process. Excess moisture is removed and the material is cooled before solvent extraction.

Solvent extraction. Oil is separated from meal fraction by solvent extraction. Currently, hexane is widely used as the solvent.

Oil refining. The term 'refining' refers to all the steps needed to produce the crude soybean oil. The refining steps include degumming, alkali refining, bleaching, hydrogenation, winterization, and deodorization. Phospholipids and free fatty acids are removed in degumming and alkali-refining step, respectively. Colors, as well as flavor compounds are removed in bleaching, and hydrogen is added to polyunsaturated fatty acids to improve texture and flavor stability. Winterizing makes oil clear when it is stored at cold temperature. Finally, unwanted flavor and other compounds are removed before packaging.

Protein products. Soybean meal is the major protein from defatted soybean flakes. The meal containing hulls has a minimum of 44% protein. Product intended for human use are called soybean flour or soybean grits. Soy protein concentrate contains a minimum of 70% protein on a dry-weight basis. It is produced by removing soluble carbohydrates. Soy protein isolates contain more than 90% protein.

Soybean proteins

Soybeans have a relatively high content of protein (38-44%) compared with other legume species (20-30%) (103). Glycinin (11S) and β -conglycinin (7S) are the major proteins found in soybeans. Glycinin comprises about 50%

of total seed protein and β -conglycinin comprises about one third of extractable proteins in soybeans (79). The amounts of glycinin and β -conglycinin in soluble soy protein (55-75% of total seed protein) in 12 soybean varieties averaged 51 and 18.5%, respectively (78).

Glycinin. Hughes and Murphy (49) investigated 10 soybean varieties and total protein content ranged from 39.4 to 44.1% and the content of glycinin was 31.4 to 38.3% total protein. Catsimpoalas (18) initially suggested that glycinin consists of six subunits with three acidic and three basic components. Glycinin has a molecular weight of 320 kDa (5) and consists of 12 polypeptide components, six acidic subunits and six basic subunits, which are packed into two identical hexagons. Staswick et al. (108) demonstrated that acidic and basic components of the glycinin subunits are specifically linked with cysteines. These disulfide bonds were formed from the precursor post-translationally (8, 109). Tumer et al. (123) purified mRNA of glycinin and found that pre-glycinin is formed as a precursor, followed by post-translational modification to produce mature glycinin. Acidic and basic components are produced from a single mRNA. Glycinin precursors have N-terminal leader sequence followed by the acidic peptide component then the basic polypeptide component (123). The amino acid sequences of the acidic and basic components of the A_2B_1 subunit of glycinin were determined as 278 amino acids (31.6 kDa) of acid subunit and 180 amino acids (19.9 kDa) of basic component (107). Also, Hirano et al. (47) determined the amino acid sequence

of the A_3 subunit, an acidic subunit of the glycinin, is 410 amino acids and 46 kDa. mRNA of glycinin A_3B_4 subunit was sequenced by Fukuzawa et al. (37) and they found that glycinin is synthesized as a precursor polypeptide which undergoes post-translational cleavage to form the nonrandom polypeptide pairs *via* disulfide bonds. Amino acid sequence analysis indicates that there is a considerable homology between the acidic and basic polypeptide of individual families of acidic and basic polypeptides, suggesting that the members of each family arose from a common ancestral gene (75). Nielsen et al. (80) found that the glycinin gene structures are highly conserved. They studied five glycinin genes which illustrated 80 to 90% homology among members of the same subfamily, whereas percent homology between members of different groups was less than 50%.

β -Conglycinin. β -Conglycinin, a trimer, has a molecular weight range of 150-175 kDa (117). There are three kinds of subunits with the designations α , α' , and β for β -conglycinin (117) and all are glycoproteins (119). All three subunits are rich in aspartate and/or asparagine, glutamate and/or glutamine, leucine and arginine, and low levels of methionine, which is associated with both α and α' , whereas the β subunit has none (79). Beta subunit is devoid of cysteine and methionine, and α , α' subunits have a higher content of hydrophobic amino acids (118). Koshiyama (58) reported that β -conglycinin is in 7S form in 2 components when pH is lower than pI at μ (ionic strength) < 0.1 M, and β -conglycinin is in the mixture of 7S and 9S form when μ is between

0.1 and 0.5 M, and stable 7S form when $\mu > 0.5$ M. Iibuchi and Imahori (51) studied the sedimentation coefficient of β -conglycinin which varies with ionic strength; β -conglycinin exists as dimer (10S) when μ is 0.1 M, and as monomer (7S) at μ of 0.5 M.

Nutritional value. The major proteins in soybean products are glycinin and β -conglycinin. Therefore, the nutritional value of soybean products with respect to protein is determined by the quantity and quality of these proteins. Some amino acids in soybean proteins are shown in Table 1.

The sulfur-containing amino acids in soy protein are limiting (Table 1), and digestibility is high when soybeans are properly processed (103). Both the quantity and quality of soybean protein are factors that make it the protein of choice for animal feedstuffs. Dehulled soy flakes are sold at 47.5 to 49.0% minimum protein content, and soybean flakes with ground hulls added have a 44% protein minimum (103).

There are many soybean-based foods (fermented or nonfermented) which are consumed in many Asian countries. Tofu is one of the traditional nonfermented foods as well as soy sprouts, soymilk, and soyfilm. Some fermented foods are soy sauce, soy paste, fermented soy curd and fermented soy pulp, which are under many different names for the same commodity by different major consumers (103).

Off-flavors. Off-flavors, expressed as beany or grassy, are major obstacle to increased human consumption, which are probably produced by

Table 1. Average amino acid content of soybean proteins and Food and Agriculture Organization (FAO)/World Health Organization (WHO) requirement (N X 6.25)

Amino acid	Soybean^a	FAO/WHO^b
Histidine	25	-
Isoleucine	45	40
Leucine	78	70
Lysine	64	55
Methionine + cystine	26	35
Phenylalanine + tyrosine	80	60
Threonine	39	40
Tryptophan	13	10
Valine	48	50

^aSource: Bodwell and Hopkins (10).

^bSource: Steinke (110).

lipoxygenase activity. Matoba et al. (69) suggested that n-hexanal is enzymatically generated through 13-hydroperoxide from free linoleic acid, but not from bound linoleic acid such as glycerides and phospholipids. Arai et al. (2) reported that n-hexanal is from degradation of hydroperoxides derived from *cis,cis*-linoleic acid or its ester. Volatile flavor constituents of defatted soy flour

were identified as 2-pentyl furan and ethyl vinyl ketone. These compounds are responsible for soybean characteristic beany, grassy, and green odors (48). Mattick and Hand (72) identified ethyl vinyl ketone causes "green bean like" odor by distillation and compared it with soymilk isolate. Endo et al. (31) reported that 10-oxo-8-octadecenoic acid, 10- and 9-hydroxy octadecanoic acid are components that cause flavor reversion. Some other off-flavor components are listed in Table. 2.

n-Hexanal is one of the major products from autoxidized soybean oil that can generate off-flavor in very small amounts. Flath et al. (34) reported that threshold of hexanal and 2-hexenal are 5 and 17 ppb, respectively. However, Eriksson et al. (32) showed that odor detectability (threshold) is 5 ppm for n-hexanal, 19 ppb for n-hexenal, and 316 ppb for *trans*-2-hexenal. Also, Dixon and Hammond (30) reported different threshold values of hexanal (6 ppm), hexenal (10 ppm), and *t,t*,2,4-hexadienal (3 ppm).

Lipoxygenase catalyzes lipid oxidation, which combines molecular oxygen with polyunsaturated fatty acids to yield hydroperoxide. Lipoxygenases have a major role in production of off-flavors and there are three lipoxygenases present: L-1, L-2, and L-3. L-1 has higher pH optimum than L-2 and L-3, and it is more reactive with free fatty acids than esterified fatty acids. Recessive mutants of L-1 could reduce volatile carbonyl compounds (45).

Davies et al. (28) reported that the removal of L-2 improved flavor of soybean (i.e., it significantly lowered scores of beany, rancid, and oily flavor).

Table 2. Some volatile components of soybeans, flours, concentrates, isolates, and textured soy proteins^a

Class	Compounds
Aldehydes	Alkanals: C1, C2, C3, C4, C5, C6, C7, C8, C9, C10
	Alkenals Δ^2 : C5, C6, C7, C8, C9
	Alkenals Δ^4 : C10
	Dienals $\Delta^{2,4}$: C6, C7, C8, C9, C10, C11
	Others: methylpropanal, 2-methylbutane, 3-methylbutane
Ketones	1-Alkanones: C3, C4
	2-Alkanones: C5, C6, C7, C8, C9, C10
	3-Alkanones: C8
	5-Alkanones: C10
	Unsaturated: butenone, 1-pentene-3-one, 3-octene-2-one, 3-nonene-2-one, 3,5-octadiene-2-one, butadione
Alcohols	Saturated: methanol, ethanol
	Unsaturated: 1-propanol, 2-propanol, 1-butanol, 2-butanol, 1-pentanol, 2-pentanol, 3-pentanol, 1-hexanol, 2-hexanol
Hydrocarbons	Saturated: C3, C4, C6, C8, C10, C15, C17
	Unsaturated: C6, 1,4-pentadiene, 1,3-octadiene, cyclohexane
	Carboxylic acid: C2, C3, C5, C6, C8, C9, C10
Other compounds	lactones, ethyl esters, benzene, toluene, benzaldehyde, benzyl alcohol

^aSource: McLeod and Ames (73).

Also, Grosch and Laskawy (43) found that the neutral lipoxygenases L-2 and L-3 form both a greater quantity and a greater range of volatile carbonyl compounds than does the alkaline isoenzyme L-1.

Matoba et al. (70) studied lipoxygenase deficient mutants (null) of soybeans and reported that the level of n-hexanal was the lowest in the L-2 null homogenate and the highest in the L-1, L-3 null homogenate. Thus, they claimed that L-2 isozyme is responsible for n-hexanal formation by using free linoleic acid as the substrate. On the other hand, Hildebrand et al. (46) demonstrated that hexanal concentration was reduced when L-3 isozyme was added. Their results suggested that nonvolatile ketodienes of hexanal were formed with linoleic acid or 13-hydroxyperoxy-9,11-octadecanoic acid. This indicated that L-3 isozyme may reduce hexanal yield in soybean seed by competing with L-1 and L-2 for the available fatty acids or fatty acid hydroxides.

Other researchers (125) reported that L-1 isozyme is most effective in the hydroperoxidation of free fatty acid, whereas L-2 and L-3 are relatively more effective in the hydroperoxidation of esterified or neutral fatty acid. Also, they found that L-2 isozyme is largely responsible for the generation of C6 aldehydes with most of the substrates tested and that the soybean line with L-3 reduced hexanal formation. Whereas, free linoleic acid yielded the highest relative levels of C6 aldehyde with L-2 isozyme, and its lipid-dependent O₂ uptake was lower than or similar to that of many other compounds.

Off-flavors and soy protein interactions. In spite of the progress made in understanding the mechanisms of off-flavor components formation in soy materials, the soy flavor problem remains. Understanding the ability of proteins to bind certain components that affect food aromas is important to the food industry. Understanding the different mechanisms of interaction between flavors and proteins is vital for increased utilization of soybeans (73).

There are two different approaches to studying flavor binding to soy protein. One is gas/solid interactions and the other is gas/liquid interactions. Arai et al. (2), using vacuum distillation and gel filtration technique, showed that the concentration of hexanal and 1-hexanol increased with increasing protein denaturation. Hexanal binds more strongly than does hexanol; this was attributed to hydrophobic interactions between protein and flavor compounds.

Franzen and Kinsella (37) examined the binding of some aldehydes and ketones by different forms of soy proteins with headspace gas chromatography analysis techniques. The presence of soy protein in the aqueous systems increased the retention of volatile components in all samples. Interactions were thought to be due to surface area and solubility effects. It was suggested that reactions between the protein and the aroma components were also involved.

Gremler (42) studied the effect of adding certain compounds to soy protein isolate in an aqueous medium, in which aldehydes strongly reacted with the soy protein, but alcohols did not. The interaction was reversible and/or irreversible and was due to chemical reactions and/or physical sorption.

However, aldehydes bind strongly to soy protein, followed by ketones and alcohols, whereas carboxylic acids showed no binding (9).

Crowther et al. (25) studied the effect of thermal processing on the binding properties of "dry" soy protein in nonaqueous systems. Based on their heat of adsorption data, it was observed that alcohols bind strongly to "dry" soy protein, whereas aldehydes and ketones bind less. Furthermore, adsorption and binding heat-coefficients escalated with increasing hydrocarbon length due to expanded van der Waals interactions. Aspelund and Wilson (4) confirmed this observation and suggested that aldehydes, ketones, and methyl esters bind to soy protein *via* one hydrogen bond plus van der Waals forces.

Damodaran and Kinsella (27) studied the interaction of carbonyls with soy protein by using an equilibrium dialysis method which equilibrates soy protein with solution containing carbonyls (e.g. 2-octanone, 2-nonanone, 5-nonanone, and nonanal) separated by dialysis membrane. They showed that there were about four binding sites for each ligand in the soy protein, assuming a molecular weight of 100 KDa. However, they used 2-nonanone as a model compound, which is not an off-flavor component (23, 82, 93).

O'Keefe et al. (82) performed the thermodynamic binding study of purified glycinin and β -conglycinin in aqueous system with butanal, pentanal, hexanal, octanal, 2- and 3-hexanone, 2- and 5-nonanone, hexanol, and hexane. All flavor ligands bound better to glycinin than β -conglycinin. Affinity for aldehydes increased with increasing chain length for glycinin, but was constant

for β -conglycinin. O'Keefe et al. (83) also investigated the equilibrium binding of hexanal to soybean glycinin and β -conglycinin. The numbers of binding sites for hexanal to glycinin and β -conglycinin were 108 and 26, respectively. It was suggested that structural changes might occur due to hexanal binding. Cooray (22) studied ^{14}C -heptanal binding to purified glycinin and β -conglycinin. ^{14}C -Heptanal binding to soy proteins was not completely reversible due to presence of tightly bound ligands.

Soybean flavor improvement. Soybean is a good protein source for the human diet. Furthermore, tocopherols found in soybeans have illustrated some anticancer properties (73). Human consumption, however, is limited due to the off-flavors. Attempts to remove or mask these off-flavors continue to be made.

Borhan and Snyder (11) treated whole soybeans with heat and ethanol to inactivate lipoxygenase and suggested that useful ranges were 15 to 45% ethanol, 40 to 60°C, and 2 to 6 hr treatment. Ashraf and Snyder (3) studied soymilk prepared from soybeans soaked in 15% ethanol and in 15% ethanol with 0.1 M NaOH, 0.1 M Na_2CO_3 or 0.1 M NaHCO_3 for various times at 60°C. Soaking in 15% ethanol with 0.1 M NaHCO_3 for 4 hr did minimize off-flavor. Srinivas et al. (105) investigated sensory and physicochemical characteristics of soy meal prepared from second extraction of soy flakes, using hexane containing 3 or 5% acetic acid and compared with those of hexane-extracted-only meal. Hexane containing 5% acetic acid treatment demonstrated total

inactivation of lipoxygenase, but solubility was reduced compared to hexane-extracted meal. Swamylingappa and Srinivas (114) prepared soy protein isolate from commercial soy meal treated with hexane containing 3% acetic acid at 28 and 58°C. Off-flavor was reduced by 66% in treated isolates.

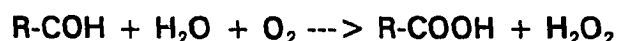
Lecomte et al. (62) reported that reduction in soybean off-flavor was observed when soy proteins were incorporated in frankfurters formulation, probably due to physical masking. They suggested that soy protein addition could improve functionality and sensory characteristics of comminuted meats.

Matoba et al. (71) reported that n-hexanal in soybean homogenates decreased during incubation at alkaline pH. They suggested that n-hexanal was converted to n-hexanol by alcohol dehydrogenase in alkaline pH and enzyme reduction was stimulated by NADH and NADPH addition. Takahashi et al. (115) studied enzymatic removal of soybean off-flavors using bovine liver aldehyde oxidase. Aldehyde oxidase removed the beany odor of the raw soybean extracts through aldehydes oxidation. Aldehyde oxidase-catalyzed reaction was almost undetectable in the initial period of incubation when soybean protein-bound aldehyde was used as the substrate. However, soybean protein-bound aldehyde was oxidized during prolonged incubation. Aldehyde oxidase was less efficient in the oxidation of soybean protein-bound aldehydes than bovine mitochondrial aldehyde dehydrogenase (21). Enzymatic off-flavor removal could be a desirable method in that it is simple and specific. Also, it may not affect protein functionality while removing soy protein-bound

aldehydes. Some characteristics of aldehyde oxidase are followed in next section.

Aldehyde oxidase

Aldehyde oxidase is an oxidoreductase that catalyzes the reaction in which aldehydes are converted to their corresponding acids. It requires molecular oxygen as an electron acceptor and the Enzyme Commission (EC) number is E.C. 1.2.3.1. A generalized reaction is:



Known sources of enzyme. Aldehyde oxidase was first isolated from porcine liver in 1940 when it was tentatively identified as flavoprotein by Gordon et al. (39). The enzyme has also been obtained from various animal sources such as rabbit liver (7, 13, 33, 40, 44, 58, 59, 94, 95, 116, 120), equine liver (17), bovine liver (15, 115), porcine liver (33, 50, 67, 84, 85, 96), guinea pig liver (124), rat liver (20, 76, 81, 99, 106) and human liver (100, 106). In addition, Krenitsky et al. (59) isolated aldehyde oxidase from wide variety of animals such as sea anemone, planaria, cat, earthworm, mealworm, lobster, oyster, snail, frog, snake, turtle, pigeon, dog, cow, and monkey. Large and Connock (61) studied aldehyde oxidase from three species of terrestrial gastropod. Aromatic aldehyde oxidase from microbial source, *Streptomyces viridosporus* T7A, was reported by Crawford et al. (24). Also, aliphatic aldehyde oxidase from *Pyrococcus furiosus* was reported by Mukund and

Adams (77).

