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The Decrease in Phenolic Content of Canola Meal and the Production of Polyphenoloxidase in Submerged and Solid State Fermentation using the White-Rot Fungus *Trametes versicolor*

by

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the degree of

Masters of Applied Sciences
in Chemical Engineering
University of Ottawa

January, 1999

Abstract

The goal of this study was to examine the production of polyphenoloxidase (PPO) and the decrease in phenolic content of canola meal during submerged and solid state fermentation with canola meal. Several parameters during submerged fermentation were examined, including addition of CM phenolics, CM sugars, Vogel's minerals and aeration. The best inducer of laccase, the inducible form of the PPO enzyme from *Trametes versicolor* was 2,5-xylidene. Only slight increases were observed due to induction of this enzyme with CM phenolics. Sucrose had a negligible effect on PPO production, however 6 mg/mL CM proteins were found to increase PPO production by 25%. The addition of Vogel's minerals solution to the culture medium had a negative effect on PPO production and at a concentration of 10 mL/L resulted in less than one-half of the amount of biomass and PPO obtained at 0.5 mL/L. Aeration had a positive effect on PPO production, however, the activity also began to decrease more rapidly.

The effect of CM, soybean meal (SBM) and sunflower meal (SFM) concentration on PPO production in a submerged process was also examined. The optimum concentration for CM and SBM was 5%, however the optimum concentration for SFM was only 3%. The maximum activity in the CM culture was 140 nkat/mL which was higher than the 118 and 102 nkat/mL measured in the SBM and SFM cultures, respectively. In all cases, the activity was higher than the 60 nkat/mL obtained in the medium without meal. When the medium without meal was supplemented with either the soluble components from a 5% CM mixture or with 5% phenolic-free CM, the enzyme activity was comparable to the media with 5% CM, indicating that it is a soluble component in the meals which is responsible for the stimulation in PPO production.

Several parameters were examined for the production of PPO and decrease in phenolic content during solid state fermentation (SSF) of *Trametes versicolor* on CM. The amount of PPO activity in the culture was found to increase with moisture content.

maximum of 500 nkat/g wet SSC at 80% moisture. There was not a large difference in PPO levels or residual levels of phenolics for a 10 day old fermentation with temperatures ranging from 24-32°C. However, above this temperature, the PPO activity decreased rapidly with no growth or PPO observed at 40°C. The addition of Vogel's minerals to the solid state culture was shown to stimulate PPO production. However, after 8 days, all cultures, regardless of Vogel's minerals concentration, had reached the same sinapine (SIN) level. After 13 days, all solid state cultures had SIN contents that were decreased by 97%. The inoculum concentration and homogenization of the inoculum was not an important parameter and in all cases had decreased the SAE content in CM by 95% after 18 days.

Preliminary flooding experiments, in which water was added to a portion of the fermenting culture to assist in the diffusion of phenolics and PPO, was shown to be useful. Upon addition of water to a 3 day culture to make a 20% suspension of culture in water, the SAE and SIN content decreased by 45% and 60%, respectively, after 16 hours. A 40% suspension was found to decrease these two components by 60% and 80%, respectively.

The maximum enzyme activity occurred for both types of fermentation at approximately 8 days. The submerged enzyme preparation had a much larger activity per gram of canola meal compared to the solid state fermentation, 2500 nkat/g vs 400 nkat/g, however when expressed in terms of volumetric reactor productivity, the activities were approximately equal.

Resumé

L'objectif de ce projet était d'examiner la production de l'oxydase de polyphénole (OPP) et la réduction du contenu phénolique des tourteaux de canola (TC) pendant la fermentation du liquide au solide. Plusieurs paramètres ont été examinés pendant le processus de fermentation à l'état liquide, incluant les phénols des TC, les sucres des TC, les minéraux Vogel et l'aération. Le meilleur générateur de lacasse, une enzyme provenant du *Trametes versicolor*, était le 2,5-xylidene. On a pu observer une légère augmentation de l'activité de cette enzyme due à l'induction des phénols des TC, en occurence, il a été observé que 6 mg/mL des protéines des TC augmente l'activité par 25%. L'addition d'une solution de minéraux Vogel à la culture a eu un effet négatif sur la production de l'OPP, en effet, une concentration de 10 mL/L a diminué par 50% la quantité de biomasse et l'activité de l'OPP obtenue était de 0.5 mL/L. L'aération a eu un effet bénéfique sur la production de l'OPP, par contre, l'activité a commencer à diminuer plus rapidement.

L'effet de la concentration des tourteaux de canola, de soya et de tournesol sur la production de l'OPP dans un processus de submersion ont été aussi étudiés. La concentration optimale de l'OPP pour le canola et le soya a été de 5%, alors que la concentration optimale pour le tournesol a été de 3%. L'activité maximale des TC était 140 nkat/mL, ce qui est une valeur nettement plus élevée que ceux obtenus avec le soya et le tournesol qui ont une valeur de 118 nkat/mL et 102 nkat/mL respectivement. Dans chaque cas, l'activité a été plus élevée que la valeur de 60 nkat/mL qui a été obtenus avec une culture sans tourteaux. Quand on fait l'addition au milieu des composants solubles contenus dans un mélange à 5% TC avec ou sans contenu phénolique réduit, l'activité de l'enzyme est comparable à celle d'un milieu avec 5% TC, ce qui indique que c'est une composante soluble dans les tourteaux qui est responsable de la stimulation de la production de l'OPP.