Characteristics of enzyme.

Structure and size. Cabré and Canela (15) reported that bovine liver aldehyde oxidase MW was 222 kDa by gel filtration and pI was 7.0 by titration curve. FAD content was 0.72 mol per mole enzyme.

Yoshihara and Tatsumi (124) reported that guinea pig liver aldehyde oxidase MW was 348 kDa by gel filtration and 300 kDa by native polyacrylamide gel electrophoresis (PAGE). They also found 148 and 134 kDa bands on SDS-PAGE similar to rabbit liver aldehyde oxidase. FAD content was 2.1 mole, whereas 8.5 g atoms of iron, and 1.0 g atom of molybdenum per mole of the native enzyme was detected. Moriwaki et al. (76) reported that rat liver aldehyde oxidase MW was 270 kDa by PAGE.

Felsted et al. (33) reported that rabbit liver and hog liver aldehyde oxidase MW were 270 and 268 kDa, by sedimentation equilibrium studies, and 270 and 260 kDa, by gel filtration, respectively. Both enzymes will spontaneously polymerize into higher MW species and the aggregation appeared to be concentration-dependent. The molecular weights for the major polymers were 460 and 540 kDa. Complete reversal as well as the prevention of the polymerization of both enzymes was achieved with thiol reagents such as mercaptoethanol, glutathione, and dithiothreitol at concentration of 60, 1.6, and 1.6 mM, respectively, and cysteine was also found to be effective at a concentration of 5 mM. Tomita et al. (120) reported that rabbit liver aldehyde

oxidase MW was 270 kDa by SDS-PAGE and was composed of identical dimers. Also, Maheshwari (66) purified two aldehyde oxidase from porcine liver using affinity chromatography and molecular weights of two isozymes were 262 and 255 kDa, respectively.

Deobald and Crawford (29) reported that bacterial aromatic aldehyde oxidase from *Streptomyces viridosporus* T7A had a molecular weight of 80 kDa as determined by nondenaturing polyacrylamide gel electrophoresis (PAGE). Mukund and Adams (77) reported that hyperthermophilic aldehyde oxidase from *Pyrococcus furiosus* had a MW of 80 and 90 kDa by SDS-PAGE and gel filtration, respectively, and the same enzyme was crystallized by Chan et al. (19).

As illustrated above, aldehyde oxidase from animal sources is about three times larger than that from the bacterial sources. Thus, bacterial aldehyde oxidase could be more accessible to soy proteins that are 160 and 320 kDa in size.

Mahler et al. (67) found that FAD and iron component were present in pig liver aldehyde oxidase, and showed molybdenum content is 0.5 g atom per mole of flavin. On the other hand, Palmer (84) reported that flavin content of aldehyde oxidase from pig liver was 5 nmoles per mg of protein and molybdenum content was 1 to 2 nmoles per mg of protein.

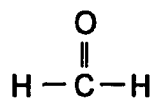
Felsted et al. (33) found 2.03 moles of FAD, 7.92 g atoms of iron, and molybdenum content ranged from 0.66 to 1.14 g atoms per mole of rabbit

enzyme. Rajagopalan et al. (96) reported that 2 molecules of FAD, 8 atoms of iron, 2 atoms of molybdenum, and 1 or 2 molecules of coenzyme Q₁₀ present per molecule of rabbit liver aldehyde oxidase.

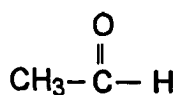
Substrate specificities. Major substrates in the review are illustrated in Fig. 1. (15, 17, 24, 39, 96). A systematic work on aldehyde oxidase specificity toward aliphatic or aromatic aldehydes has not been reported. Some aldehyde oxidase catalyzed aliphatic aldehydes at higher rate than aromatic aldehydes. Thus, the enzymes could be divided into two groups depending on substrate specificity. It is a tentative classification of following enzymes because it may not be definite until a variety of substrates are tested.

Aliphatic aldehyde oxidase. Gordon et al. (39) found that increase in carbon chain length of substrate decreased the catalytic velocity by pig liver enzyme. Substrate preference by the enzyme was highest for acetaldehyde followed by crotonaldehyde, benzaldehyde, propanal, butanal, glycolic aldehyde, and salicylaldehyde. In addition, Cabré and Canela (15) reported that propanal was preferably catalyzed by aldehyde oxidase from bovine liver, followed by furfural (67% compared with propanal as 100%), acetaldehyde (45%), benzaldehyde (32%), crotonaldehyde (26%), chloroacetaldehyde (16%), and formaldehyde (9%). Mukund and Adams (77) investigated aldehyde oxidase from *Pyrococcus furiosus* and crotonaldehyde showed the highest in specific activity (27 units; 1 international unit was defined as μ mole substrate oxidized/min/mg protein), followed by acetaldehyde

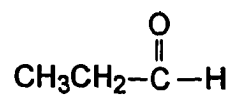
Aliphatic aldehydes



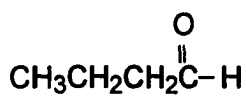
formaldehyde



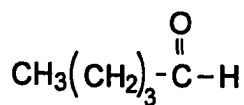
acetaldehyde



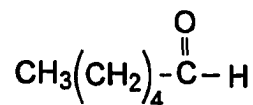
propanal



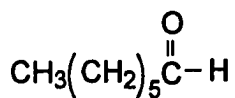
butyraldehyde



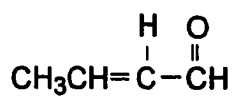
valeraldehyde



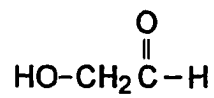
hexanal



heptanal



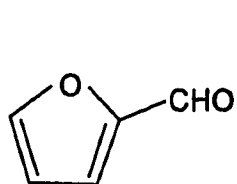
crotonaldehyde



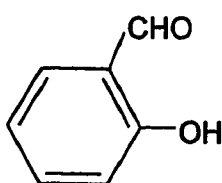
glycolic aldehyde

Figure 1. Structure of some substrates for aldehyde oxidase

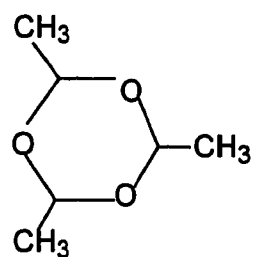
Aromatic aldehydes



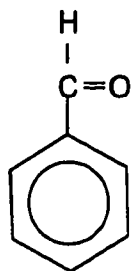
furfural



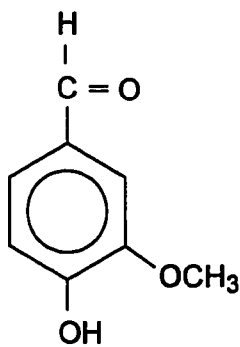
salicylaldehyde



paraldehyde



benzaldehyde



vanillin

Figure 1. (continued)

(19 units), formaldehyde (9.2 units), butyraldehyde (7.4 units), and glyceraldehyde (4.8 units).

Aromatic aldehyde oxidase. Carpenter (17) used formaldehyde, acetaldehyde, heptaldehyde, furfural, salicylaldehyde, paraldehyde, benzaldehyde, and crotonaldehyde as substrates for horse liver enzyme. Salicylaldehyde and furfural were catalyzed at higher rates than other substrates. On the other hand, Crawford et al. (24) reported that *m*-hydroxybenzaldehyde was oxidized at highest rate, followed by salicylaldehyde, vanillin, and benzaldehyde by bacterial aldehyde oxidase from *Streptomyces viridosporus* T7A. Large and Connock (61) tested several aromatic aldehydes as substrates for three species of terrestrial gastropod, and found that benzaldehyde was used at highest rate, followed by 3-methylbenzaldehyde, 3-methoxybenzaldehyde, cinnamaldehyde, veratraldehyde, and salicylaldehyde. Rodrigues (100) tested benzaldehyde for human liver aldehyde oxidase and average activity was 22.4 ± 10.4 nmole/min/mg protein from 6 samples.

Other aldehyde oxidase. Krenitsky et al. (58) investigated some purine, pyrimidine and their derivatives as substrates for rabbit liver aldehyde oxidase. The numbers in parenthesis for the following substrates are relative rate compared with that of purine as 100%. 6-Cyanopurine (280%) was highest among C-monosubstituted purine, followed by 2-hydroxypurine (140%), and 6-purinecarboxamide (94%). Other purine derivatives are 6-methylpurine (82%), 6-chloropurine (56%), 6-bromopurine

(58%), 6-iodopurine (32%), 2-aminopurine (38%), and 2-mercaptapurine (43%). 2-Hydroxypyrimidine (280%) was highest for pyrimidine derivatives followed by 4-hydroxypyrimidine (134%) and 2-methylpyrimidine (18%). On the other hand, 3-methylhypoxanthine (710%) was highest for N-substituted purines followed by 7-methyladenine (68%) and 1-methylhypoxanthine (34%). Generally, C-monosubstituted purines were good substrates for rabbit liver aldehyde oxidase and C-disubstituted purines were not good for the enzyme. The oxidation rate of these hydroxyl derivatives were lower compared with those of the parent unsubstituted compounds, except with pyrimidine itself and 6-substituted purines. Overall, aldehyde oxidase hydroxylated these substrates.

Felsted et al. (33) found that rabbit and hog livers aldehyde oxidase catalyzed the oxidation of N¹-methylnicotinamide to both N¹-methyl-2-pyridone-5-carboximide and N¹-methyl-4-pyridone-3-carboximide in a ratio (2-pyridone to the 4-pyridone) of 100 to 3.8 for rabbit and 3.8 for hog enzymes, respectively. Also, Stanulović and Chaykin (106) showed that aldehyde oxidases from human liver and rat liver catalyze N¹-methylnicotinamide to the formation of N¹-methyl-2-pyridone-5-carboximide and N¹-methyl-4-pyridone-3-carboximide.

Rajagopalan et al. (96) also reported that the purified rabbit liver aldehyde oxidase was found to oxidize acetaldehyde and salicylaldehyde, as well as N¹-methylnicotinamide and quinine. Rajagopalan and Handler (95) found N¹-methylnicotinamide, N-methylquinolinium, N-ethylquinolinium,

phenazine methosulfate, 7-methylhypoxanthine, purine, quinoline, acetaldehyde, and salicylaldehyde could serve as substrates for rabbit liver aldehyde oxidase.

Hall and Krenitsky (44) tested many purines (66 compounds) and pyrimidines (26 compounds) as substrates for rabbit liver aldehyde oxidase. These will be discussed in the next section.

Kinetics. Palmer (85) reported that kinetic mechanism was noncompetitive for pig liver aldehyde oxidase when double reciprocal plot of acetaldehyde versus cytochrome C was drawn, using cytochrome C as one substrate and acetaldehyde as the second substrate. He found ternary complex formation with independent binding of each substrate.

Takahashi et al. (115) reported that K_m values for n-hexanal and acetaldehyde were 6 μM and 20 mM, respectively, when reacted with bovine liver aldehyde oxidase. On the other hand, Gordon et al. (39) reported the K_m value of 7 mM for crotonaldehyde with pig liver enzyme.

Knox (56) reported K_m values of 17 and 0.17 mM for crotonaldehyde and quinine, respectively, when catalyzed by rabbit liver aldehyde oxidase. On the other hand, Felsted et al. (33) reported that the K_m of the rabbit aldehyde oxidase for N¹-methylnicotinamide was found to be 0.66 and 0.36 mM for hog liver enzyme.

Mukund and Adams (77) reported that the apparent V_{max} and K_m values were 10.5 units and 1.0 mM for glyceraldehyde at 65°C, whereas, V_{max} and K_m

values were 67 units and 40 μM for crotonaldehyde at 80°C. Large and Connock (61) reported the apparent K_m for benzaldehyde were 1.8, 11.5, and 5.6 μM for three species of terrestrial gastropod.

Palmer (84) reported V_{max} and K_m values for several aldehydes as substrates for pig liver enzyme (Table 3). Butyraldehyde and 2-methyl butyraldehyde have highest V_{max} ($\mu\text{mole aldehyde oxidized/min/mg protein}$) value of 20.6, followed by 2-ethylbutyraldehyde (V_{max} 10.0) and propanal (V_{max} 8.7), whereas valeraldehyde and 2-ethylbutyraldehyde have lowest K_m value of 1.25 mM, followed by heptaldehyde (1.3 mM) and crotonaldehyde (6.7 mM). The values of other aldehydes are in Table 3.

Aldehyde oxidase from various sources demonstrated a broad substrate specificity for both aliphatic and aromatic aldehydes. Bovine liver aldehyde oxidase had a higher affinity to hexanal than did acetaldehyde (115). Therefore, it could be very useful to use aldehyde oxidase in removal of some soybean off-flavors because hexanal gives a major contribution.

Inhibitors. Palmer (85) observed that 1,10-phenanthroline inhibits pig liver enzyme in partially competitive manner and found that the inhibitor bound adjacent to the substrate binding site. On the other hand, Takahashi et al. (115) found that menadione (3.3 μM) and Triton X-100 (800 μM) exhibit inhibition to bovine liver enzyme, and Banks and Barnett (7) reported that potassium cyanide (0.2 mM) is inhibitor for rabbit liver aldehyde oxidase.

Table 3. V_{max} and K_m values for various aldehydes for pig liver aldehyde oxidase^a

Substrate	V_{max} ^b	K_m (mM)
formaldehyde	1.7	380
acetaldehyde	7.9	100
propanal	8.7	30
butyraldehyde	20.6	25
valeraldehyde	4.1	1.25
heptaldehyde	0.54	1.3
2-methyl propanal	1.47	40
2-hydroxy butyraldehyde	4.1	28
2-methyl butyraldehyde	20.6	25
2-ethyl butyraldehyde	10.0	1.25
crotonaldehyde	13.0	6.7

^asource: Palmer (84).

^b μ mole aldehyde oxidized/min/mg protein.

Tatsumi and Kitamura (116) showed that N-hydroxy-2-acetylaminofluorene has noncompetitive inhibition and N-hydroxy-4-acetylaminobiphenyl, N-hydroxyphenacetin, and N-hydroxy-2-propionylaminofluorene were inhibitory to rabbit liver aldehyde oxidase. Also, Igo and Mackler (50) reported that quinacrine inhibits flavin nucleotide containing enzymes and aldehyde oxidase.

Knox (56) found that cyanide (10 mM), propamidine (0.4 mM), and plasmochin (0.4 mM) inhibit rabbit liver enzyme when cinchonidine and crotonaldehyde were used as substrates, and 8-hydroxyquinoline (2.4 mM) and atabrine (2 mM) when crotonaldehyde was used. In addition, Yoshihara and Tatsumi (124) reported that allopurinol, menadione, estradiol, and dithiothreitol were inhibitors for guinea pig liver aldehyde oxidase.

Branzoli and Massey (13) found that inactivation of rabbit liver aldehyde oxidase by cyanide is due to the cyanolysis of a persulfide group essential for the catalytic activity of the enzyme. Rajagopalan et al. (96) reported that benzoquinone, menadione, hydroquinone, amytal, antimycin A, estradiol, progesteron, 2,4-dinitrophenol, and Tritron X-100 are inhibitors for rabbit liver aldehyde oxidase. Also, Rajagopalan and Handler (95) showed that N-methylpyridinium inhibits the enzyme. Quinacrine and arsenite were found to be competitive inhibitors, and *p*-mercuribenzoate was competitive type. In addition, methanol in large concentration caused progressive inactivation of enzyme and 0.33 mM and 20 μ M dinitrofluorobenzene caused complete

inactivation of enzyme in two minutes and in five minutes, respectively. This suggested that molybdenum component was present at the substrate-binding site, participated in the hydroxylating event, and was the first component of these enzymes to be reduced.

Mahler et al. (67) studied a variety of compounds and found that *p*-chloromercuribenzoate, arsenite, and iodoacetate are sulfhydryl inhibitors, whereas EDTA, 8-hydroxyquinoline, *o*-phenanthroline, thenoyltrifluoroacetone, citrate, cyanide, and azide are metal-binding agents as inhibitors for pig liver aldehyde oxidase. Also, quinacrine was found to be a flavin inhibitor. They suggested that sulfhydryl group on the enzyme was involved in catalysis. They also found that metal is required for cytochrome C interaction, but not for interaction with dyes or oxygen.

Hall and Krenitsky (44) tested chlorpromazine as an inhibitor for rabbit liver aldehyde oxidase, using 6-methylpurine as the variable substrate and oxygen as the electron acceptor. In addition, Gormley et al. (40) reported that anilinoacridine is extremely potent competitive inhibitor for rabbit liver aldehyde oxidase but not xanthine oxidase.

Chang et al. (20) reported that mercaptoethanol, dithiothreitol, and allopurinol were effective inhibitors to rat aldehyde oxidase. Robertson and Gamage (99) found that methadone was a strong inhibitor to rat aldehyde oxidase.

Electron acceptors. Yoshihara and Tatsumi (124) compared

the kinetics of the reductions of diphenylsulfoxide (DPSO) and other classical electron acceptors such as oxygen and ferricyanide. The double-reciprocal plot of 2-hydroxypyrimidine-linked DPSO reduction with the highly purified enzyme was biphasic. Similar biphasic plots were obtained with the reductions of other electron acceptors.

Rajagopalan et al. (96) assayed molecular oxygen, ferricyanide, 2,6-dichlorophenolindophenol, methylene blue, phenazine methosulfate, silicomolybdate, and cytochrome C as electron acceptors with either aldehyde or N¹-methylnicotinamide. Also, Rajagopalan and Handler (94) tested molecular oxygen, 2,6-dichlorophenolindophenol (DCIP), nitroblue tetrazolium (NBT), trinitrobenzenesulfonic acid (TBS), ferricyanide, methylene blue, silicomolibdate, and cytochrome C as electron acceptors. DCIP was insensitive to inhibitors and amytal inhibited transfer of electron to ferricyanide, methylene blue, phenazine methosulfate, and silicomolybdate. Antimycin A had no effect on these activities, but inhibited reduction of molecular oxygen, cytochrome C, NBT, and TBS. Oligomycin was also found to be an effective inhibitor of aldehyde oxidase. Antimycin A had anaerobically no effect on the reduction of ferricyanide, methylene blue, DCIP, silicomolybdate, or phenazine methosulfate. The markedly different effects of several inhibitors suggested the existence of multiple points of electron egress from the enzyme, and the complexity of the internal electron transport system of rabbit liver aldehyde oxidase. They concluded that these observations indicated four sites of electron egress from

the enzyme to the electron acceptors. This suggested an internal sequence of four carriers which mediate electron transport from substrate to oxygen.

Tomita et al. (120) also tested cytochrome C, ferricyanide, DCIP, and NBT as electron acceptors for rabbit aldehyde oxidase.

Krenitsky et al. (59) tested ferricyanide as electron acceptor for aldehyde oxidase from a wide variety of animals, and found that ferricyanide was an efficient electron acceptor in all but few species. They did not test oxygen as an electron acceptor. However, ferricyanide was not effective for extracts of snake tissues, dogfish liver, and the intestine of rat, mouse, and guinea pig. They suggested that actual electron acceptor could be different *in vivo*.

Physiological role of aldehyde oxidase. Krenitsky et al. (59) investigated a variety of sources and concluded that wide distribution of aldehyde oxidase suggests that its primary metabolic function is one of rather fundamental importance to animals. In addition, Hall and Krenitsky (44) studied rabbit liver aldehyde oxidase and found that the commonly occurring nucleobases (hypoxanthine, xanthine, and adenine) are readily oxidized by xanthine oxidase but not by aldehyde oxidase. They suggested that aldehyde oxidase might play a less important role in the oxidation of endogenously generated purines than does xanthine oxidase. This view is consistent with the finding that levels of aldehyde oxidase vary more from species to species than does xanthine oxidase (59). However, the net effect is that many nucleoside analogues are much more efficiently oxidized by aldehyde oxidase than by

xanthine oxidase (44). Characteristics of aldehyde oxidases from various sources are summarized in Table 4.

Streptomyces

Streptomyces are filamentous bacteria that are a member of the *Actinomycetales* family. These bacteria are most known for their numerous antibiotic production (26). *Streptomyces* are also known for some unique enzymes. Some of these enzymes are amylases (26, 36), cellulases (36), hemicellulase (36), proteases (26, 36), glucose isomerase (26, 36), lignin peroxidase (1, 98), polyethylene-degrading extracellular enzymes (90), and more.

The life cycle of *Streptomyces* includes spore formation and vegetative mycelium growth. A mass of vegetative hyphae is referred to as mycelium, and hyphae are branched or unbranched filaments. Two kinds of mycelium exist: aerial mycelium and substrate mycelium. Aerial mycelium projects above the medium, whereas substrate mycelium occurs on the medium surface. Streptomycetes are similar to fungi in that they produce long filaments, they settle rapidly in solution, and they are easily recovered by filtration, but they are a true procaryote. Their spores are resistant to 5 to 10 min at 60 to 80°C (26).

Streptomyces have been used in industrial fermentations for decades and

Table 4. Characteristics of various animal liver aldehyde oxidase

Source	MW (KDa)	K _m (mM) (substrate)	V _{max} ^a	Inhibitor	e ⁻ Acceptor	Reference
pig	-	7 (crotonaldehyde)	-	-	methylene blue	Gordon et al. (1940)
		380 (formaldehyde)	1.7			
		100 (acetaldehyde)	7.9			
		30 (propanal)	8.7			
pig	-	25 (butyraldehyde)	20.6	1,10-phenanthroline	ferricyanide DCIP ^b	Palmer (1962a)
		1.25 (valeraldehyde)	4.1			
		1.30 (heptanal)	0.54			
		6.7 (crotonaldehyde)	13.0			
Guinea pig	348 ^c 300 ^d	-	-	menadione, amytal, estradiol, cyanide, dithiothreitol potassium, Triton X-100	ferricyanide DCIP ^b	Yoshihara & Tatsumi (1985)
bovine	-	0.006 (n-hexanal) 20 (acetaldehyde)	-	menadione Triton X-100	oxygen	Takahashi et al. (1979)
bovine	222 ^c	-	-	-	DCIP ^b	Cabre & Canela (1987)
hog	268 ^d	0.36 (NMN ^o)	-	-	oxygen	Flested et al. (1973)
rabbit	270 ^d	0.66 (NMN ^o)	-	-	-	-
rabbit	-	17 (crotonaldehyde) 0.17 (quinine)	-	cyanide, propamidine, plasmochin, atabrine 8-hydroxyquinoline	oxygen methylene blue	Knox (1946)
rabbit	-	1.3 (purine) 0.21 (hypoxanthine)	100 167	-	ferricyanide	Hall & Krenitsky (1986)

^aμmole aldehyde oxidized/min/mg protein.

^bDCIP: dichlorophenolindophenol.

^cBy gel filtration.

^dBy gel electrophoresis.

are, therefore, acceptable for commercial production. Some are considered generally recognized as safe (GRAS)(12), which permits the use of their products in foods. For example, *Streptomyces natalensis* produces natamycin which is used as a food preservative for strawberries and raspberries, and *Streptomyces griseus* produces pronase, a heat stable protease (60°C for 10 min)(53).

Lignin-degrading *Streptomyces setonii* 75Vi2 and *Streptomyces viridosporus* T7A form white-yellowish and grey-green aerial spores, respectively, and grow optimally at 37°C. These microorganisms are involved in several aromatic compounds metabolism, producing different dioxygenases (111).

L-Phenylalanine and L-tyrosine were completely catabolized through homogentisate 1,2-dioxygenase by *S. setonii*, but partially degraded by *S. viridosporus* (87). Catechol 1,2-dioxygenase was inducible in both *S. setonii* and *S. viridosporus* grown with catechol (91), *trans*-cinnamic acid, vanillin (112) and benzoic acid (113). Also, protocatechuate 3,4-dioxygenase was inducible with *p*-coumaric, or ferulic acids and *m*-hydroxybenzoic acid in *S. setonii* and *S. viridosporus*, respectively (91, 112). Whereas, gentisate 1,2-dioxygenase was induced when *S. viridosporus* was grown with gentisate. *S. setonii* 75Vi2 is best known for its aromatic aldehyde and acid biodegradation.

trans-Cinnamic acid was metabolized *via* benzaldehyde, benzoic acid to catechol, and *p*-coumaric acid was catabolized to protocatechuic acid by *S.*

setonii (112). Vanillic acid was metabolized *via* guaiacol and catechol to *cis,cis*-muconic acid by *S. setonii* (91) and cytochrome P-450 was involved (111). Vanillin (aromatic aldehyde) was converted to vanillic acid by *S. viridosporus* in a whole-cell bioconversion (88). Vanillic acid is not degraded by *S. viridosporus*. Also, ferulic acid was converted to vanillin, vanillic acid then guaiacol to catechol by *S. setonii* (112). *S. viridosporus* metabolized *p*-hydroxybenzaldehyde *via* *p*-hydroxybenzoic acid and benzaldehyde, *m*-hydroxybenzaldehyde, *p*-hydroxybenzaldehyde, and protocatechualdehyde (24). All aromatic compounds were catabolized *via* the β -keto adipate and gentisate pathways.

Investigation of lignocellulose biodegradation by *S. viridosporus* T7A has been very extensive. Endoglucanase, xylanase, and lignin peroxidase were identified for *S. viridosporus* incubated with lignocellulose in a lignocellulose slurry with 0.6% yeast extract medium (1). The optimal lignocellulose degradation by *S. viridosporus* was observed in the pH range of 8.4 to 8.8, with an optimum of pH 8.5 (86). Lignin degradation by *S. viridosporus* was oxidative and involved demethylations, ring cleavage reactions, and oxidative attack on phenylpropanoid side-chains (24). Cellulase activity was higher for mutants than the wild type *S. viridosporus* (29). Also, a UV-irradiated mutant and a protoplast fusion recombinant had higher and more persistent peroxidase, esterase, and endoglucanase activities than did the wild-type *S. viridosporus*, whereas higher xylanase was shown in a UV-irradiated mutant (97). Four

isoforms of peroxidase were identified by PAGE and individually purified (98). Three isoforms were found to be immunologically related to one another (65).

S. viridosporus and *S. setonii* were also reported to degrade heat-treated polyethylene film containing prooxidant and 6% starch (63). The presence of extracellular enzyme(s) produced by these two *Streptomyces* species was demonstrated (89, 90). These unique enzyme(s) were produced in 0.6% yeast extract medium.

Experimental Design

Aldehyde oxidase was initially induced in an *S. setonii* 75Vi2 shake-flask study and was scaled-up *via* 800-ml continuous stirred reactor (CSR), 5-L, and 15-L, to 50-L batch fermentation. Aldehyde oxidase activity was monitored during each step of scale-up procedure. In order to get high aldehyde oxidase activity, amount of inducer, number of inducer additions, time interval between inducer additions, time of addition, and presence or absence of pH control were tested. Also, effect of spore and vegetative cell inoculation on enzyme activity were compared. Initially propanal was used as an inducer for aldehyde oxidase; however, aromatic aldehydes (vanillin and *trans*-cinnamaldehyde) were used as inducers for *S. viridosporus* T7A because of inconsistent aldehyde induction pattern. For the detection of product by aldehyde oxidase reaction, high-performance liquid chromatography (HPLC), thin-layer chromatography

(TLC), and 2,4-dinitrophenylhydrazine were used. Also, polyacrylamide gel electrophoresis (PAGE) was performed to determine aldehyde oxidase molecular weight and H_2O_2 production on zymogram. Experiments were performed in duplicate and statistical analysis was done by least significance difference (LSD) at the level of $P < 0.05$.

Statement of the Problem

Aldehyde oxidase from various sources has a broad substrate specificity as illustrated above. Soybean's use for human consumption is limited due to some off-flavors despite its nutritional benefit. Thus, it is desirable to remove or reduce those off-flavors to expand soybean as a protein source for human consumption.

Lipoxygenase inactivation by solvent modification (3, 11, 105, 114) seems to be effective, but it often denatures soy protein, making it less soluble. Enzymatic off-flavor removal could be a better way to improve this problem. Chiba et al. (21) used aldehyde dehydrogenase from bovine liver to remove the green beany flavor in soy protein isolate solution. However, they suggested that it is impractical and uneconomical because of NAD^+ requirement in the enzyme reaction.

Most aldehyde oxidases reported are from other than bacterial source. Production of aldehyde oxidase by fermentation would be preferred for a

bacterial enzyme source because mass production is possible in a relatively short time. Bovine liver aldehyde oxidase was reported to have a high affinity for hexanal (115), which is a major off-flavor compound. Bacterial aldehyde oxidase might have similar characteristics and possibly it could attack hexanal bound to soy protein. The next section addresses the production of aldehyde oxidase by *Streptomyces* species in small and large fermentations.

MATERIALS AND METHODS

Microorganisms

The actinomycetes used were *Streptomyces viridosporus* T7A (ATCC 39115) and *Streptomyces setonii* 75Vi2 (ATCC 39116). *S. viridosporus* T7A is reported to produce an aromatic aldehyde oxidase (29) and *S. setonii* 75Vi2 is also known to produce an aliphatic aldehyde oxidase when propanal (3.9 g/L medium) was used as an inducer (unpublished data by Erwin Affandi). *S. viridosporus* and *S. setonii* were maintained at 4°C on yeast extract-malt extract-glucose agar and 0.6% (w/v) yeast extract agar, respectively (87). *S. viridosporus* spores are very shelf-stable and the culture was transferred every 3-6 weeks. In contrast, *S. setonii* spores are not very shelf-stable and require transferring every 2-4 weeks.

Medium

The medium used was 0.6% (w/v) yeast extract (Difco Laboratories, Detroit, MI) in nitrogen-free salts solution (5.03 g of Na₂HPO₄, 1.98 g of KH₂PO₄, 0.20 g of MgSO₄·7H₂O, 0.20 g of NaCl, 0.05 g of CaCl₂·2H₂O, plus 1 ml of trace elements solution [71] per liter of deionized H₂O; pH 7.1 to 7.2)

This medium has been used previously for the production of unique enzymes like lignin-peroxidases (1, 98) and polyethylene-degrading enzymes by *Streptomyces* (63, 89).

Optimization of Enzyme Production

Shake-flask studies

A cotton plugged 2-L Erlenmeyer flask containing 1 L of sterile 0.6% (w/v) yeast extract medium was inoculated with a loopful of *S. setonii* 75Vi2 spores (10^7 spores/ml). The culture was incubated with shaking at 125 rpm and 37°C. After a 48, 60, or 72 hr incubation, 3.9 g propanal (5 ml; 97%; density = 0.805 g/ml) (Aldrich Chemicals Co., Milwaukee, WI) was added to 1-L medium and after an additional 12 hr incubation, a second 3.9 g propanal was added to each bioreactor for enzyme induction. Propanal was stored in a N₂ atmosphere to prevent autoxidation. The pH was checked after 24 hr incubation then monitored every 12 hr until harvest. Cell-mass was harvested by filtration (Whatman no. 54 harden filter paper, Whatman Co., Hillsboro, OR), scraped from the paper, suspended in 0.1 M phosphate buffer (pH 7.2), cells disrupted *via* French press (SLM Instruments, Inc., Urbana, IL) (24) then supernatant was recovered by centrifugation 27,000 x *g* at 4°C. The enzyme activity was assayed by oxygen consumption measurement after substrate addition by using a YSI oxygraph (YSI model 5300, Yellow Springs Instruments

Inc., Yellow Springs, OH). Enzyme activity was determined for cultures with various incubation times (12 and 24 hr) after a 3.1 or 3.9 g propanal/L addition.

pH study in 800-ml continuous stirred reactor (CSR)

Initial CSR fermentations were performed in customized 1.2 L Fleaker Beakers (Corning Glass Works, Corning, NY). *S. setonii* 75Vi2 spores were inoculated into 800 ml of sterile 0.6% (w/v) yeast extract medium, stirred with a magnetic bar, and incubated in a 37°C waterbath, with aeration and manual antifoam control with Antifoam A (Sigma Chemical Co., St. Louis, MO). Four reactors were operated simultaneously with pH controlled with 2 N NaOH or HCl, at pH 7.0, 7.5, 8.0, and 8.5. After a 36 or 48 hr incubation, 1.6 g propanal/L was added to determine the optimal time for propanal addition for enzyme induction. Aldehyde oxidase activity for different cell-free extracts was compared with one propanal addition after 36 or 48 hr incubation and two propanal additions to the culture. The time interval (6 or 12 hr) between two propanal addition was also compared.

5-L batch fermentation

Spore vs. vegetative cell inoculum. *S. setonii* 75Vi2 spores were inoculated into 100-ml of sterile 0.6% (w/v) yeast extract medium in a 250-ml Erlenmeyer flask then incubated with shaking at 37°C for 48 hr. This vegetative cell inoculum was then aseptically transferred into 5-L of sterile

0.6% (w/v) yeast extract medium. The spores from slants were also used as an inoculum and the results were compared with vegetative cell inoculum.

Fermentation was done in a 7.5-L Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, NJ). The pH was adjusted to 7.0 with 2 N HCl or NaOH before inoculation and agitation was 250 rpm at 37°C with continuous aeration and automatic antifoam control with Antifoam A. The propanal concentrations tested for enzyme induction were 0.8, 1.6, and 2.4 g/L medium. Growth curve was constructed by measuring dry cell-mass and absorbance at 600 nm. For dry cell-mass weight measurements, a 100-ml sample was taken every four hours, cell-mass was collected on preweighed filter paper (Whatman no. 1), then dried at 45°C overnight. The dry cell weight was determined by subtracting preweighed filter paper from dried filter paper with cell-mass. For wet cell-mass weight determination, a 100-ml of culture sample was taken every 4 hr for a 76 hr fermentation after an initial 20 hr incubation. After a 36 hr incubation, 3.9 g/L propanal was added to the bioreactor for a maximum of four times in 6 hr intervals to determine the optimal propanal addition for aldehyde oxidase induction.

15-L batch fermentation

Fermentation was done in a 19-L Bioengineering fermentor (Wald, Switzerland) with dissolved oxygen, temperature, antifoam and agitation control. Fifteen-liters of 0.6% (w/v) yeast extract medium with a modified

nitrogen-free mineral salts solution in which the phosphate concentration was changed to 0.02 M (1.006 g of Na_2HPO_4 and 0.396 g of KH_2PO_4) and sterilized *in situ*. The pH was adjusted to 7.0 then inoculated with a spore suspension of *S. setonii* 75Vi2 (3 slants) and the fermentor operated with agitation (250 rpm), continuous aeration (0.7 vvm), and antifoam control (Antifoam A) at 37°C, as previously done in 5-L fermentation. The pH was monitored and dissolved oxygen was controlled at 80%. Propanal (1.6 g/L) was added when pH increased to 7.8 (24 hr incubation), indicating ammonium release from amino acid biodegradation (86), and dissolved oxygen (DO_2) dropped to 20% then started to increase which indicated the cells were entering late-log phase. A second 1.6 g propanal/L addition was made after 6 hr incubation without pH control and the culture was harvested when DO_2 returned to 100% which indicates late stationary phase. During the course of the fermentation wet cell-mass weight was determined by filtration (Whatman no. 54) of 100-ml culture samples every 4 hr. The aldehyde oxidase activity was also determined for each sample *via* oxygraph.

50-L batch fermentation

Fermentation was done in a Braun U-50 Biotech 72-L fermentor (B. Braun, Allentown, PA) with a 50-L working volume, temperature, aeration, and antifoam controls. The fermentation was maintained at 37°C. Dissolved oxygen was controlled by change in agitation speed and it was set for 80%

saturation. However, during log phase growth, dissolved oxygen dropped to 20% saturation. The pH was adjusted to 7.0 before inoculation then continually monitored but not controlled during the fermentation.