Plusieurs paramètres ont été examinés pour la production de l'OPP et la réduction du contenu phénolique durant la fermentation en phase solide (FPS) du *Trametes versicolor* sur les TC. L'activité de l'OPP a augmenté avec l'humidité de la culture. L'enzyme a été détecté à 50% d'humidité et l'activité a augmenté rapidement jusqu'à un maxima de 500 nkat/g pour une valeur d'humidité de 80%. Il n'y a pas de différences significatives dans la valeur de l'activité de l'OPP ou dans le niveau résiduel des phénols dans une fermentation de 10 jours entre 24 et 32 C. Cependant, au-dessus de cette température, l'activité a diminué rapidement sans qu'il n'y ait de croissance ou d'OPP observée, même à 40 C. L'ajout de minéraux Vogel à la culture solide a semblé stimuler la production de l'OPP, mais après 8 jours, toutes les cultures ont atteint le même niveau de sinapine (SIN). Après 13 jours, toutes les cultures solides avec ou sans minéraux Vogel, ont vue diminuer leur niveau de SIN de 98%. La concentration et l'homogénéisation de la biomasse initiale n'ont pas été des paramètres importants et, dans tous les cas étudiés, la concentration d'esters d'acide sinapique (EAS) a diminué de 98% après 18 jours.

Les expériences d'inondation, dans lesquelles l'eau a été ajoutée à une partie de la culture pour aider la diffusion des phénols et de l'OPP, a été utile. Après l'ajout d'eau à une partie de la culture âgée de 3 jours, le contenu d'EAS et de SIN ont diminué de 45% et de 60%, respectivement. Une suspension de 40% permet de diminuer la concentration d'EAS et de SIN de 60% et 80% respectivement. L'activité maximale de l'OPP s'est produite après 8 jours. La préparation submergée de l'enzyme a démontré une plus grande activité par gramme de TC, en comparaison avec la fermentation du solide, 2500 nkat/g contre 400 nkat/g. Cependant, quand elles sont comparée à la productivité volumétrique, les activités étaient approximativement égales.

Acknowledgments

I would like to express my gratitude to Dr. Z. Duvnjak for his guidance and financial support throughout the duration of this project. I am also very thankful to Dr. Karol Łacki for his much needed help with the methodology as well as his insights into many of the initial problems encountered during this project. I would also like to thank my colleagues Dr. Sameer Al-Asheh, Mr. Hasan Atiyeh and Ms. Jinghua Hu for their help, encouragement and companionship during these last two years.

It is with great joy that I express my thankfulness for the support and encouragement of my parents and family. And last, but not least, I would like to thank my wife Nathalie, without whose encouragement, support, love and understanding this project could not have been possible.

Nomenclature

Abbreviations

ATCC	- American Type Culture Collection
CM	- Canola Meal
Da	- Daltons
DAD	- Dehydroxydisinapic Acid Dilactone
DNS	- 3,5- Dinitrosalicyclic acid
EAS	- d'esters d'acide sinapique
FPS	-fermentation en phase solide
HPLC	- High Performance Liquid Chromatography
LiP	- Lignin Peroxidase
MnP	- Manganese Peroxidase
nkat	- Nanokatal or Nanomole/s
OPP	- l'oxydase de polyph nole
PDMS	- Polydimethylsiloxane
PPM	- Parts per Million or mg/L
PPO	- Polyphenoloxidase
SA	- Sinapic Acid
SAE	- Sinapic Acid Esters
SBM	- Soybean Meal
SFM	- Sunflower Meal
SIN	- Sinapine
SSC	- Solid State Culture
SSF	- Solid State Fermentation
SWM	- Safflower Meal
TC	- tourteaux de canola

Symbols

$A_{initial}$ - initial absorbance

A_{final} - final absorbance

Abs_{530} - absorbance at 530 nm

Dil - dilution factor for Equation (4-1)

k - rate constant for sinapine decrease in first order equation (d^{-1})

k_s - rate constant for sinapine decrease in logistic model (g SSC^2/g X^2/ d)

k_v - rate constant for enzyme production (nkat/g SSC/ d)

M_{bio} - mass of biomass in sample (g)

M_{CM} - mass of canola meal used for extraction of SAE (g)

M_{sample} - dry mass of sample including biomass and canola meal (g)

S - sinapine concentration (g/kg SSC)

S_0 - initial sinapine concentration (g/kg SSC)

SAE - SAE content of the sample (g/kg)

t - time (d)

t - length of spectrophotometric test to measure PPO activity (s)

v - rate of PPO reaction (nkat/mL or nkat/g SSC)

v_0 - initial PPO activity (nkat/g SSC)

V_{enz} - volume of enzyme (mL)

v_{ext} - volume of the methanol extract (mL)

V_{sys} - volume in cuvette (3mL)

V_{MeOH} - total volume of methanol used for extraction of CM or culture (mL)

x - moisture content of the sample (g/g)

X - biomass concentration (g/g SSC)

X_m - maximum biomass concentration (g/g SSC)

X_0 - initial biomass concentration (g/g SSC)

Greek Symbols

β	- parameter defined by Equation (3-3)
ε	- extinction coefficient (mol^{-1} cm^{-1})
μ_m	- maximum specific growth rate (d^{-1})

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Chapter 1: Introduction

Canola is a very important oilseed crop and is the second most important agricultural crop in Canada (Shahidi and Naczk, 1992). After the extraction of the oil, the canola seed residue is called canola meal (CM). Due to its favourable nutritional composition, CM is used as protein supplement for livestock feeding. It has a well-balanced array of amino acids and contains many vitamins and minerals. Unfortunately, CM also contains a significant amount of phenolics, glucosinolates and phytic acid, which limit its utilization in a livestock diet. Decreasing these components would allow the meal to be used to a greater extent as well as replacing the more expensive soybean meal as the main protein source in livestock diets.

The main phenolic fractions in CM are the free phenolic acids and the free phenolic esters. The main component of these two fractions is sinapic acid, SA, which can constitute up to 65-86% of the free and 71-97% in the esterified phenolics in CM (Naczk *et al.*, 1992). There have been a variety of methods used to decrease the phenolic content of CM. These include solvent extraction, heat degradation and chemical reaction. However, the most effective method to date is an enzymatic process using a polyphenoloxidase from the white-rot fungus *Trametes versicolor* (Lacki, 1997). This enzyme was able to decrease the phenolic content by over 98%.