Three slants of *S. viridosporus* T7A (4 to 6 weeks old culture) or *S. setonii* 75Vi2 (fresh, 1 to 4 weeks old culture) were used for inoculation. The medium contained 0.6% (w/v) yeast extract (Difco) with or without 1.0% (w/v) malt extract (Difco) in the modified nitrogen-free mineral salts solution with 0.02 M phosphate. Malt extract was heat sterilized in water separately, then was aseptically added to the sterile fermentor and pH adjusted to 7.1. During culture growth, the pH increased to 7.6-7.8 with a corresponding oxygen consumption drop to 30-50% saturation. A 0.2% (v/v) propanal, 0.2% (w/v) vanillin (Aldrich) or 0.02-0.2% (v/v) *trans*-cinnamaldehyde (Aldrich) was added for enzyme induction. Two-liters of culture broth were removed before vanillin or *trans*-cinnamaldehyde addition for *S. viridosporus* and *S. setonii* culture, respectively. The culture was incubated for an additional 6 hr and the fermentor was cooled to 17°C when an 80% decrease of vanillin or *trans*-cinnamaldehyde was observed spectrophotometrically (Beckman DU^o-50 spectrophotometer, Beckman Instruments Inc., Fullerton, CA) at 345 nm and 286 nm, respectively (88). Then, the culture was collected in a 50-L carboy and stored at 4°C. Reduction in aromatic aldehyde concentration was continually monitored for two to three days during storage at 4°C and the culture was harvested when the aromatic aldehyde concentration dropped to 0-

5% of initial vanillin added for *S. viridosporus* or *trans*-cinnamaldehyde for *S. setonii*.

Enzyme Assay and Partial Purification

Cell-free crude-extract preparation

The cell-mass was harvested by filtration (Whatman no. 54), washed with 0.1 M phosphate buffer, mixed with 0.1 M phosphate buffer to form a paste and then disrupted *via* a French press at 1,200 psi (24). These cracked cells were then centrifuged for 20 minutes at 27,000 x *g* at 4°C and the cell-free extract evaluated for aldehyde oxidase activity. Crude-extracts were freeze-dried for 72 hours in a Virtis freeze-dryer (Unitrap II, Gardiner, NY) at room temperature and were stored in a closed container at 4°C until analysis. For enzyme assay, freeze-dried crude-extract was resuspended at a concentration of 20 to 40 mg/ml in 0.1 M phosphate buffer. Freeze-dried extracts contained 0.22 to 0.26 mg protein/ml.

Ammonium sulfate precipitation

Ammonium sulfate (Sigma Chemical Co.) was slowly added to continuously stirred crude-extract at 4°C. Serial continuous ammonium sulfate saturation from 25 to 60% was evaluated and precipitate was collected by centrifugation at 27,000 x *g*. The pellet was resuspended in 0.1 M phosphate

buffer (pH 7.1). Each fraction (supernatant and pellet) was assayed for aldehyde oxidase activity.

Enzyme assay

For aldehyde oxidase activity, the consumption of molecular oxygen upon the addition of 500 mM propanal, 10 mM vanillin or 10 mM *trans*-cinnamaldehyde was measured *via* oxygraph (YSI Inc.) with recorder (Omega, Stamford, CT), in a temperature-controlled reaction chamber at 37°C and continually stirred. Oxygraph reaction mixture procedure is described in Figure 2. The reaction mixture contained 3.6 ml of 0.1 M phosphate buffer, 200 μ l of 3% H₂O₂ (Fisher Scientific Co., Pittsburgh, PA) and 100 μ l of cell-free extract. Hydrogen peroxide was added to the reaction mixture in an effort to fatigue catalase activity, which interferes with the aldehyde oxidase assay. After equilibrating the reaction mixture for 5 minutes at 37°C, the reaction was initiated by substrate addition (0.1 ml of 500 mM propanal, 131 mM vanillin, or 10 mM *trans*-cinnamaldehyde). The initial oxygen decrease in the reaction mixture was measured. Background changes in oxygen consumption were determined for each enzyme extract after a 10-min treatment in boiling water. This boiled extract was used as control. A 0.1 μ mole of oxygen change for the oxygraph was calibrated by using catechol 1,2-dioxygenase produced by *S. setonii* 75Vi2 grown in benzoic acid medium (112). One unit of enzyme activity was expressed as 0.1 μ mole of O₂ consumed per minute per mg of

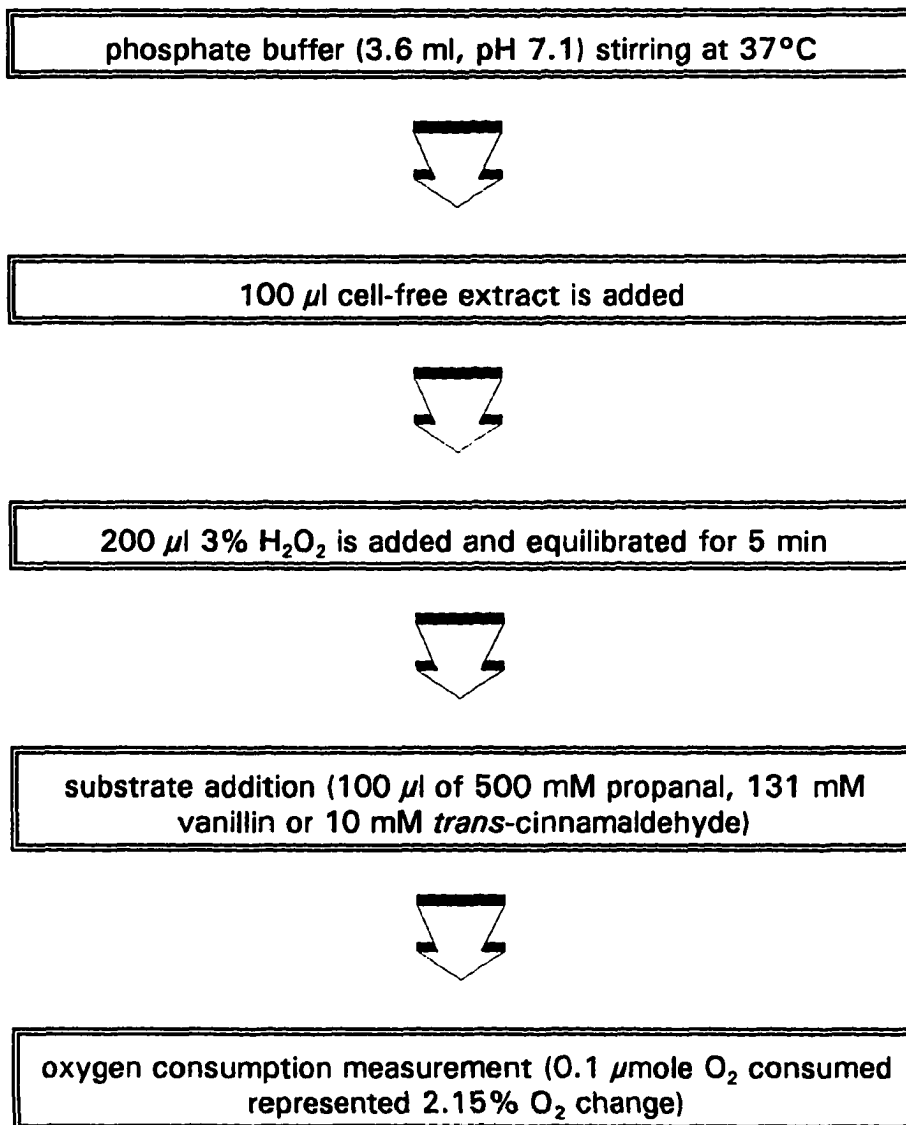


Fig 2. Flow diagram of aldehyde oxidase assay

protein (87). Protein concentration was measured by the method of Lowry et al. (64).

Product analysis. Propanal bioconversion to propionic acid was confirmed by using a Waters high performance liquid chromatograph (HPLC) (Milford, MA), equipped with Waters Model 401 refractive index detector. A Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) was used with a 20- μ l injection loop and 0.012 M H₂SO₄ as a mobile phase at a flow rate of 0.8 ml/min at 65°C. Propanal and propionic acid had retention times of 17.5 and 14.2 min, respectively.

The conversion of vanillin to vanillic acid was detected by absorbance at 345 nm. *trans*-Cinnamaldehyde and vanillin have absorbance maxima at 286 and 345 nm, respectively, which is not present for *trans*-cinnamic or vanillic acid. Samples from each fermentation were analyzed in replicates of two or more.

Another method evaluated for aldehyde detection was a 2,4-dinitrophenylhydrazine assay. The mixture contained 0.1 ml of 2,4-dinitrophenylhydrazine, 1.9 ml of 95% ethanol, 1.0 ml of 0.2 N NaOH, and 1.0 ml of various concentration of propanal or 1.0 ml water for the blank. The 2,4-dinitrophenylhydrazine reacts with aldehyde groups to produce a yellow to red precipitate, which had an absorption maximum at 520 nm.

For aldehydes detection, thin-layer chromatography (TLC) was also used. Glass plate (20 x 20 cm) coated with silica gel G and mobile phase of

hexane:ether:acetic acid (50:50:1) was used. The 3.6 ml reaction mixture after propanal or hexanal addition and oxygraph measurement was acidified to pH < 3 with concentrated HCl, then extracted with 1.0 ml ether, which was dewatered by the addition of anhydrous Na₂SO₄. A 5 µl sample was applied to activated TLC plate and R_f values were measured. Aldehydes were identified by spraying the plate with 0.5% (w/v) 2,4-dinitrophenylhydrazine solution in 2 N HCl which gave yellow to red spot and their corresponding acids were identified under longwave ultraviolet-light after a 0.2% (w/v) 2',7'-dichlorofluorescein solution in 95% ethanol was sprayed over the TLC plate.

Polyacrylamide gel electrophoresis (PAGE)

For preparative sodium dodecylsulfate (SDS) PAGE, the discontinuous gel buffer system of Laemmli (60) was used. All chemicals used were purchased from Sigma Chemicals Co. Stacking gel was 4% acrylamide and separating gel was 10 or 12% acrylamide (29.2 acrylamide:0.8 N,N'-bis-methylene-acrylamide, w/w). The polymerization reaction was started with addition of 0.05% ammonium persulfate and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED) and placed at room temperature for 45 minutes. Sample buffer (SDS reducing buffer) contained 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol and 0.00125% (w/v) bromophenol blue. The sample was diluted with sample buffer at 4:1 and was heated at 95°C for 4 minutes before loading. The gels were 7 cm long and 8 cm wide (Mini-PROTEAN®II

Electrophoresis cell, Biorad, Hercules, CA) and were electrophoresed for an hour at constant 64 V. The gels were stained with 0.1% (w/v) Coomassie Blue R-250 in the solution containing methanol:acetic acid:water (40:10:50) and were destained with the same solution without stain.

Nondenaturing gel electrophoresis was performed in a 5 cm, 12% acrylamide (29.2 acrylamide:0.8 N',N'-bis-methylene-acrylamide, w/w) separating gel, and a 2 cm, 4% acrylamide stacking gel. About 50 to 150 μ g of protein were loaded into each well depending on the gel thickness. The gels were electrophoresed for an hour in buffer (0.025 M Tris-glycine; pH 8.3) at constant 64 V. The gels were submerged in 0.02% (w/v) vanillin solution, washed in buffer, then placed in 0.002% (w/v) horseradish peroxidase and 0.02% (w/v) of *o*-dianisidine dihydrochloride solution (24). The H₂O₂ produced during oxidation of vanillin to vanillic acid oxidized *o*-dianisidine to yellow or brown which identified the aldehyde oxidase band on the gel. The protein bands which demonstrated positive enzyme activity were cut out of the gel, frozen then ground by a pestle in mortar with cold phosphate buffer to extract aldehyde oxidase for SDS-PAGE molecular determination as above.

RESULTS AND DISCUSSION

Fermentation

Shake-flask studies

Aldehyde oxidase in *S. setonii* 75Vi2 was induced by adding 3.9 g propanal/L in shake-flask study by a former colleague Erwin Affandi (unpublished data). This study was performed to further characterize aldehyde oxidase induction and production by *S. setonii*. Initial pH was around 7.1 for all three flasks before inoculation and pH increased slowly. The first propanal (3.9 g/L) addition was after 48, 60, or 72 hr incubation for aldehyde oxidase induction (Table 5). Generally propanal addition (3.9 g/L) caused a decrease in medium pH, which could be the result of autoxidation to propionic acid. Propanal addition at the beginning of fermentation resulted in no cell growth, probably due to its toxic effect (unpublished data by Erwin Affandi).

After a 48 hr shake-flask incubation of *S. setonii*, 3.9 g of propanal was added and cells were harvested after 60 or 72 hr incubation to see the effect of incubation time after propanal addition. Final pH was 6.4 and 6.0 for 3.1 and 3.9 g propanal/L addition to 48 hr culture of *S. setonii*, respectively, which was harvested after 72 hr incubation. Also, pH decreased after propanal addition and pH decreased during incubation which will affect the cell growth and

Table 5. pH changes of *S. setonii* 75Vi2 culture in shake-flask study after 3.9 g propanal addition to 1 L culture medium at different time of incubation

Flask #	Incubation time				
	0	24 hr	48 hr	60 hr	72 hr
1	7.07	7.07	7.15 ^a	ND ^{ab}	5.12
2	7.06	7.09	7.12	7.29 ^a	6.53 ^a
3	7.07	7.08	7.24	7.46	7.60 ^a

^aTime of propanal addition.

^bND: not determined.

enzyme production. Thus, it was decided that pH controlled fermentation was required.

The 800-ml continuous stirred reactor study

Mass transfer of oxygen into culture broth is limited in shake-flask cultures compared to CSR. Therefore, CSR was employed for further studies and initially four-customized fleaker-beaker reactors with pH control were available and utilized. *S. setonii* cultured in 800-ml CSR with and without pH control was employed. To each bioreactor, 3.9 g propanal/L medium was

added after 24 hr incubation and a second propanal addition was made after 36 hr incubation to fermentors with and without pH control. Figure 3 illustrates pH changes during CSR fermentation with two propanal (3.9 g/L) addition without pH control. A first propanal addition caused pH drop from 7.5 to 6.5 and a second propanal addition demonstrated about 0.5 pH unit drop. As seen in shake-flask study, the pH drop after each propanal addition was probably due to autoxidation of propanal to propionic acid .

5-L batch fermentation

The amount of propanal was decided as 1.6 g/L in previous 800-ml CSR study. However, the CSRs were home-made with limited control of agitation, thus it had a potential mixing problem. Therefore, it was necessary to confirm aldehyde oxidase production in 5-L fermentation with better agitation and aeration control.

After 36 hr incubation, 3.9 g propanal/L was added up to four times in 6 hr intervals to determine optimal number of propanal addition for maximum enzyme induction. Samples were removed prior to each propanal addition and cell-free extracts were prepared for aldehyde oxidase activity. Different propanal amount of 0.8, 1.6, and 3.1 g/L was also investigated.

Growth curve of *S. setonii* 75vi2 was determined by measuring dry cell weight and absorbance at 600 nm (Fig. 4) to determine late-log and stationary phase for propanal addition. *Streptomyces* are filamentous bacteria that settle

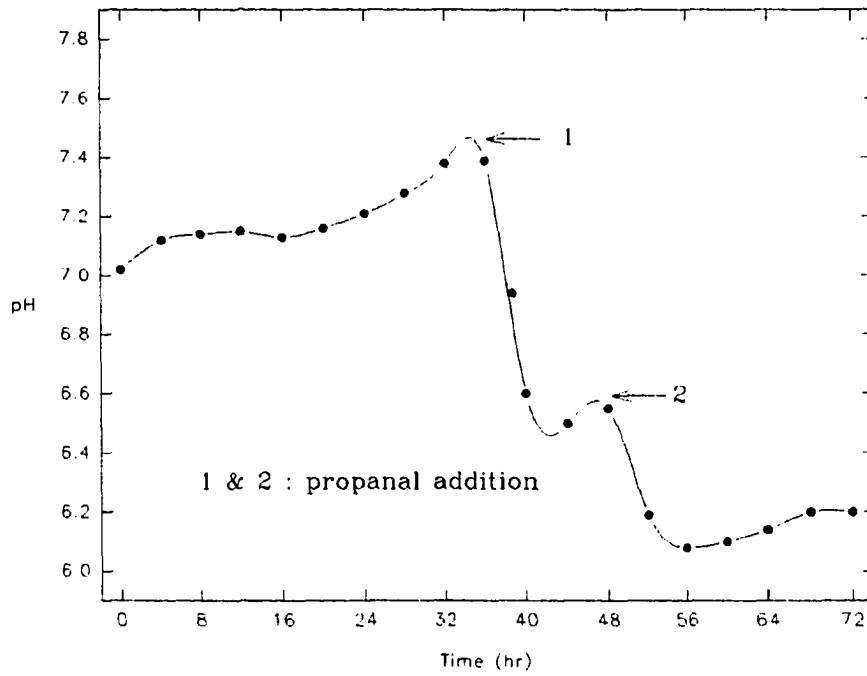


Figure 3. Changes in pH with inducer added after 36 and 48 hr incubation for continuous stirred reactor (CSR) fermentation of *S. setonii* 75Vi2 in 0.6% yeast extract medium without pH control

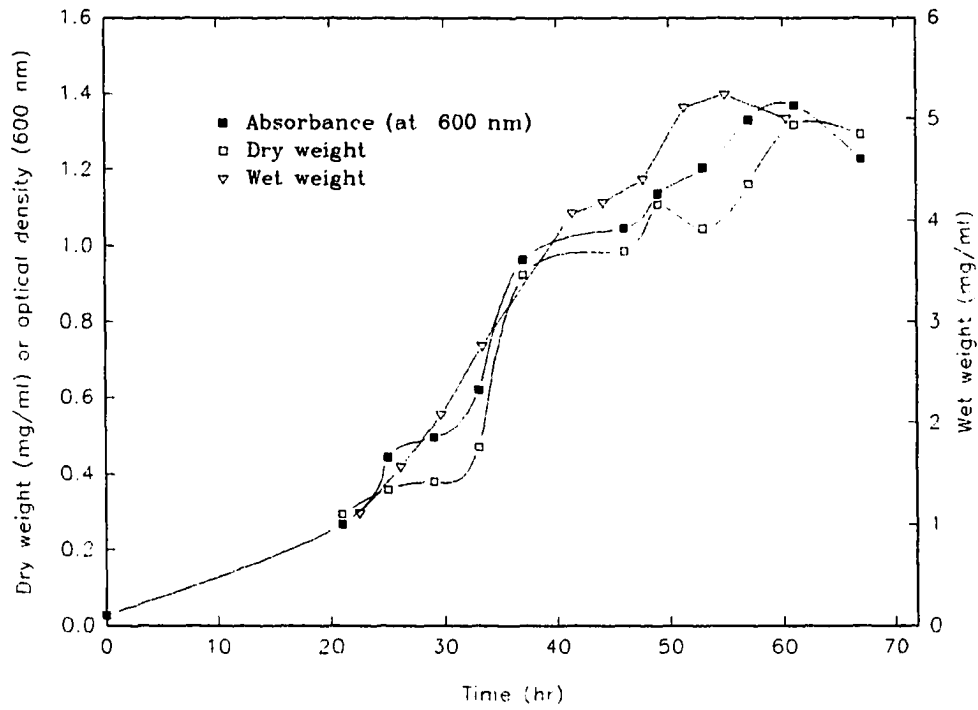


Figure 4. Growth curve for 5-L batch fermentation of *S. setonii* 75Vi2 without pH control

rapidly making cell measurement spectrophotometrically very difficult. However, absorbance measurement at 600 nm was tried for a quick and easy cell-mass determination. The late-log and stationary phase occurred after 48 to 56 hr incubation. All three methods illustrated the same growth patterns, which indicated they adequately measured cell production and dry cell-mass correlated with cell growth in 15-L batch fermentation (in next section). Similar results were demonstrated by Granade et al. (41) for filamentous fungi and by Flowers and Williams (35) for *Streptomyces* sp. Also, wet cell-mass weight measurements were useful in rapid growth estimation for filamentous microorganisms, compared with dry cell-mass weight measurements (16).

To *S. setonii* culture in stationary phase (62 hr incubation), 1.6 g propanal/L medium was added and a second propanal addition after 6 hr incubation. Generally, pH dropped after propanal was added to *S. setonii* 5-L batch fermentor. This was probably due to propanal autoxidation to propionic acid and to possible cell lysis. The culture medium pH of 5-L fermentor increased faster than that of shake-flask culture medium. This difference between fermentor and shake-flask cultures were probably due to a lower dissolved oxygen in shake-flask which depends on diffusion of O₂ through the liquid's surface (6) which consequentially results in slow growth rate.

Spore vs. viable cell inoculum. In an effort to reduce lag time, viable cells were evaluated as an inoculum for 5-L batch fermentation. *S. setonii* spores were inoculated into 100-ml sterile 0.6% (w/v) yeast extract medium in

250-ml Erlenmeyer flask and incubated with shaking at 37°C for 48 hr. For viable cell inoculum, a fermentor with 5-L of 0.6% (w/v) yeast extract medium was inoculated with 50 ml of shake-flask culture after cell-mass settled to the bottom. For a spore inoculum, a fermentor with 5-L of 0.6% (w/v) yeast extract medium was inoculated with aerial spores from two stock slants suspended in 10 ml of sterile water. For enzyme induction, propanal (1.6 g/L) was added after 50 hr incubation followed by a second propanal addition after 56 hr incubation, then cell-mass was harvested after 62 hr incubation. The pattern for pH change in the fermentor with viable cell inoculum was similar to the spore inoculated 5-L batch fermentations. However, aldehyde oxidase was not induced when viable cells were used as an inoculum, and no difference in lag time was observed when spores or viable cells were used as inoculum. Therefore, a spore suspension was used to inoculate all future fermentation.

15-L batch fermentation

This 15-L fermentation was done to correlate pH change, cell growth, and enzyme activity between 5-L and 50-L batch fermentation. *S. setonii* cell density was maximum at about 21 hr incubation for 15-L batch fermentations. Despite efforts of the dissolved oxygen (DO₂) controller during the period of most rapid growth, DO₂ concentrations decreased (Fig. 5). This decrease in DO₂ correlated to a rapid increase in cell-mass (Bottom frame; Fig. 5), because oxygen is relatively insoluble in water (<10 mg/L) and quickly becomes limiting

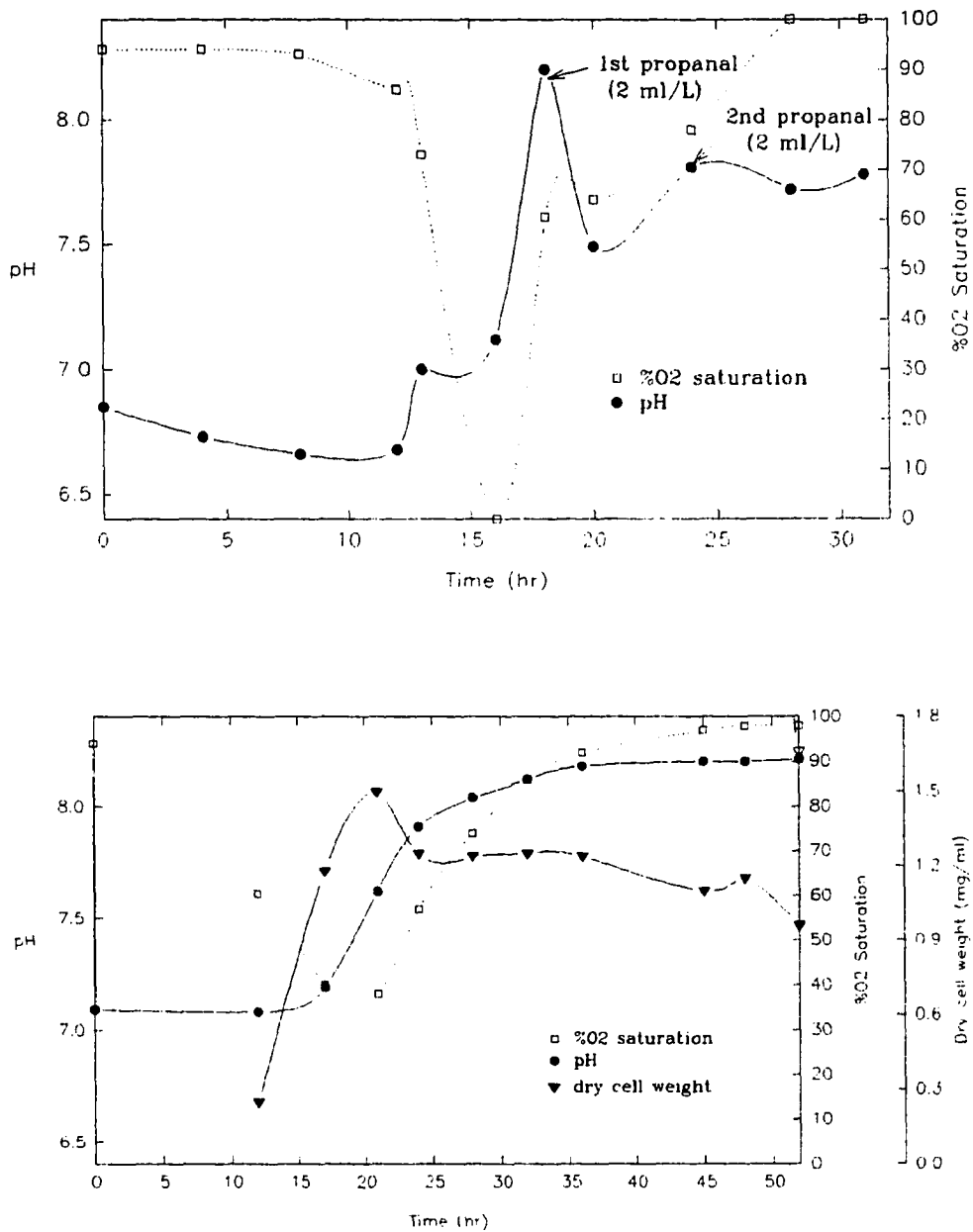


Figure 5. Changes in pH and %O₂ saturation with inducer added after 18 and 24 hr incubation (Top frame) and growth curve without inducer (Bottom frame) for 15-L batch fermentation of *S. setonii* 75Vi2 in 0.6% yeast extract medium

in liquid bacterial cultures (14). The pH constantly increased until about 36 hr incubation, then plateaued after 37 hr and a final pH was 8.2. For propanal addition, DO₂ and pH were used as indicators of late-log phase growth because reduced oxygen consumption correlates to reduced cell growth (6) and pH increase correlates to amino acid catabolism (Fig. 5)(86). It was determined that pH increase to 7.5 and a DO₂ of 10% higher than lowest DO₂ concentration achieved during log-phase growth was a good indicator for enzyme inducer addition (1.6 g propanal/L medium). A similar medium and procedure were used by Ramachandra et al. (97) and Pometto et al. (87) for the induction of lignin peroxidase and polyethylene-degrading enzymes, respectively, when pH increased ≥ 8.0 . Cell clumping was also observed in the 5-L fermentor but not in the 15-L fermentor possibly due to differences in mechanical shear of impeller blade size, reactor diameters, and baffles between the two reactors with the same agitation speeds.

50-L *S. setonii* batch fermentation

The medium contained 0.6% (w/v) yeast extract in a modified nitrogen-free mineral salts solution with 0.02 M phosphate. The first and second propanal (1.6 g/L) addition was after 19 and 25 hr incubation. Cells were harvested after additional 6 hr incubation followed by cooling down in the fermentor to 17°C. Dissolved oxygen and pH patterns were similar to 15-L batch fermentations (Fig. 5). Initially, *S. setonii* fermentation showed positive

aldehyde oxidase activity with good cell-mass production (6.5 to 7.8 g/L wet weight). However, three subsequent fermentations using the same induction patterns showed marginal specific activity (≤ 0.002 units) with cell densities of 5.5 to 7.0 g/L (wet weight). The dissolved oxygen and pH patterns were repeatable (Fig. 5), which implies fermentations were consistent. No contamination was observed in any fermentation when culture was examined microscopically.

Enzyme Activity for *S. setonii* 75Vi2

Our initial studies focused primarily on optimization of enzyme production with *S. setonii*, while most of the enzyme partial purification studies focused on *S. viridosporus*. *S. setonii* demonstrated poor enzyme stability and an irregular enzyme induction pattern, which forced us to change microorganisms.

Aldehyde oxidase activity was monitored during scale-up process from shake-flask to 50-L batch fermentation. However, enzyme activity was determined at culture termination for shake-flask, 800-ml CSR, and 5-L, and 15-L batch fermentation which indicated repeatable aldehyde oxidase induction in each step. Enzyme activity was monitored continuously after propanal addition for 50-L batch fermentations by collecting 1 to 2 liter aseptic samples before inducer addition then every 1 to 2 hr prior until harvest to establish a protocol for maximum aldehyde oxidase production.

Shake-flask cultures

Cells were harvested after 60 or 72 hr incubation when 3.9 g propanal was added after 48-hr incubation; aldehyde oxidase specific activities were 0.154 and 0.034 units, respectively. This five-fold difference in enzyme activity suggested that enzyme induction must be timed properly. It was statistically significant ($P < 0.05$) among data collected through 5-L batch fermentation when analyzed by Least Significant Difference (LSD). The culture harvested after 72 hr incubation with two propanal (3.9 g/L) additions after 48 and 60 hr incubation showed 0.017 unit of specific activity. Culture without propanal addition consistently showed no aldehyde oxidase activity. Finally, no difference in specific activity (0.02 units) was observed when 3.1 or 3.9 g propanal/L was used as the enzyme induction in 1-L shake-flask culture. Since shake-flask cultures have low dissolved oxygen and limited control, more controlled fermentation was needed to test variables such as concentration of and number of propanal additions.

The 800-ml continuous stirred reactors

pH effect. Aldehyde oxidase specific activity was 0.020 unit for pH 7.1 controlled culture, whereas pH uncontrolled culture demonstrated 0.022 unit with a final pH of 6.10 (Table 6). On the other hand, pH controlled at 8.0 and 8.5 showed no activity and poor growth, whereas, pH controlled at 7.5 demonstrated marginal activity. Possibly cell growth was in log phase when

Table 6. Aldehyde oxidase activity of *S. setonii* 75Vi2 culture in 800-ml CSR with and without pH control when propanal (3.9 g/L) was used as an inducer

pH	Specific activity^a
uncontrolled	0.022
7.1	0.020
7.5	NAD ^b
8.0	NAD
8.5	NAD

^aActivity was measured by oxygraph with propanal as a substrate and specific activity was defined as 0.1 μ mole O₂ per minute per mg protein.

^bNo activity detected by oxygraph.

propanal was added or culture was affected by pH localization due to indirect culture mixing with a magnetic bar and stir plate. However, some ligninolytic peroxidases were produced in alkaline pH (pH 8.5) (86) and polyethylene-degrading enzyme(s) were produced when pH was above 8.0 without pH control (90). Thus, it was decided not to control culture pH.

Amount of propanal used for inducer. The concentration of propanal tested was 0.8, 1.6, or 3.1 g/L medium in 800-ml CSR in an effort to optimize propanal concentrations for enzyme induction. Aldehyde oxidase

specific activity was 0.02 units when 1.6 g propanal/L was added, whereas marginal activity was detected when 0.8 or 3.1 g propanal/L was added. Probably, 0.8 g/L was too small for enzyme induction and 3.1 g/L was toxic to the culture due to autoxidation. Therefore, it was decided to use 1.6 g propanal/L medium. This resulted in less pH decrease compared with 3.9 g/L propanal addition as demonstrated in shake-flask and 800-ml CSR cultures.

Number of propanal addition effect. Specific activity was 0.003 unit of aldehyde oxidase for *S. setonii* in 800-ml CSR treated with propanal (1.6 g/L) after 36 hr incubation with cells harvested 6 hr later (Table 7). After 36 hr incubation with 6 hr interval between propanal addition, two propanal treated cell-free extracts demonstrated specific activity of 0.017 unit. On the other hand, after 36 hr incubation with 12 hr interval between the second propanal addition, two propanal treated cell-free extracts demonstrated specific activity of 0.02 unit. Two propanal additions showed better aldehyde oxidase activity with propanal as the substrate, whereas time interval between the second propanal addition demonstrated no difference probably due to limited mixing by the magnetic stir bar. Therefore, it was decided to use two propanal treatments at 1.6 g/L for *S. setonii* enzyme induction.

5-L batch fermentation

Propanal concentration and number of propanal addition were evaluated again in 5-L batch fermentation to confirm previous 800-ml CSR cultures.

Table 7. Aldehyde oxidase activity of *S. setonii* 75Vi2 culture in 800-ml CSR with propanal (1.6 g/L) added as an inducer^a

Number of propanal additions	Time interval	Specific activity ^b
1	not applicable	0.003
2	6 hr	0.017
2	12 hr	0.020

^aA first propanal was added after 36 hr incubation.

^bActivity was measured by oxygraph with propanal as a substrate and specific activity was defined as 0.1 μ mole O₂ per minute per mg protein. Average of duplicate.

Direct drive agitation control ensured excellent culture mixing in 5-L fermentor. Specific activity was 0.01 unit for the enzyme extract after the first 3.9 g/L propanal addition, whereas all the other enzyme extracts with up to 4 propanal additions in 6 hr intervals did not generate noticeable activity (Table 8). Culture with 0.8 and 3.1 g/L propanal addition showed no activity as seen in 800-ml CSR studies. Whereas, after 36 hr incubation, two propanal treatments (1.6 g/L) in 6 hr intervals between propanal additions showed specific activity of 0.026 unit, which was not statistically significant ($P < 0.05$) by LSD from the one 3.9 g/L addition. However, it was decided to add two propanal in 6 hr

Table 8. Aldehyde oxidase activity of *S. setonii* 5-L batch fermentation with various propanal amounts (0.8, 1.6, 3.1, and 3.9 g/L) and up to 4 times of propanal additions as an inducer in 6 hr intervals

Propanal amount (g/L)	Number of propanal additions	Specific activity ^a
0.8	1 - 2	NAD ^b
1.6	1	NAD
1.6	2	0.026
3.1	1 - 2	NAD
3.9	1	0.01
3.9	2 - 4	NAD

^aActivity was measured by oxygraph with propanal as a substrate and specific activity was defined as 0.1 μ mole O₂ per minute per mg protein.

^bNo activity detected by oxygraph.

intervals because of the higher overall specific activity.

Specific activity was 0.021 units for 1.6 g propanal/L added to *S. setonii* culture in stationary phase (62 hr incubation). Culture in late-log phase (48 hr incubation), when treated with propanal (1.6 g/L), showed a two-fold increase in specific activity (0.05 units). However, it was not significantly different by LSD, but this specific activity was the best for 5-L fermentation. In shake-flask cultures, the best specific activity was 0.017 units with two propanal additions

(1.6 g/L) in a 12 hr interval between propanal additions to stationary phase culture. Thus, propanal addition to late-log phase showed better activity than stationary phase culture additions. This might be a result of detoxification mechanism (68). However, explanation is not simple because the correlation of mycelium production and changes in mycelium structure is complex (26).

Spore vs. viable cell inoculum. No enzyme activity was detected for any fermentors inoculated with viable cells, which was performed in an effort to reduce lag time. Vegetative cells of *S. setonii* from shake-flask cultures were long filaments versus aerial spores which represent single cells. Aerial and substrate mycelium are physiologically different from vegetative cells; however, data on metabolic activities of hyphae are not readily available (55). Therefore, we concluded that growth from spores might be essential for aldehyde oxidase induction. This is similar to citric acid production by *Aspergillus niger* which requires a spore inoculum (26).