Despite the fact that this enzymatic process was very effective, there are some limitations, namely the loss of soluble, nutritional components due to the removal of the water by decanting. Furthermore, although some of the compounds resulting from the transformation of SA and its esters were examined, there are some unidentified phenolics. In addition, since a single enzymatic process can only take a transformation so far, it is possible that a fermentation process may be more beneficial. Due to the fact that white-rot fungi can grow using lignin, which is comprised of polymerized phenolic monomers, as a carbon source, the fungi could be able to use the phenolics for growth. This could

completely eliminate the phenolics from the meal. In addition, the presence of biomass within the meal could also improve the nutritional composition of the meal. One of the main objectives of this project is to decrease the phenolic content of CM using fermentation processes.

A second goal of this project was to examine the production of polyphenoloxidase, PPO. This enzyme has been used previously for the decrease in phenolic content of CM (Lacki and Duvnjak, 1996a,b,c). PPO is a constitutive enzyme and it is presumed that it is produced as part of the metabolic pathway to degrade lignin. The enzyme is also responsible for the degradation of SA and its esters. Furthermore, since the culture conditions play an important role in PPO production, with certain conditions or compounds stimulating or inhibiting its production, the influence of several culture parameters on PPO production will be presented in chapters 5 and 6.

Chapter 2: Literature Review

This section will provide a comprehensive review of the literature related to this project. There will be two key sections: a review of oilseed meals and a review of the relevant submerged and solid-state fermentation literature. The first section will focus on canola meal, since this is the main oilseed meal studied but will also highlight two other important oilseed meals, soybean and sunflower meals. The fermentation review is equally important since this will be of assistance when examining enzyme production and the subsequent reduction in phenolic content of the canola meal.

2.1 Canola Meal

Canola is the genetically-improved variety of rapeseed containing less than two percent of the total fatty acids in the oil as erucic acid and less than 30 mmoles of alkenyl glucosinolates per gram of oil-free dry matter of the seed (Bell, 1993). Rapeseed is among the world's most important oilseed crops (Klockeman *et al.*, 1997). In Canada, rapeseed/canola is second only to wheat in value and area planted (Shahidi and Naczk, 1991). The world-wide production of CM was 17.6 million metric tonnes in 1996-97, with the Canadian production being 1.1 million metric tonnes (Statistics Canada, 1998). Furthermore, although oil is a highly valued commodity, extraction operations rely on revenues obtained from selling the meal in order to remain profitable (Howard, 1993). In addition, the feed accounts for the majority of the total cost of livestock production, of which the major cost of the feed is the protein component. As a result, improving the nutritional value of the meal would undoubtedly increase its monetary value as well as providing a larger market for CM.

2.1.1 Canola Meal- Nutritional Aspects

Canola meal contains approximately 32-38% crude protein and is rich in vitamins, minerals and amino acids (Bell, 1993; Klockeman *et al.*, 1997; Stanford *et al.*, 1996). The meal also contains up to 2-3% starch, up to 7-8% sucrose and approximately 5% cellulose (Bell, 1993). CM is an excellent source of protein for dairy and beef cattle; however its use in the diet of poultry and swine is limited by protein quality and low digestible energy (Mustafa *et al.*, 1996).

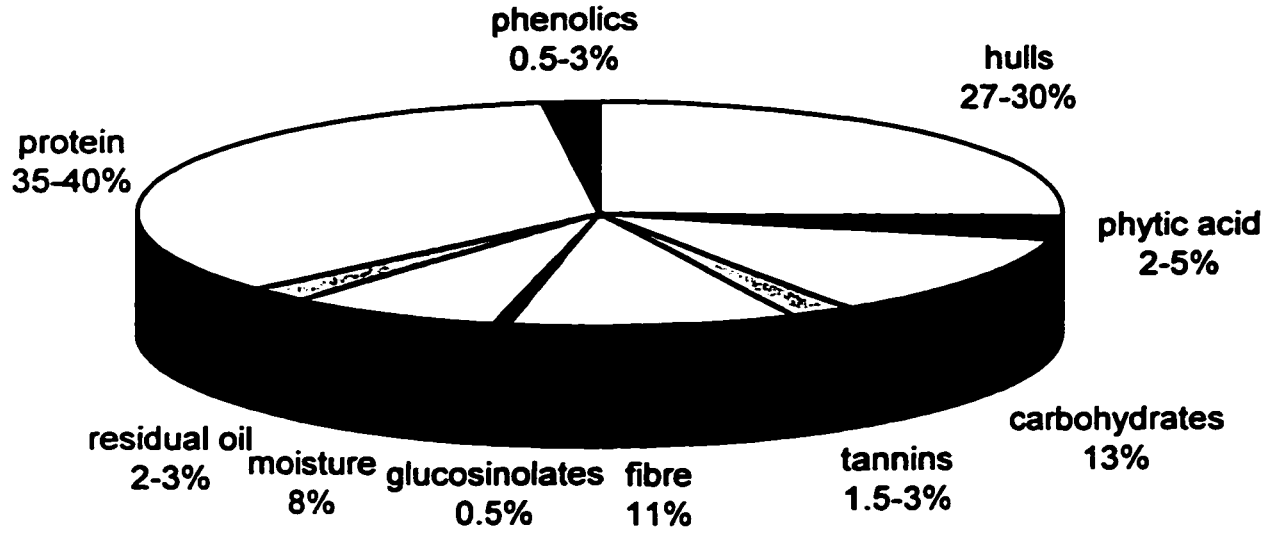

Figure 2-1: Composition of Canola Meal

The nutritional value of the meal is worsened by high levels of phytic acid, glucosinolates and certain phenolic compounds. Phenolic compounds bind with proteins, rendering them unavailable for digestion and can give bitter, unpleasant tastes to the meal (Shahidi and Naczk, 1992). Furthermore, the presence of sinapine, the major phenolic ester in CM, has been linked to fishy odours found in the eggs from some brown-egg-

laying hens (Hobson-Frohock *et al.*, 1973). This usually restricts the amount of CM to less than 10% of the poultry diet. However, when the sinapine content in the diet is less than 0.1%, the fishy odour does not develop, hence a meal with significantly reduced sinapine concentrations would increase the value of the meal (Bell, 1993).