15-L batch fermentation

This fermentation was done to correlate 5-L and 50-L batch fermentation and specific activity was 0.10 for cell-free extracts after harvest, which was two-fold higher than that in 5-L batch fermentation with the same enzyme induction pattern. Therefore, the 5-L batch fermentation was reproducible in 15-L batch fermentation in terms of aldehyde oxidase specific activity.

50-L *S. setonii* batch fermentation

Control (boiled extract) demonstrated no activity, whereas resuspended 25 and 40% ammonium sulfate saturation precipitate demonstrated specific activities of 0.21 and 0.13 units, respectively. This same cell-free crude extract was heat-treated (70°C) for 10 min and it showed positive activity (0.46 unit). However, it was not significantly different among specific activities by statistical analysis (LSD). A heat treatment (70°C) of cells or cell-free extract could provide a simple method for destroying undesirable proteases. However, heat-treatment of cells or cell-free extracts from repeated fermentation, demonstrated marginal activity. Furthermore, detectable aldehyde oxidase activity was not consistently present in propanal induced cells (Table 9).

Subsequent three fermentations with the same procedure demonstrated marginal or no activity and results were fluctuating and inconsistent. However, the pattern of changes in pH and dissolved oxygen were similar to previous 50-L batch fermentation. Possibly, propanal concentration based on cell density and not working volumes would have helped to stabilize some these differences with the range of cell-mass production being 5.5 to 7.0 g/L (wet weight). These results suggest that the aldehyde oxidase production by *S. setonii* was not consistent, it has a short half-life in the cells, or that propanal or propionic acid was toxic to the bacteria. When examined microscopically, cell morphology in 50-L fermentations was less aggregated filaments compared to

Table 9. Aldehyde oxidase specific activity of cell-free extracts prepared from *S. setonii* 50-L batch fermentation after a second 1.6 g propanal/L medium addition with propanal as the enzyme substrate

Sample^a	Specific activity^b
1 hr	0.017 ^c
2 hr	0.165 ^{de}
5 hr	0.042 ^{cd}
6 hr	0.034 ^c
8 hr	0.185 ^{ef}

^aTime after the second propanal addition.

^bActivity was measured by oxygraph and specific activity was defined as 0.1 μ mole O₂ per minute per mg protein. Substrate used was 500 mM propanal. Average of duplicate.

^{c,d,e,f}Values with different letter in the column means statistically significant at P < 0.05 by LSD.

somewhat extended long filaments aggregation in shake-flask and 800-ml CSR, probably due to mechanical shear difference by agitation. However, proper agitation was needed to supply dissolved oxygen to fast-growing culture. Short or fragmented hyphae are common at the end of fermentation in late stationary phase for some *Streptomyces* (16). *S. setonii* in continuous stirred tanks does fragment more than some *Streptomyces* (personal observation by

Dr. Pometto). Some heat-treated (70°C) extracts showed positive activity but results were again fluctuating and inconsistent. Enzyme activity varied from 0.16 to 0.46 unit for crude cell-free extract and 30% ammonium sulfate saturation precipitate, respectively, but values were fluctuating and inconsistent. Aldehyde oxidase specific activity was not statistically significant ($P < 0.05$) when analyzed by LSD.

Enzyme cofactors. Because of inconsistent and fluctuating results, some metal ions, EDTA (100 mM), and NAD (5 mg) were added to cell-free extracts, however, no benefit was observed.

Temperature effects. Cell-free extracts were evaluated at different temperatures (20 to 47°C)(Table 10) and the initial rates were higher at 42 and 47°C. Relative activity was lower for the extracts held at 42°C for 30 min than for extracts held for 5 min. Just the opposite result was observed for the extract at 47°C. Probably, temperatures tested were adverse to the oxygraph system because the proper sample temperature range is specified as 5 to 40°C in the manual. Also, oxygen probe and membrane permeability might be affected by chamber sample temperature. However, relative enzyme activity remained the same for the extracts held at 5 or 30 min at other temperatures tested.

In 50-L batch fermentation of *S. setonii*, temperature was reduced from 37 to 30°C after the first propanal addition (1.6 g/L) in an effort to stabilize maximum aldehyde oxidase intracellular concentrations longer. However, no

Table 10. Temperature effect on aldehyde oxidase prepared from *S. setonii* 50-L batch fermentation with propanal as the inducer^a

Temperature	Relative activity ^b
20°C	0
27°C	0
32°C	0
37°C	100
42°C	190
42°C (30 min ^c)	134
47°C	176
47°C (30 min ^c)	310

^aEnzyme activity was measured by oxygraph with propanal as a substrate and specific activity was defined as 0.1 μ mole O₂/min/mg protein.

^bRelative activity compared with activity measured at 37°C as 100% (0.87 units).

^cSubstrate was added to the enzyme mixture after equilibrating for 30 min.

improved enzyme activity was observed. *S. setonii* 75Vi2 mycelia storage in the coldroom (5°C), after harvest from 50-L fermentation, did demonstrate increased enzyme activity (Table 11). Maybe optimum growth temperature was needed for detoxification mechanism during fermentation, and intracellular protease activity was less active when cells were stored at 5°C.

Other enzyme substrates. Among enzyme substrates tested, 131 mM vanillin and 10 mM *trans*-cinnamaldehyde demonstrated specific activities of 0.42 and 0.49 units, respectively, for 80% ammonium sulfate saturation supernatant. Also, enzyme activity for hexanal was demonstrated (Table 11). Whereas, 18% (w/v) D-glucose, 95% ethanol, 10 mM catechol, 0.45 mM xanthine and 10 mM phenylacetaldehyde as substrates were negative.

Comparison of propanal and hexanal as substrates. From *S. setonii* 50-L batch fermentation, enzyme activity for the extracts taken every 2 hr after a first and second propanal addition (1.6 g/L) was determined (Table 11). Peak activity for both substrates was 4 hr after first propanal addition to the fermentation broth (Fig. 6). Again, aldehyde oxidase activity appeared, disappeared then reappeared after storage in the coldroom. Similar aldehyde oxidase activity was observed for propanal and hexanal. For most cell-free extract propanal illustrated a slightly higher enzyme activity than hexanal with 4 hr extract demonstrating the highest.

Peak enzyme activity measurement for crude cell-free extract. From

Table 11. Specific activity of aldehyde oxidase produced from *S. setonii* 50-L batch fermentation with propanal and hexanal as the substrates and propanal as the inducer^a

Sample ^b	Specific activity ^c	
	Propanal	Hexanal
control (zero time)	0.383 ^d	0.825 ^d
2 hr after 1st addition	0.406 ^d	0.488 ^d
4 hr after 1st addition	4.89 ^e	3.00 ^e
6 hr after 1st addition	0.494 ^d	0.646 ^d
2 hr after 2nd addition	0.488 ^d	0.381 ^d
4 hr after 2nd addition	0.27 ^d	ND ^f
final extract	0.361 ^d	0.787 ^d
Cold storage final extract ^g	1.53 ^d	1.54 ^d

^aTotal volume of 3.6 ml and 1.0 ml of extract in 0.1 M phosphate buffer (pH 7.2) at 37°C. Hydrogen peroxide was not added to the mixture.

^bSample time before and after propanal addition (1.6 g/L).

^cActivity was measured by oxygraph and specific activity was defined as 0.1 μ mole O₂ consumed per min per mg protein. Substrate concentration was 500 mM for propanal and 0.83 mM for hexanal.

^{d,e}Values with different letter represents statistically significant (P<0.05).

^fND: Not determined.

^gCells stored in the coldroom for four hours due to the processing time for final extract.

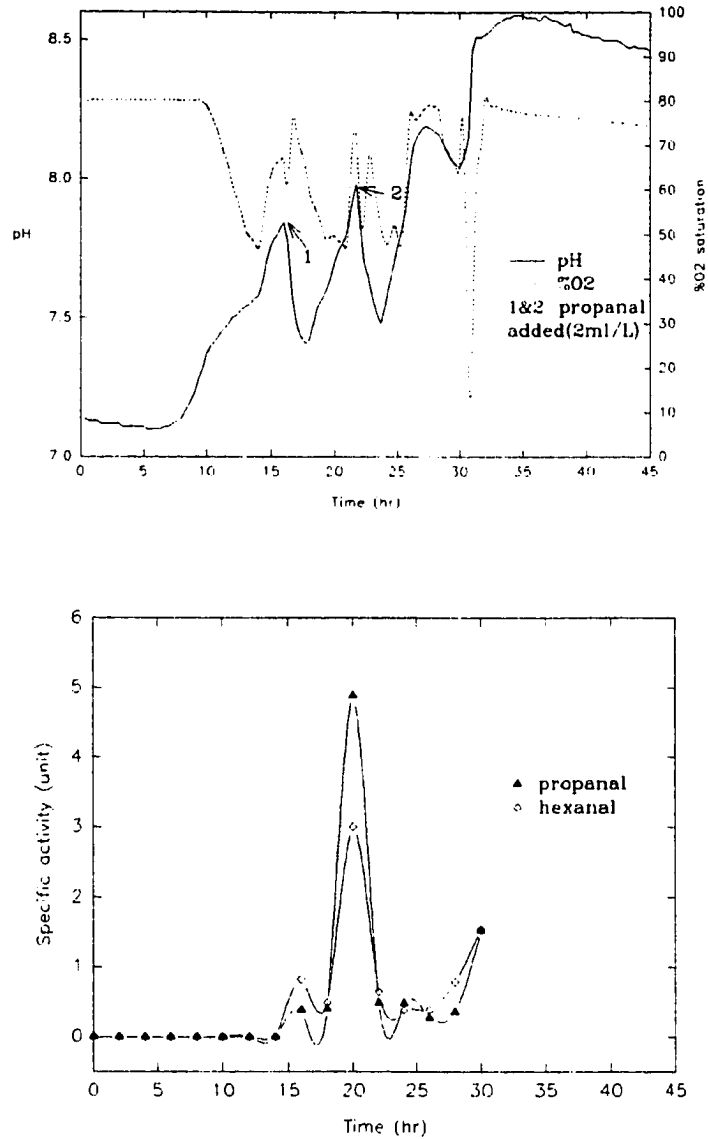


Figure 6. Change in pH and %O₂ saturation (Top frame), and aldehyde oxidase specific activity (Bottom frame) for 50-L batch fermentation of *S. setonii* 75Vi2 in 0.6% (w/v) yeast extract, 1.0% (w/v) malt extract medium and 1.6 g propanal/L medium was added at 16 and 22 hr incubation

S. setonii 50-L fermentation, one liter samples were taken every hour for six hours, after the first and second propanal addition (1.6 g/L) to determine enzyme activity profile. Enzyme activity exhibited a similar fluctuating pattern as observed previously (Table 12) and values were not significantly different ($P < 0.05$) from each other when analyzed by LSD.

Summary of attempted scale-up. Table 13 illustrates specific activity, cell mass, and protein produced in each step through attempted scale-up from shake-flask to 50-L batch fermentation. Aldehyde oxidase specific activity and total protein production in 50-L batch fermentation were increased to 9- and 65-fold, respectively, compared with those of shake-flask. Thus, scale-up procedure was proper in terms of specific activity and protein production.

Product Analysis

HPLC analysis of enzyme reaction mixture for propionic acid

Reaction mixture from positive oxygraph assay with propanal as the substrate was collected and analyzed by HPLC. Also, fermentation broth samples taken every hour after the first propanal addition (1.6 g/L) were analyzed for propanal conversion to propionic acid. Propionic acid was detected by HPLC at 0.46 and 0.89 g/L, 2 hr after the first propanal addition (1.6 g/L) and 2 hr after a second propanal addition, respectively. Most reaction solutions contained 0.04 to 0.13% (v/v) propionic acid. However, buffer

Table 12. Percentage oxygen consumed by cell-free extracts prepared from 1-L sample from 50-L batch fermentation of *S. setonii* with propanal as the substrate and the inducer

Sample	%O₂/min	Sample	%O₂/min
zero	0.94	6 hr/1	0.34
1 hr/1 ^a	0.88	1 hr/2 ^b	0.38
2 hr/1	1.57	2 hr/2	0.8
3 hr/1	1.05	4 hr/2	0
4 hr/1	0.93	6 hr/2	0
5 hr/1	1.00	final	0.90

^aHours after a first propanal addition (1.6 g/L).

^bHours after a second propanal addition (1.6 g/L).

solution containing no cell-free extract (control) showed 0.18% (v/v) propionic acid which results from autoxidation. This lower propionic acid concentration for enzyme reaction mixtures from crude cell-free extract is difficult to explain. Longer chained aliphatic aldehydes and acids were not detectable by the HPLC column and mobile phase used. These results illustrate some of the difficulties associated with aliphatic aldehydes as substrates.

Table 13. Summary of aldehyde oxidase production by *S. setonii* scale-up attempt

Fermentation	Sp. act. ^a	Cell mass (g/L)		Total protein (g) ^b
		Dry	Wet	
shake-flask (1-L)	0.02	ND ^c	ND	0.08
CSR (800 ml)	0.02	ND	2.5	0.22
5-L	0.05	1.2	4.7	1.70
15-L	0.10	1.2	4.6	3.24
50-L	0.18	1.7	7.0	7.24

^aSpecific activity was measured for crude cell-free extracts with propanal as a substrate and one unit was defined as 0.1 μ mole O₂/min/mg protein. Values represent the highest specific activity.

^bTotal protein produced based on 40 ml crude cell-free extracts per 1-L harvest at the end of fermentation. Protein concentration was measured by the method of Lowry et al. (64).

^cNot determined.

2,4-Dinitrophenylhydrazine assay for aldehyde detection

The 2,4-dinitrophenylhydrazine assay was investigated in an effort to develop a spectrophotometric assay for measuring aldehyde oxidation to support the oxygraph and HPLC results. The reaction of aldehydes and 2,4-dinitrophenylhydrazine produces a yellow to red color (121). Different concentrations of propanal solution were scanned from 350 to 520 nm to

identify the optimal absorbance that would generate a linear standard curve. However, absorbance values at different wavelengths showed no linear relationship (Fig. 7). Different compositions were attempted, but they were not effective. Also, no variation in pH was observed. This illustrates 2,4-dinitrophenylhydrazine may not be suitable for quantitative analysis but qualitative analysis as in TLC.

Thin-layer chromatography (TLC) for aldehyde detection

TLC was used in an effort to support HPLC results and confirm aldehyde to acid enzymatic conversions. The R_f values for propanal, propionic acid, and hexanal standard sample were 0.675, 0.583, and 0.540, respectively. Hexanal standard, however, showed four spots and it was difficult to detect on TLC plate. Aldehydes were identified by spraying 2,4-dinitrophenylhydrazine solution (0.4% [w/v] in 2 N HCl) which gave yellow to red spots and their corresponding acids were identified under ultraviolet light (longwave) after 2',7'-dichlorofluorescein (0.2% [w/v] in 95% ethanol) was sprayed over the plate. However, this procedure also proved ineffective. This chronic detection problem stems from aliphatic aldehydes volatility and enzyme reaction solution containing low concentrations (30 to 100 ppm) of each compounds. Thus, quantitative analysis was not possible in TLC and more sensitive method (e.g., gas chromatography) might be needed for detection of corresponding acids produced by enzyme.

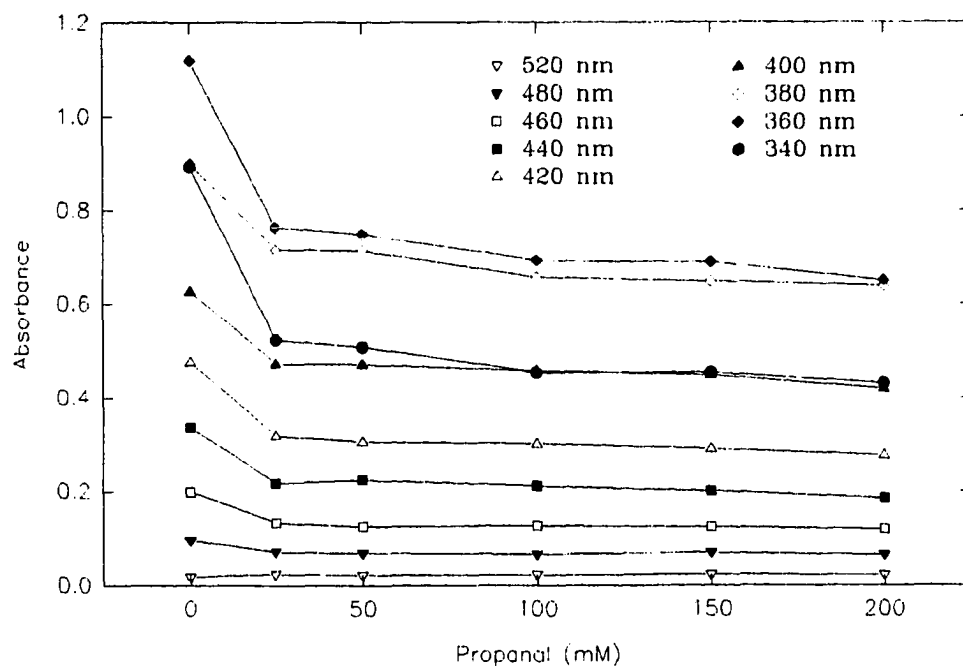


Figure 7. Absorbance of propanal and 2,4-dinitrophenylhydrazine (DNPH) solution at different wavelength (The solution contained 0.1 ml of DNPH, 1.0 ml of various amount of propanal in 0.1 M phosphate buffer, and 2.0 ml of 95% ethanol)

Aromatic Aldehyde as an Inducer

From each fermentation, aldehyde oxidase production pattern was inconsistent and not reproducible when propanal was used as the inducer. Therefore, *trans*-cinnamaldehyde, which is more stable than propanal, was evaluated as an aldehyde oxidase inducer. *trans*-Cinnamaldehyde level was monitored by UV spectrophotometer at 286 nm for *trans*-cinnamaldehyde or 268 nm for its corresponding acid.