2.1.2 Phenolic Compounds in Canola Meal

Phenolic compounds consist of a phenolic ring and often contain other substituted groups such as carboxyl, amino or hydroxyl groups. There are many different types of phenolic compounds that occur naturally and these include phenolic acids, lignin, flavonoids and tannins. Lignin and tannins are complex polymeric phenolics, made up of repeating phenolic units, and have high molecular weights (>500). Simple phenolics have much lower molecular weights and include substituted benzoic and cinnamic acid derivatives as well as flavonoid compounds.

A common subdivision of the phenolics in rapeseed/canola meal is as free, esterified or insoluble-bound phenolics. Since the phenolic composition and distribution for rapeseed and canola are similar, the two will be called canola for the purposes of this literature review. The free acids and esterified phenolics are both readily soluble in aqueous media whereas the insoluble-bound phenolics are, as the name implies, attached to the meal and are not water-soluble (Krygier *et al.*, 1982a). The vast majority of the phenolics found in canola meal, up to 80%, are in the esterified form (Shahidi and Naczk, 1992). The next largest fraction are the free phenolic acids (up to 15%) and the remainder are insoluble-bound phenolics.

Sinapic acid (SA), 3,5-dimethoxy-4-hydroxy cinnamic acid, is the most abundant phenolic acid in canola meal. Naczk *et al.* (1992) found that for several varieties of canola meal, SA constituted 65-86% of the free and 71-97% of the esterified phenolic acids. Fenton *et al.* (1980), Kozlowska *et al.* (1983), Dabrowski and Sosulski (1984) and Shahidi (1992) also found that the predominant phenolic acid in canola meal was SA.

There are several other phenolic acids in canola meal and these include *p*-hydroxybenzoic acid, vanillic acid, syringic acid and ferulic acid (Dabrowski and Sosulski, 1984). The structures are given in Figure 2-2.

As mentioned above, the esterified phenolics constitute the major fraction of phenolics in CM. These esters usually consist of a phenolic acid esterified to another group, such as a glycoside or choline group. The predominant esters are the sinapic acid esters (SAE) and, in 4 different varieties of rapeseed, constitute over 90% of the total soluble esters (Krygier *et al.* 1982b). Sinapine (SIN), 3,5-dimethoxy-4-hydroxy cinnamoylcholine, is the choline ester of sinapic acid and was shown to constitute 59-79% of all SAE in 15 varieties of rapeseed meal (Bouchereau *et al.*, 1991). SIN is accumulated during seed development, to be later saponified into choline, which is used for membrane synthesis (Taylor *et al.*, 1993). Cinnamic acid is transformed into carbohydrate esters (*ibid*).

2.2 Soybean Meal

A soybean bushel, 27.3 kg, yields approximately 21.8 kg of 44% crude protein and 5 kg of extracted oil (Brumm and Hurburgh, Jr., 1990). The resulting soybean meal (SBM) is primarily used as a protein source for livestock or human consumption. SBM has a wide range of amino acids and contains approximately 47% crude protein (O'Quinn *et al.*, 1997). It is also very rich in choline, approximately 2400-3050 mg/kg and is believed to be more bioavailable than CM choline (Menten *et al.*, 1997). SBM has a biological value similar to CM for pig feeding but contains approximately 20-30% more protein (Siljander-Rasi *et al.*, 1996). Furthermore, since the fibre content, which decreases the energy and protein content of the meal, is about one-half that of CM (*ibid.*), SBM is sold at a higher price (Statistics Canada, 1996). The total world production of soybean seed in 1996-97 was 92.4 million metric tonnes (Statistics Canada, 1998).

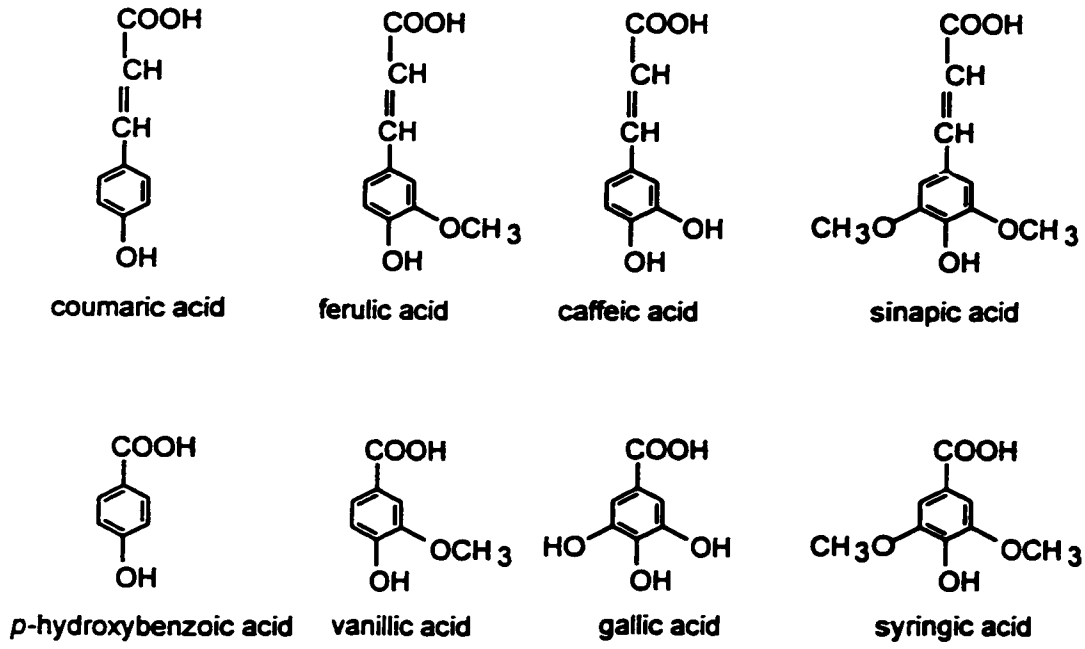

Figure 2-2: Chemical structures of phenolics found in canola and other oilseed meals