5-L *S. setonii* batch fermentation

Shake-flask and 800-ml CSR studies were skipped based on our previous findings for enzyme induction with propanal. Aromatic aldehyde would be a more stable enzyme inducer than aliphatic aldehyde, if the aldehyde oxidase is induced with *trans*-cinnamaldehyde addition in 5-L batch fermentation, and if the aldehyde oxidase induced would oxidize aliphatic aldehydes as well as aromatic aldehydes. After a 48 hr incubation in 0.6% (w/v) yeast extract and 1.0% (w/v) malt extract medium, 0.10 g *trans*-cinnamaldehyde/L medium (0.01% v/v) was added. Average cell-mass yield, after 72 hr incubation, was 9.4 g/L (wet weight) and the conversion of all the *trans*-cinnamaldehyde to *trans*-cinnamic acid was monitored spectrophotometrically at 286 nm. Enzyme induction, however, was not improved. Probably aromatic aldehyde like *trans*-

cinnamaldehyde is toxic to cells because no growth was observed when *trans*-cinnamaldehyde was present prior to inoculation. Also, 50% inhibition was reported when *trans*-cinnamaldehyde (70 $\mu\text{g/ml}$) was added to a 72 hr *Saccharomyces cerevisiae* culture (74). A combination of *trans*-cinnamaldehyde (0.2 g/L) and propanal (1.6 g/L) as inducers was also evaluated, but no improvement was observed.

50-L *S. setonii* batch fermentation

Since dissolved oxygen, pH, and cell-mass changes correlated between 15-L and 50-L batch fermentations in previous study, 15-L batch fermentation was not performed. When pH was about 7.8 and a reduction in DO_2 was observed, 0.5, 1.0, or 2.0 g of *trans*-cinnamaldehyde/L medium was added. *trans*-Cinnamaldehyde oxidation was monitored by UV absorbance at 286 nm. The advantage of aromatic aldehyde as an inducer is that it is possible to monitor the inducer oxidation rapidly which should correspond to enzyme induction.

Bacterial bioconversion of *trans*-cinnamaldehyde to corresponding acid in 3 to 4 hours was observed when 0.02 to 0.1% (w/v) aromatic aldehyde was added. However, *trans*-cinnamaldehyde was not decreased when 0.2% (w/v) was added probably due to its toxicity. Ultraviolet absorbance (286 nm) initially and after 2 hr incubation was 0.828 and 0.177, respectively, which indicated acid production. The aldehyde oxidase induced by *trans*-

cinnamaldehyde addition to late-log phase cells in 50-L batch fermentation did oxidize propanal (Table 14) and this suggests a wide substrate specificity of this enzyme. The heat treatment (70°C) of cell-free extract showed excellent results compared with other extracts for both propanal and *trans*-cinnamaldehyde as substrates. Crude cell-free extract was active for 2 to 3 days at 4°C, whereas whole cells lost activity in cold storage (5°C) in 2 or 3 days. Ammonium sulfate precipitation of aldehyde oxidase extended its shelf-life to a week when stored at 4°C. It was difficult to deal with *S. setonii* due to inconsistent enzyme induction and intracellular stability in fermentation. Stability of bacterial enzyme preparations, however, was extended by freeze-drying cell-free extract. Like propanal, the *trans*-cinnamaldehyde treated *S. setonii* fermentation demonstrated inconsistent enzyme induction and recovery. Therefore, our efforts changed to *S. viridosporus* batch fermentation.

Enzyme Activity for *S. viridosporus* T7A

Crawford et al. (24) demonstrated aromatic aldehyde oxidase activity in *S. viridosporus* T7A using 0.15% yeast extract medium in shake-flask studies. For our study, the medium used contained 0.6% (w/v) yeast extract and 1.0% (w/v) malt extract medium. When pH was about 7.8 and after DO₂ reduced, 1 or 2 g vanillin/L was added as an inducer. Vanillin level was monitored by UV spectrophotometer at 345 nm or 250 (282) nm for its corresponding acid.

Table 14. Specific activity of aldehyde oxidase produced by *S. setonii* 50-L fermentation with *trans*-cinnamaldehyde (0.2 g/L) as the inducer

Sample	Specific activity ^a	
	Propanal	<i>trans</i> -Cinnamaldehyde
crude extract	0.19	0.34
0-25% precipitate	0.30	0.31
70°C heated (10 min) crude extract	0.46	0.35

^aActivity was measured by oxygraph and specific activity was defined as 0.1 μ mole O₂ consumed per minute per mg protein. Substrate concentration was 500 mM propanal or 10 mM *trans*-cinnamaldehyde.

Average cell-mass yield of two fermentations was 69 g/L (wet weight) and harvested cells were stored in the coldroom. Cell-mass (wet weight) was much higher for *S. viridosporus* due to the production of larger mycelium clumps than *S. setonii* in cell suspension producing visibly large spherical cell-aggregations.

Crude cell-free extract harvested after two vanillin treatments (1 g/L) demonstrated excellent aldehyde oxidase activity for propanal (Table 15), whereas boiled extracts were negative. Specific activity was highest for 80% ammonium sulfate saturation supernatant (2.27 units). However, it was not significantly different ($P < 0.05$) among different ammonium sulfate %

saturation fractions by LSD. When hexanal and *trans*-cinnamaldehyde were used as substrates, specific activities of 5.6 and 0.49 units were observed, respectively. Two fermentations with vanillin addition (2 g/L) were performed and activity values were inconsistent even those from the same ammonium sulfate fractions (data not shown). Oxygraph measurements of enzyme activity were not always conclusive, because of the pervasive presence of catalase in every ammonium sulfate fraction, even after treatment with 3% H₂O₂.

Hydrogen peroxide was added to enzyme reaction mixture to fatigue catalase that catalyzes H₂O₂ to O₂ and H₂O. This treatment improved aldehyde oxidase assay 4.4-fold when measured by oxygraph. Aldehyde oxidase oxygraph assay is based on oxygen consumption measurement. However, H₂O₂ is a co-produced of the enzyme reaction. So, as O₂ was consumed by aldehyde oxidase, an equimolar amount of O₂ was produced by catalase and H₂O₂. Therefore, catalase contamination was a reoccurring problem for all our oxygraph measurement. The molecular weight range of bacterial catalases is reported as 220 to 350 kDa (101).

Temperature effect. *S. viridosporus T7A* mycelia storage in the coldroom (5°C) up to three days after harvest from 50-L fermentation did slowly increase enzyme activity. Residual vanillin was present and the lower temperature might decrease cell activity and intracellular enzyme degradation by proteases.

Table 15. Purification of aldehyde oxidase produced by *S. viridosporus* 50-L fermentation with vanillin as the inducer

Ammonium sulfate ^a	Specific activity ^b	Purification (fold)
original	0.67	1
31-40P	1.20	1.8
31-40S	1.12	1.7
71-80S	2.27	3.4

^aAmmonium sulfate saturation fraction evaluated were supernatant (S) and precipitate (P).

^bOne unit was defined as 0.1 μ mole O₂ consumed per minute per mg protein. The reaction mixture contained 3% H₂O₂, 0.1 M phosphate buffer, and total volume was 3.6 ml at 37°C. After H₂O₂ consumption by catalase, 500 mM propanal was added.

50-L *S. viridosporus* batch fermentation

Enzyme production was more consistent for *S. viridosporus* than for *S. setonii*. Changes in pH and %O₂ saturation during fermentation are illustrated in Fig. 8. Aldehyde oxidase activity was 4.05 units for cell-free extract from the fermentation with agitation speed of 200 rpm and upon microscopic examination, cell morphology exhibited about 1 mm diameter. In the next fermentation, agitation speed was increased to 250 rpm in an effort to supply more dissolved oxygen. However, aldehyde oxidase activity decreased to 2.33

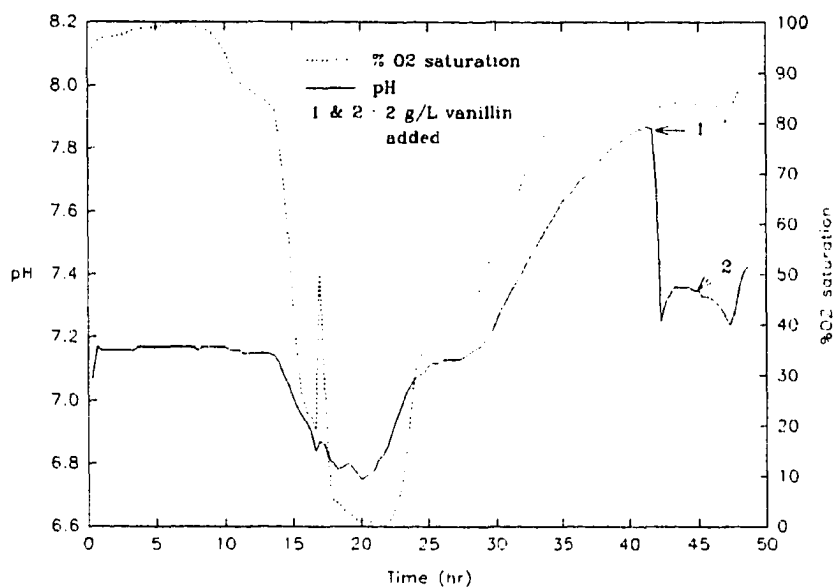


Figure 8. Changes in pH and %O₂ saturation for 50-L batch fermentation of *S. viridosporus* using vanillin (2 g/L) as an inducer added at 42 and 45 hr incubation

units for cell-free extract and observed cell aggregation size was about < 0.5 mm. This suggested that cell aggregation size was an important factor in fermentation, because a larger cell aggregation size gave better enzyme activity. Cell aggregation size was controlled by inoculum size and agitation speed by using 3 stock slants of spores and 200 rpm, respectively.

Enzyme stability was better when it was cold stored or freeze-dried. Also, whole cells showed positive activity even after four weeks of coldroom storage (5°C), suggesting a longer intracellular half-life for the aldehyde oxidase for *S. viridosporus*, compared with *S. setonii*. The resuspended freeze-dried crude extract was also quite stable (Table 16) up to 24 days and specific activity remained about the same as original. Thus, freeze-drying could be used as a good preservative method.

Table 17 presents enzyme purification with ammonium sulfate for *S. viridosporus* cell-free extracts. The specific activity was 71 units for 70% ammonium sulfate saturation supernatant and 40 units for 30% ammonium sulfate saturation supernatant. However, propionic acid was not detected by HPLC. The amount of propanal converted to propionic acid by these extracts were possibly too small to be detected by HPLC. On the other hand, propionic acid was detected for the 30% ammonium sulfate saturation precipitate, whereas boiled 30% ammonium sulfate saturation precipitate and supernatant showed no propionic acid peak. This product detection by HPLC confirms aldehyde oxidase activity (Fig. 9).

Table 16. Storage stability of freeze-dried crude cell-free extract produced by *S. viridosporus* 50-L fermentation with vanillin as an inducer and propanal as the substrate^a

Days stored at 4°C	Specific activity ^b	Relative activity ^c
0	13.4	100
3	13.9	104
14	12.0	90
18	14.8	110
24	14.0	104

^aThe freeze-dried crude extract was prepared from whole cells stored for 8 days in a coldroom.

^bActivity was measured by oxygraph and one unit was defined as 0.1 μ mole O₂ consumed per minute per mg protein. Propanal used was at 500 mM.

^cThe values were compared to the freeze-dried extract at 0 day as 100%.

Total activity and % activity recovery of 30S (supernatant) and 70S were greater than original cell-free extract (Table 17). The discrepancy of the oxygraph might be due to the presence of catalase activity which was detected in all ammonium sulfate fraction. Catalase has a wide pH optimum range (3 to 9) and it is difficult to remove. Many known catalase inhibitors (e.g., azide, pyrazole), will only partially inactivate the enzyme (52). Also, cyanide, a

Table 17. Ammonium sulfate fractionation of aldehyde oxidase produced by *S. viridosporus* cell-free extract prepared from 50-L fermentation with vanillin as an inducer and propanal as the substrate^a

AS ^b %	Vol. (ml)	Prot. ^c (mg/ml)	Total prot.	Total activity	Spec. act.	% Act. rec. ^d	Purif. (fold)
0	100	5.28	528	3722	7.05	100	1.0
0-30S	106	1.94	206	8343	40.5	224	5.7
51-70S	118	0.74	87	6177	71.0	166	10
0-30P ^e	3.5	5.60	19.6	425	21.7	11	3.1

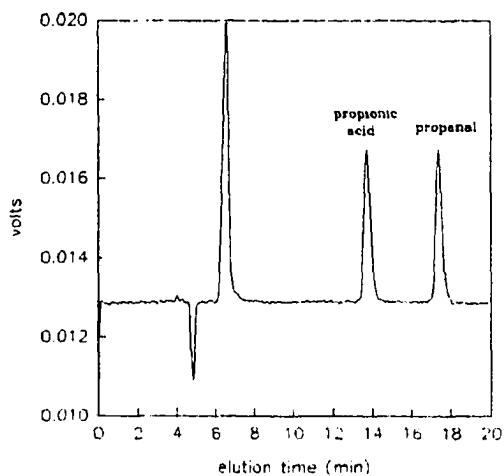
^aThe values were subtracted by boiled extract background. Specific activity was defined as 0.1 μ mole O₂ per min per mg protein. Original extract was from the whole cells stored for 8 days at 5°C, freeze-dried, then stored for 7 weeks at 4°C. For enzyme assay, 20 mg was resuspended in 1 ml of 0.1 M phosphate buffer. Vanillin (0.2% w/v) was used as an inducer and 1 M propanal as a substrate.

^bAmmonium sulfate (AS) % saturation with S for supernatant and P for precipitate.

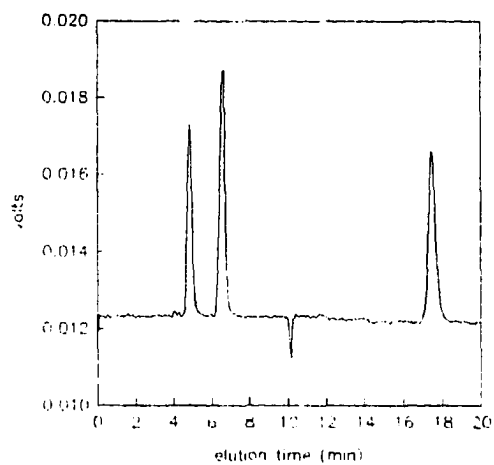
^cProtein determined by the method of Lowry et al. (64).

^dPercentage activity recovered.

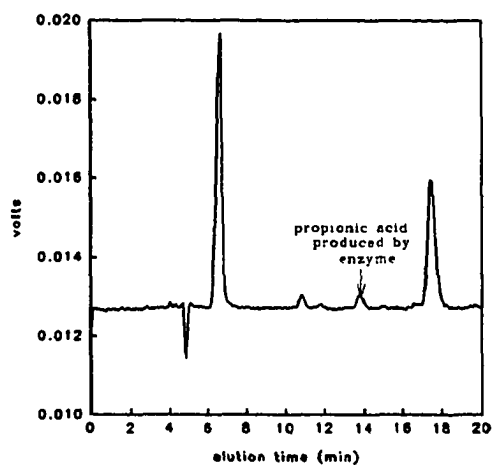
^ePropionic acid production was confirmed by HPLC (Fig. 9).



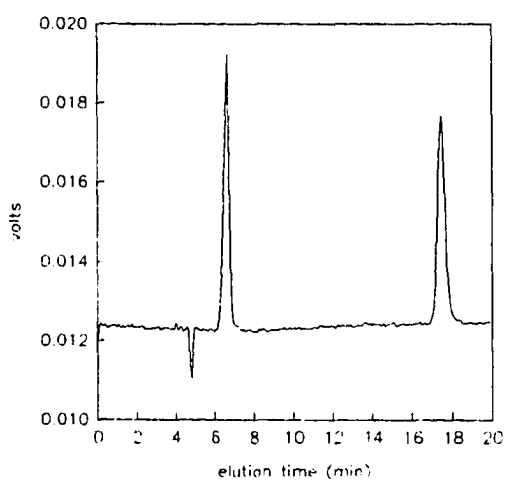
a. Standard for propanal and propionic acid



b. 30% ammonium sulfate supernatant



c. 30% ammonium sulfate precipitate



d. Boiled 30% precipitate

Figure 9. HPLC chromatograms for propanal and corresponding propionic acid produced by aldehyde oxidase from *S. viridosporus* cell-free extracts

catalase inhibitor, was not tested because it is also an aldehyde oxidase inhibitor (7, 13).

A rapid assay for aldehyde oxidase activity such as the oxygraph is needed to monitor enzyme purification and optimization. Furthermore, propanal chemical autoxidation could also be a problem. Therefore, a more stable substrate to follow enzyme activity was needed, and vanillin was selected. The advantage of using aromatic aldehyde was that bioconversion of vanillin to vanillic acid can be monitored spectrophotometrically at 345 nm (88), which can be used to support oxygraphic measurement.

Comparison of Enzyme Activity and Residual Vanillin

This time aldehyde oxidase activity was detected in all ammonium sulfate fractions by oxygraph and by residual vanillin-concentration spectrophotometrically, which correlated enzyme activity with substrate bioconversion (Table 18). The best results were for the 30 and 40% ammonium sulfate saturation precipitate with 75% vanillin converted to vanillic acid as determined spectrophotometrically. Specific activity determined by oxygraph showed some variation compared with residual vanillin concentration. However, in the future, oxygraphic assay with spectrophotometric residual-substrate measurement can provide rapid enzyme activity measurements, and would be the preferred method as an aldehyde oxidase assay using aromatic

Table 18. Ammonium sulfate fractionation of aldehyde oxidase produced by *S. viridosporus* 50-L fermentation with vanillin as the substrate and an inducer^a

AS ^b (%)	Volume (ml)	Protein (mg/ml)	Total protein	Total activity	Specific activity ^c	% Activity recovery	Purification (fold)	Vanillin ^d (mg/ml)	% Conversion ^e
0	200	14.0	2800	14	0.005	100	1.0	24.1	21
30S	212	12.8	2714	ND ^f	ND	ND	ND	21.8	29
40S	221	11.3	2492	ND	ND	ND	ND	24.5	20
50S	224	7.21	1615	ND	ND	ND	ND	25.6	16
60S	226	6.64	1501	120	0.08	857	16	23.3	24
70S	229	4.9	1134	147	0.13	1050	26	17.3	43
80S	232	4.55	1056	444	0.42	3170	84	21.1	31
30P	15	7.34	110	53	0.48	377	96	7.54	75
40P	5	4.93	24.7	4	0.16	28	32	7.54	75
50P	5	1.50	7.5	ND	ND	ND	ND	14.3	53
60P	5	3.71	18.6	ND	ND	ND	ND	22.2	27
70P	4	1.68	6.72	ND	ND	ND	ND	21.1	31
80P	4	1.66	6.64	ND	ND	ND	ND	24.5	20

^aVanillin (40 mg/ml) was added to the reaction mixture and about 25 mg/ml of freeze-dried extract was resuspended in 0.1 M phosphate buffer. Zero control contained 30.6 mg of vanillin per ml and the background was subtracted.