Unfortunately, the use of SBM is also hindered by certain anti-nutritional factors including phytic acid, phenolics and fibre (Rozan *et al.*, 1997). These components interfere with protein hydrolysis, and hence decrease the availability of amino acids, as well as decreasing the adsoprtion of minerals. Furthermore, the oil extraction process, which includes heat treatment, also decreases the amounts of certain amino acids, in particular lysine, threonine and methionine (Siljander-Rasi *et al.*, 1996).

2.2.1 Soybean Meal Phenolics

The phenolic content of SBM is approximately 90% lower than in CM and with a different assortment of free phenolic acids (Dabrowski and Sosulski, 1984). The majority of the phenolic acids liberated from the soluble esters and insoluble residue of CM are cis- and trans- sinapic acid (98%) followed by ferulic acid (1.3%) and *p*-hydroxy benzoic acid (*ibid.*). On the other hand, the predominant phenolic acids found in SBM are: syringic acid (39%), ferulic acid (21%), *p*-hydroxy benzoic acid (19%), *p*-coumaric

acid (13%) and caffeic acid (8%). Furthermore, SA was not detected, which means that SIN is not a problem for SBM. Ramakrishna *et al.* (1989) also reported significant amounts of chlorogenic acid in several varieties of SBM. Maga and Lorenz (1974) found, in addition to the phenolics mentioned above, salicyclic, vanillic, genistic and protocatechuic acids in one type of SBM, as well as low quantities of SA.

To the best of our knowledge, there have been no fermentation processes used to decrease the phenolic content of SBM. However, a biotechnological process using an enzyme preparation from the fungus *Trametes versicolor* has been successfully used to decrease the phenolic content of SBM (Nielsen, 1998).

2.3 Sunflower Meal

The 1990 global production of sunflower meal (SFM) was 8.6 million metric tonnes, ranking this as the 4th largest oilseed meal behind soybean, cottonseed and canola meal (World Oilseed Situation and Outlook, 1992). SFM and SBM are similar protein sources (Leitgeb *et al.*, 1996). When supplemented with lysine and methionine, SFM can be used to replace SBM in poultry diets without adverse effects on performance or feed efficiency (Mohme *et al.*, 1997). It was also found that SFM, in concentrations of up to 33%, could be included in the feed requirements of bovine diets without any changes in growth rates and metabolizable energy utilization (Siebrits, 1996). SFM is also very high in the major nutritional metals, in particular potassium, magnesium, calcium and iron (Sotillo and Hettiarchchy, 1994). While the phenolic content can be as high as 5.8% (Prasad, 1990) it is typically much less than this.

The protein content of the meal depends on whether the hull is removed prior to extraction. For example, Niazi *et al.* (1991) reported that SFM with hulls contained 37% protein and 17% fibre, whereas when the hulls were removed first, the protein and fibre contents were 51% and 7%, respectively. The amino acid composition of the meal is not influenced by the amount of hull removed (Siebrits, 1996). The reported protein content

of SFM is approximately 50% (Prasad, 1990; Ibrahim *et al.*, 1992; Niazi *et al.*, 1991; Zhang and Parsons, 1994). However, a value of 61% was measured by Sotillo and Hettiarachchy (1994). The amino acid profile is considered to be well-balanced (Ibrahim *et al.*, 1992); however, the low levels of lysine are often limiting and are required to be supplemented.

2.3.1 Sunflower Meal Phenolics

The total phenolic content of SFM is comparable to CM, however the major phenolic in SFM is chlorogenic acid, which comprises of a caffeic acid and a quinic acid moiety. Mikolajczak *et al.* (1970) estimated that the phenolic content was 2.4%, with 2% comprising of chlorogenic acid, 0.2% caffeic acid and 0.2% other phenolics. Sabir *et al.* (1974a) analyzed three types of SFM and each contained approximately 3.3% phenolic acids. Chlorogenic acid and a chlorogenic acid isomer were found to represent 62-66% of the total phenolic acids, with the remainder being SA-like (14-17%), isoferulic acid-like (4-5%), caffeic acid (4-5%) and coumaric acid-like (3%) phenolics. Leung *et al.* (1981) found a similar profile, but also detected *p*-hydroxybenzoic acid, vanillic and syringic acids. Finally, Dabrowski and Sosulski (1984) found that caffeic acid represented 97.5% of the free phenolic acids in SFM, with 1% each of ferulic and *p*-hydroxybenzoic acid and traces of vanillic, syringic and coumaric acids. Since chlorogenic acid was hydrolyzed into caffeic and quinic acids using the method of Dabrowski and Sosulski (1984), it could not be measured separately. However, they estimated it spectrophotometrically to be 2.85%.

Due to the favourable amino acid composition of SFM, there is interest in using this meal for human consumption. However, the presence of chlorogenic acid, which has been implicated in the enzymatic browning of fresh fruits and vegetables, poses problems. Under neutral or alkaline conditions, which are often used for protein separation, the solutions develop a dark green or brown colour due to protein bonding

with the oxidized phenolic compounds, in particular, chlorogenic acid (Sabir *et al.*, 1974b). Therefore, although the meal can be used to supplement livestock diets, the dark colour is unacceptable for most food purposes. It has been suggested that if the concentration of chlorogenic acid in the meal was less than 0.3%, the protein concentrate (or flour) prepared would have acceptable white colour characteristics (Dabrowski and Sosulski, 1984).

2.4 Submerged Fermentation Using Fungi

Three classes of fungi are usually considered when discussing the preferential degradation of wood or wood components, of which phenolics are a major constituent (Sklarz *et al.*, 1989): soft-rot, brown-rot and white-rot fungi. Soft-rot and brown-rot fungi preferentially degrade the cellulose (carbohydrate) fraction of wood whereas white-rot fungi can degrade both lignin (phenolic) and cellulose. As a result, there have been numerous studies using white-rot fungi for degradation of lignin in pulping operations (biobleaching), reduction of tannin contents and degradation of phenolics. In this thesis, the process is considered to be a submerged process when the bulk phase is liquid. The concentration of meal in the liquid is less than 10%.

2.4.1 Enzyme and Microbe Used in This Study

The fungus used in this project was *Trametes versicolor*, ATCC 42530. It is the same fungus studied by Khan and Overand (1990), Lacki and Duvnjak (1996a,b,c) and Lacki (1997). This microbe was chosen since it produced an oxygenase system capable of degrading a wide range of phenolic compounds including SIN and SA, the main phenolics in CM (Lacki and Duvnjak, 1996a,b,c). The constitutive form of the enzyme was used by Lacki and Duvnjak (1996a,b,c) and was considered to be a polyphenoloxidase (PPO) instead of a laccase since it did not require an inducer. This

enzyme, PPO, was studied extensively in terms of optimum reaction conditions, and many of its reaction products have been determined.

2.4.2 Enzyme Production by Trametes versicolor

Trametes versicolor has been frequently studied due to its role in delignification. Many of the enzymes produced by this fungus have been characterized, with the most attention focused on cellulase, laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). The last three are important since they differentiate the white rot fungi from other types of fungi.

It has been found that the conditions under which the fungus is grown have an influence on enzyme production and even the types of enzymes produced. Furthermore, even different strains of the same fungus can produce different amounts of enzymes under the same conditions (Szklarz *et al.*, 1989). Under nitrogen- limiting conditions LiP was observed, however under nitrogen-rich conditions, there was no LiP activity (*ibid.*). Similarly, Collins and Dobson (1995) found that supplementing the medium with veratryl alcohol increased the amounts of LiP formed but not MnP. Conversely, Dehorter and Blondeau (1992) found that in a culture medium that did not initially show any LiP or MnP activity, the addition of the surfactant Tween 80 induced the presence of MnP, but not LiP. Furthermore, the addition of humic acids from soil, essentially lignin after much decay and alteration, stimulated the production of both LiP and MnP (*ibid.*).

2.4.3 Laccase from Trametes versicolor

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is an extracellular enzyme produced as part of the metabolic pathway during the degradation of lignin or lignin-like compounds. There are considered to be 2 forms of laccase (Asiegbu *et al.*, 1996): constitutive and inducible. The non-induced enzyme is usually called

polyphenoloxidase, PPO (Lacki, 1997). There is not a clear distinction in the literature between PPO and laccase since these two enzymes perform similar functions. However, the two can be distinguished experimentally since PPO can catalyze the hydroxylation of *p*-cresol, while laccase cannot (Butt, 1980). Furthermore, upon purification, PPO is a colourless enzyme whereas laccase is a blue enzyme (*ibid.*).

Bollag and Leonowicz (1984), using polyacrylamide gel electrophoresis, found two enzyme bands in the presence of 2,5-xylidene, an inducer used to promote laccase production, whereas there was only one band otherwise. The sole band, whose molecular weight corresponded to approximately 61000 Daltons was the constitutive enzyme and the additional band, with a molecular weight of approximately 70000 Da was from the induced enzyme. Morohoshi *et al.* (1987) was able to separate three forms of laccase, depending upon the culture conditions. Since the majority of the literature makes no distinction between laccase and PPO, for the purposes of this review section, the two will be considered collectively as laccase.

Laccase is responsible for the oxidation of phenolic and non-phenolic compounds as well as for aromatic ring cleavage (Khan and Overand, 1990). It possesses an excess of acidic amino acids (Fahraeus and Reinhammar, 1967) and is active at lower pH levels (Lacki and Duvnjak, 1996a). It is also believed that the enzyme is a combination of two identical subunits each containing a Cu^{+2} and Cu^{+} atom (Fahraeus and Reinhammar, 1967). The nitrogen content of laccase is much lower than most proteins, due to the abundance of carbohydrate covalently bonded to the enzyme (Fahraeus and Reinhammar, 1967).

2.4.4 Influence of Culture Conditions on Laccase Production

Enzymes are usually produced either parallel to biomass growth or as a result of a secondary metabolite during the stationary phase, often accompanied by significant mass

loss (Szklarz *et al.*, 1989). Both types of response have been observed for laccase production by different researchers for different strains of *Trametes versicolor*. Furthermore, Szklarz *et al.* (1989) examined a strain newly isolated from a forest and one being cultured in their lab. It was found that the newer strain exhibited the first type of behaviour whereas the cultured strain exhibited the latter behaviour. Despite the fact that the two strains produced laccase with the same isoenzyme pattern (i.e., same enzyme), the activity of the cultured strain was 88% lower than the locally obtained strain. This indicates that fungi can lose their potential for enzyme production when cultured for long periods of time. Furthermore, the cultured strain grew very fast initially, however it began to autolyze after 7 days and the maximum activity was at 19 days. The recently isolated strain grew slower and did not begin to autolyze until 14 days. Its maximum laccase activity was at 13 days.