^bAmmonium sulfate (AS) % saturation with S for supernatant and P for precipitate. AS fractionation was performed in the sequence presented.

^cEnzyme activity was measured by oxygraph and one unit was defined as 0.1 μ mole O₂/min/mg protein.

^dResidual vanillin concentration in mg/ml after enzyme reaction for oxygraph analysis was determined spectrophotometrically at 345 nm. Initial vanillin concentration was 40 mg/ml.

^ePercentage vanillin converted to vanillic acid.

^fND: not determined due to the background was greater than these data.

aldehyde as a substrate.

Another set of measurement was done for the residual vanillin from a cell-free extract to confirm previous results (Table 19). Low vanillin concentration correlated with good enzyme activity for the reaction mixtures from 25 to 45% ammonium sulfate saturation as expected. Therefore, the combination of oxygraphic and spectrophotometric measurement could be a good aldehyde oxidase assays.

Polyacrylamide Gel Electrophoresis (PAGE)

On nondenaturing gel, 30 to 50 μ g of crude cell-free extract, heat-treated (70°C) for 5 and 10 min, and 45% ammonium sulfate saturation precipitate was loaded into each well. All these proteins demonstrated positive enzyme activity, with vanillin as substrate as indicated by visual brown color formation on zymogram with native-PAGE (Fig. 10). This confirms the presence of aldehyde oxidase in these extracts. On the other hand, boiled extract (control) and other 25 or 45% ammonium sulfate saturation supernatant did not show detectable color formation. Figure 11 illustrates the protein patterns as determined by SDS-PAGE for *S. viridosporus* cell-free extracts and different ammonium sulfate fractions. Crude cell-free extract, 25 and 45% ammonium sulfate saturation supernatant showed similar protein patterns (lanes 1, 2, and 3). Some additional proteins appeared in 25% ammonium sulfate saturation

Table 19. Residual vanillin concentration after enzyme reaction for cell-free extracts prepared by *S. viridosporus* 50-L fermentation with vanillin as the substrate and an inducer

Extract ^a	Vanillin (mg/ml) ^b	% Conversion ^c
zero (without stirring)	7.60	0
inactivated enzyme	7.25	0
0	6.77	7
0 - 25P	7.42	0
25 - 45P	0.51	93

^aEnzyme extracts were freeze-dried, stored in a coldroom and 25 mg/ml was resuspended in 0.1 M phosphate buffer from various ammonium sulfate precipitation. P and S are for precipitate and supernatant, respectively.

^bInitial vanillin concentration was 7.80 mg/ml (0.2 ml 1 M vanillin, 100 μ l enzyme and 3.6 ml buffer) and residual vanillin was determined by absorbance at 345 nm spectrophotometrically. Average of duplicates.

^cPercentage vanillin converted to vanillic acid by residual vanillin measurement at 345 nm spectrophotometrically.

precipitate (lane 4) which were not present in 45% ammonium sulfate saturation precipitate (lane 5). Just a few protein bands were detected in heat-treated (70°C) cell-free extracts for 10 min (lane 6 and 7). Thus, heat treatment (70°C) could be an excellent procedure to remove some undesirable proteins which precipitated after heat treatment.

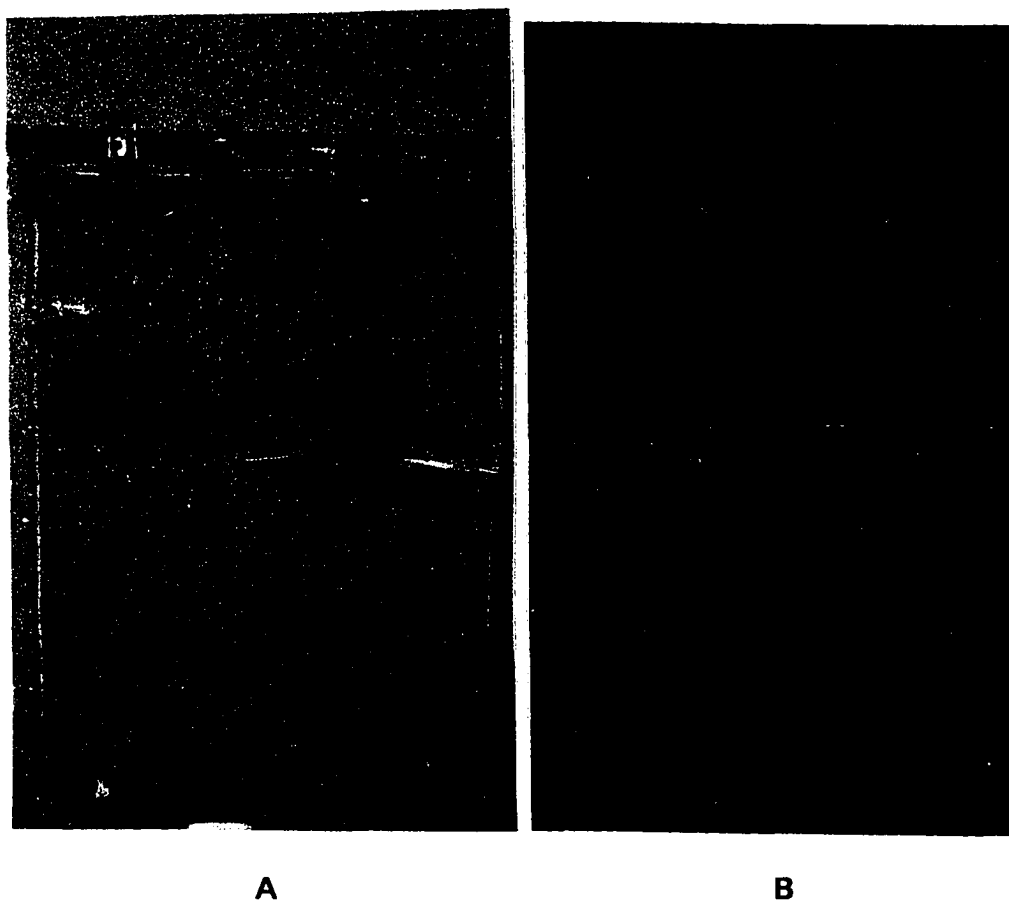


Figure 10. Nondenaturing gel electrophoresis to detect aldehyde oxidase activity. A. active extracts B. boiled extracts 1; crude cell-free extract, 2 and 3; heat-treated (70°C) for 5 and 10 min, respectively, 4; 45P^a (^aAmmonium sulfate with P for precipitate)

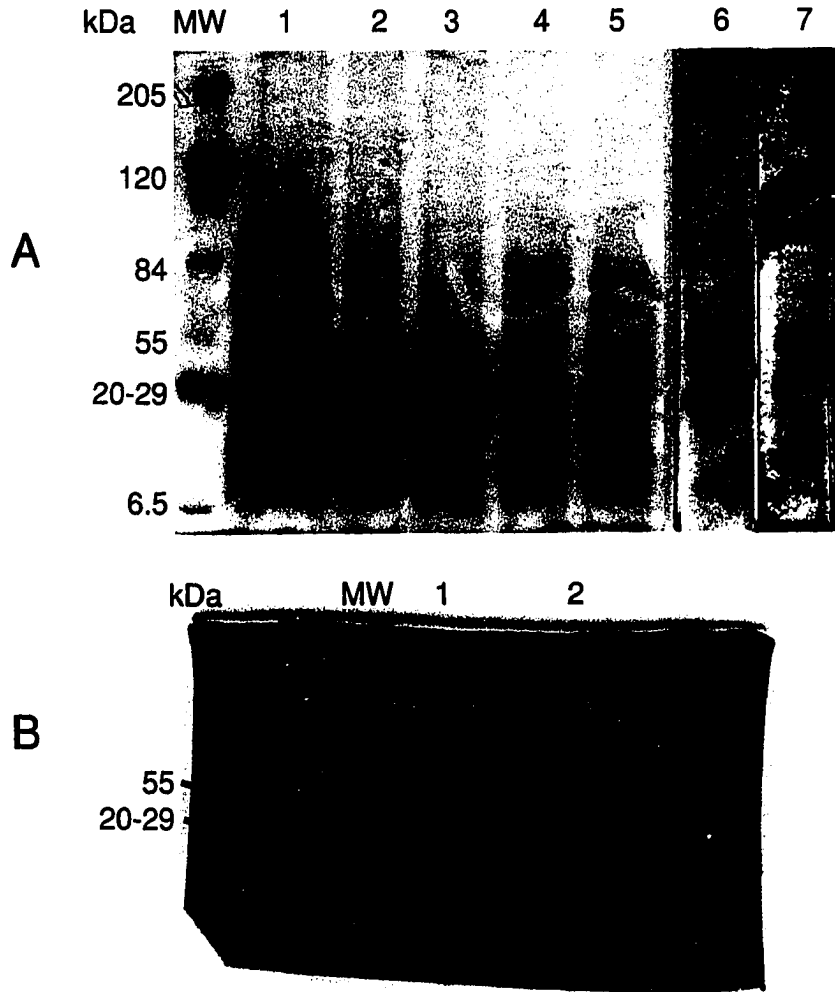


Figure 11. Polyacrylamide gel electrophoresis (PAGE) A. SDS-PAGE 1; crude cell-free extract 2; 25S^a 3; 45S^a 4; 25P^a 5; 45P^a 6, 7; heat-treated (70°C) for 10 min. B. SDS-PAGE 1, 2; extracts from positive enzyme activity bands on nondenaturing gel (^aAmmonium sulfate saturation with S for supernatant and P for precipitate)

The protein bands which demonstrated positive enzyme activity on non-denaturing gel were cut from the gel, frozen, and extracted in phosphate buffer, then ran on SDS-PAGE (Fig. 11 b). Two broad protein bands were detected near molecular marker of 20 and 55 kDa. These were similar to those observed in heat-treated (70°C) extract (Fig. 11 a; lanes 6 and 7). About 50% of the proteins in crude cell-free extract as determined by the method of Lowry et al. (64) was denatured by heat-treated (70°) for 10 min. These data suggest that aldehyde oxidase has a molecular weight range of 20 to 55 kDa, and possibly could be a dimer with the complete enzyme being the sum of both bands (about 75 kDa). Molecular weight from animal sources varies from 222 to 348 kDa for bovine liver (15) and guinea pig (124), respectively.

Mukund and Adams (77) reported that hyperthermophilic aldehyde oxidase from *Pyrococcus furiosus* had a molecular weight of 80 and 90 kDa by SDS-PAGE and gel filtration, respectively. Also, Deobald and Crawford (29) reported that for the *S. viridosporus* T7A, aromatic aldehyde oxidase had a molecular weight of 80 kDa as determined by gel filtration. This correlates to the sum of our two bands of 20 and 55 kDa.

CONCLUSIONS AND RECOMMENDATIONS

Aldehyde oxidase production from *S. setonii* 75Vi2 was initiated in 2-L flask and scaled-up *via* 800-ml CSR, 5-L and 15-L to 50-L batch fermentations. For *S. setonii*, enzyme induction with propanal and *trans*-cinnamaldehyde was inconsistent and not reproducible. *S. viridosporus* T7A illustrated more consistent enzyme activity when vanillin was used as an inducer. However, specific activities were not significantly different ($P < 0.05$) among different ammonium sulfate percentage saturation fractions when analyzed by LSD for both *S. setonii* and *S. viridosporus*.

It is recommended to use aromatic aldehydes when possible as enzyme inducers. Aromatic aldehydes are more stable and their corresponding oxidation to aromatic acid is easily monitored spectrophotometrically. A first inducer addition (0.2% [w/v]) at late-log phase and a second addition after 6 hr more incubation are needed for proper enzyme induction in 50-L *S. viridosporus* fermentation. Spore inoculum was essential for enzyme induction and late-log phase of the culture was determined by pH 7.8 and dissolved oxygen.

Enzyme induction and half-life were inconsistent in the bacteria. Therefore, bacterial mutants enhanced in intracellular aldehyde oxidase stability with increased concentration are needed.

Aldehyde oxidase activity was continuously detected by oxygraph in

each scale-up step, which illustrated that enzyme activity was reproducible. The previous works by other researchers were performed in shake-flask. This study was the first reported scale-up to 50-L batch fermentation. Also, this was the first time that aldehyde oxidase was detected in *S. setonii*.

The average enzyme peak activity was observed when harvested cells were stored in coldroom at 5°C for 2 hr and shelf-life was extended when crude cell-free extract was freeze-dried. Catalase presence was a problem for enzyme assays. However, catalase addition with aldehyde oxidase will be essential for removing H₂O₂ from soybean products treated with this enzyme to remove off-flavors. Oxygraphic and spectrophotometric methods with vanillin were good enzyme assays. However, additional quick and reliable aldehyde oxidase assays are needed. Autoxidation and low water solubility of medium-chain aldehydes seem to be the major problems. Reported aldehyde oxidase assays were mainly spectrophotometric methods and compounds other than molecular oxygen were used as an electron acceptor (32, 56). Furthermore, most researchers used other substrates than aldehydes (e.g., purines, pyrimidines), probably due to better solubility and less autoxidation than aliphatic aldehydes.

Bacterial aldehyde oxidase produced by *Streptomyces* species was confirmed by organic acid and H₂O₂ production by HPLC and PAGE zymogram, respectively. However, more sensitive product analysis is required. Currently, 25 to 45% ammonium sulfate precipitate was the best fraction and heat

treatment (70°C) for 10 min could facilitate enzyme purification.

Bacterial aldehyde oxidase is a smaller enzyme (80-90 kDa) than those previously reported from animal sources (220-350 kDa). Aldehydes bound to soy protein were oxidized much slower rate than free aldehydes by aldehyde oxidase (112) or aldehyde dehydrogenase (20) from animal source because aldehydes were buried or trapped in soy proteins that the enzyme can't reach (20). This suggests that it would potentially outperform these larger enzyme in soy off-flavor reduction because of the competition for off-flavor bindings to the two major soy proteins (160 and 320 kDa).

Further purification steps (e.g., ion-exchange, gel filtration, or affinity chromatography) would be desirable for enzyme kinetics study to determine its practical use in removal of some off-flavors. Also, catalase removal would greatly enhance enzyme assays and optimizations. Finally, any evaluation of off-flavor by the addition of bacterial aldehyde oxidase will require a sensory evaluation of treated products. Soybeans contain high quality protein with anticancer properties. However, human consumption is limited due to some off-flavors. Enzymatic off-flavor removal could be one method to expand soybean utilization in human foods.

Recommended Research Plan Modifications

Followings are some modifications I would do if I started this research all over again.

Fermentation. The age of each culture's stock slants needs to be controlled strictly for inoculum preparation, based on specific culture age for homogeneous spores preparations. Inducer addition to the cells in late-log phase looks appropriate for the enzyme induction. Concentration of inducer addition should be proportional to wet cell-mass/L medium rather than medium volume.

Scale-up procedure would be in three steps: shake-flask, 5-L, and 50-L batch fermentation. Initial enzyme induction will be done in shake-flask study and the cell growth curve with pH and dissolved oxygen change will be determined in 5-L batch fermentation. Enzyme activity change during fermentation with inducer addition would be monitored throughout the fermentation. Also, different aeration and agitation rates are worth testing because dissolved oxygen and cell aggregation size were important factors for enzyme activity. All crude cell-free extracts should be stored frozen and freeze-dried until proper enzyme assays are developed.

Also, medium modification might be effective for enzyme induction. Different amount of yeast extract (e.g., 0.1 to 0.6% [w/v]) could be used for fermentations to determine the best enzyme activity. All parameters will be tested in 5-L batch fermentation and key parameters confirmed in 5-L fermentation would be followed in 50-L batch fermentation for reproducible enzyme activity and for large-scale enzyme production.

Enzyme assay. A reliable and simplified enzyme assay needs to be

developed. Oxygraph is easy and quick, but inactivation or removal of catalase is essential for accurate aldehyde oxidase assay.

A further purification step (e.g., ion-exchange, gel filtration, or affinity chromatography) is needed to separate aldehyde oxidase from catalase. Once this is done, oxygraphic enzyme assay would be more useful than before. Currently, vanillin with oxygraph and spectrophotometer assay seem to be OK. However, correlation between aliphatic and aromatic aldehydes as substrates needs further testing. Maybe other compounds other than aldehyde could be used as a model compound that is ideally stable, reliable, readily water-soluble, easily detected, easily degraded by enzyme, and correlates well to aldehydes, especially hexanal.

Further purification steps after ammonium sulfate are needed prior to PAGE for separation of aldehyde oxidase from catalase to perform enzyme kinetics. The optimum temperature and pH for the enzyme activity and stability needs to be determined.

Others. Since aliphatic aldehydes aqueous solubility is a problem, reverse-phase HPLC column would be suitable for these aliphatic aldehyde detection. At very low concentrations, GC is also needed for propanal or hexanal detection. Mutant development by chemical or UV mutagenesis for enhanced and stabilized enzyme activity needs to be developed after enzyme assay is set up, since aldehyde oxidase induction and activity were inconsistent and unstable.

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