In addition to fungal strain, the culture conditions also influence enzyme production. *Phanerochaete chrysosporium*, a different type of white-rot fungus that does not normally secrete laccase, has been found to produce this enzyme in a high nitrogen medium provided cellulose is supplied as the carbon source (Srinivasan *et al.*, 1995). Similar observations were noticed by Eggert *et al.* (1996) in which laccase production by *P. chrysosporium* was repressed in the presence of glucose but was detectable when grown on cellulose. The opposite effect was observed for *T. versicolor* during the brightening of kraft pulp (Kirkpatrick *et al.*, 1989). In a low N-medium (2.5 g/L soytone) the fungus was able to produce the enzymes (including laccase) required to de-lignify the wood pulp. However, under high N conditions (10 g/L soytone), the measured brightness increase was due strictly to dilution with the white fungus and not to enzymatic degradation of lignin. It was assumed that decreasing the amounts of soytone, an enzymatic hydrosylate of soybean meal, caused nitrogen limiting conditions, however that may not be the case. It is possible that since the soytone also supplies many other

nutrients, including vitamins, minerals and carbohydrates, one of these could have been the limiting factor.

Farhaeus *et al.* (1952) found that the enzymatic activity of *T. versicolor* depended upon the kind of medium used. When using glucose as the carbon source in a synthetic medium, laccase was not produced until after 9 days, at which point almost all of the glucose had been consumed and cell autolysis had begun. After 9 days, the activity began to increase rapidly over the next 10 days. However, when using malt extract as the carbon source, the situation was very different. Laccase activity began almost immediately and reached a maximum by 8 days; however within 13 days, the activity had dropped by almost 50%. The biomass continued growing linearly until 18 days, while the activity continued to decrease rapidly. It was postulated that the fungus may have begun to produce a protease(s) which decomposed the laccase. Two additional findings from this study are important. The first is that there was very little laccase remaining entrapped within the biomass. This means that it was not stored and released during cell lysis but actually produced and secreted as a result of a secondary metabolite or removal of an inhibitor. The second finding was that other natural extracts, including yeast and potato extracts, also had a strong positive effect on laccase formation in young cultures. It was postulated that one or more of these nutritional factors could be important for stimulating laccase production: (1) a vitamin or amino acid in the malt; (2) some component of the synthetic medium could inhibit enzyme production; (3) changes during fermentation could promote more favourable conditions (e.g. decrease in pH); (4) autolysis releases nutrients in synthetic media (i.e. vitamins, proteins etc.) and (5) shortage of carbohydrates stimulates autolysis.

Finally, the addition of oxygen to a culture medium also stimulated the production of laccase. Reid and Seifert (1982) examined lignin degradation by *T. versicolor* in shake and SSF cultures in air and oxygen environments. It was found that higher levels of

oxygen stimulated both carbohydrate consumption and lignin degradation. This is reasonable since oxygen is a required co-substrate of laccase (Lacki and Duvnjak, 1996b). The oxygen-enriched cultures produced more biomass than the air cultures. However they also underwent rapid autolysis after reaching the maximum biomass concentration. Ziomek *et al.* (1991) also examined pulp brightening using *T. versicolor*, however instead of using oxygen, they added an oxygen carrier. Polydimethylsiloxane (PDMS, also called simethicone) increases the solubility of oxygen 30-40 times over that in pure water and is reported not to have any adverse effects on the growth and metabolism of microbial cells. PDMS provided higher levels of dissolved oxygen during the critical growth phase and resulted in faster biomass production rates; however the ultimate biomass yields were identical to the control culture.

2.4.5 Effect of Inducers on Laccase Production

An inducer is a substance that promotes the synthesis of a specific enzyme, usually by acting on the genetic level, but does not otherwise affect the microorganism (Bailey and Ollis, 1986). The rate of production of inducible enzymes such as laccase, is very sensitive to environmental conditions, as was discussed in the previous section. The most effective inducer of laccase production by *T. versicolor* is 2,5-xylidene; however other inducers have been used. Many of these inducers are toxic to biomass in large dosages, hence they are usually added once the cultures have begun to grow.

Fahraeus and Reinhammar (1967) induced laccase production in a 3 day culture with 0.2 mM 2,5-xylidene and found immediate increases in this enzyme compared to the control, to a level of 2 times the control. It was found that this inducer is very specific and resulted in most of the additional extracellular protein being laccase. Furthermore, the addition of glucose at the same time as the 2,5-xylidene resulted in an even greater increase in laccase production. Bollag and Leonowicz (1984) also added 0.2 mM 2,5-xylidene to a young culture of *T. versicolor*. It was found that the control produced only

one type of laccase whereas the 2,5-xylidene culture produced two, indicating that induction resulted in the formation of a new enzyme. In this case, the induced culture contained almost 5 times the activity of the non-induced culture.

Similar results were obtained by Rogulski *et al.* (1990), however it was determined that the optimum time for the addition of the inducer was much later (after 10 days) when the mycelium had reached its stationary growth phase and the glucose content was almost exhausted. The laccase in the non-induced cultures had begun at about 5 days; however upon addition of the inducer at 10 days, the activity doubled. It should be noted that the authors did not test earlier additions, which could have an influence. Furthermore, Bollag and Leonowicz (1984) re-induced laccase production in a culture late in its growth cycle, which also caused an additional increase in activity. Finally, Lacki (1997) also induced production of this enzyme with 2,5-xylidene and found a 3 fold increase in the laccase activity.

Ferulic acid, a phenolic monomer of lignin, has also been used as an inducer. It was found that a 0.1 mM solution of ferulic acid increased the laccase activity in several varieties of fungi, including *T. versicolor*, however the results were not quantified (Leonowicz and Trojanowski, 1975). Furthermore, the addition of ^{64}Cu at induction demonstrated that all the ^{64}Cu was incorporated into the induced form of laccase. The molecular weights of the constitutive and induced forms were 71000 and 61000 Daltons, respectfully, which was the opposite as found by Bollag and Leonowicz (1984).

A recent study examined the effect of ferulic acid concentration on laccase production by *T. versicolor* (Asiegbu *et al.*, 1996). At 0.5 g/L (2.57 mM), in a medium containing 1% cellulose, the activity was 5 times greater than the control without ferulic acid. An increase to 1 g/L resulted in an 8 times increase compared to the control. A concentration of 5 g/L inhibited biomass growth and hence no activity was observed. The pattern of laccase production was also different in the induced and non-induced

cultures. The former exhibited two activity maxima, at 6 and 14 days, with a drop of at least 50% in between. The latter exhibited a slow and continual increase with a maximum activity at 12 days.

Another study showed the effect of a plant hormone, 2,4-dichlorophenoxyacetic acid on laccase production by *T. versicolor* (Tsujiyama *et al.*, 1993). Increasing the amounts of this compound, up to a level of 50 ppm, produced the greater amounts of biomass. At a concentration of 10 ppm, 2.6 times more biomass was produced than the control. This study also showed that the cultures with the inducer had a maximum laccase activity at 10 days, whereas the control had a maximum at 20 days. Furthermore, the maximum activities of the two cultures were identical.

2.5 Solid State Fermentation

In solid state fermentation (SSF) the growth of the microorganism occurs on the surfaces of solid substrates, with low moisture contents (Mudgett, 1986). Filamentous fungi are often used in SSF since solids with low moisture contents resemble their natural habitats (Smits *et al.*, 1998). These solids are often agricultural wastes and are rich and complex sources of nutrients (Mudgett, 1986). Furthermore, these substrates are often more complex carbon and energy sources than those used in submerged media, but may or may not provide complete nutritional requirements (*ibid.*, Penaloza, 1991). Furthermore, enzymatic activity is often higher during SSF (Ramakrishna *et al.*, 1994).

Valmaseda *et al.* (1991) have considered 2 distinct growth phases for *T. versicolor* during SSF of straw: (1) colonization phase and (2) degradation phase. The first stage results in a strong increase in the respiratory activity and a decrease in free sugars and extractives. This phase was found to last approximately 7 days. The second phase represents the fungal attack of the lignin and polysaccharides. This phase is often associated with an increase in protein content of the culture.

The main advantages of SSF are the inexpensive nature of the equipment and materials, growth conditions similar to the natural habitat of the fungus and the fact that aeration is often easy to provide (Morin, 1992). However, there are usually longer growth cycles required, difficulties in monitoring fermentation parameters such as temperature, pH and substrate and biomass concentrations as well as limited varieties of microbes available due to low moisture levels. Submerged fermentations have the advantages of shorter growth cycles, established technologies and ease of monitoring fermentation parameters. However they also require sophisticated equipment, have higher capital and operating costs and aeration may be problematic with high biomass levels.

2.5.1 General Considerations for SSF

As in submerged fermentation, there are many parameters that influence biomass growth and enzyme production. These include temperature, inoculum size, moisture content, aeration and the effects of different substrates. Unlike submerged fermentation, the direct measurement of biomass during SSF is usually impossible due to the presence of the support material. As a result, an indirect analysis is normally used and a common method will be discussed in section 2.5.2. However, due to difficulty in continuously monitoring the biomass throughout a fermentation, many researchers ignore biomass measurement unless a simple method is used, such as those discussed in this section.

The effect of temperature on the SSF growth of *Trichodermi reesei* on wheat bran has been studied by Smits *et al.* (1998). The temperature dependence of the growth rate of this fungus was also modeled by measuring the increasing radial diameter of the culture placed in the centre of a Petri dish. It was found that the growth rate slowly increased linearly with temperature over the range of 20°-35°C (.01 mm/h/°C). Above this temperature, the growth rate decreased rapidly (-.06 mm/h/°C) until 41°C, at which point there was no growth.

The effect of temperature on the growth of *T. versicolor* on wheat straw has been examined by Yadav and Tripathi (1991). The purpose of the study was to upgrade cereal straw for use in animal feed. The fungus was found to de-lignify the meal and increase the protein content and *in vitro* dry matter digestibility. The optimum temperature was found to be 30°C. However, there was not a large difference between the results at 22° and 30°C.

The amount of inoculum, or initial biomass, can also affect the fermentation process of a solid state culture (SSC). During the solid state production of glutamic acid on sugarcane bagasse by a *Brevibacterium* sp., increasing the inoculum size from 2.5% to 10% (mL/g SSC) did not significantly increase the productivity (Nampoothri and Pandey, 1996). However, using a larger inoculum size, 20%, initially increased the rate of production, but the ultimate yield eventually decreased to below the levels obtained by the smaller inoculum sizes. This was attributed to the shortage of nutrients due to the larger number of cells. On the other hand, the rate of decrease of phytic acid during a SSF process with the fungus *Apergillus carbonarius* using canola meal increased as the amount of inoculum increased (Al-Asheh, 1993).

The effect of moisture content is also important. During the growth of *T. versicolor* on wheat straw, the optimum moisture content was found to be 55% (Yadav and Tripathi, 1991). Decreases in moisture content to 50% and 40% resulted in a 38% and 55% decrease in the *in vitro* digestibility as well as 22% and 63% decreases in the crude protein content of the wheat straw. Similarly, an increase to 70% moisture resulted in decreases of these two parameters by 27% and 84%, respectively. It was believed that the changes in the moisture content can affect the gas exchange so that the fungus suffers. Contrary evidence was found during the production of glutamic acid by a *Brevibacterium* sp. (Nampoothiri and Pandey, 1996). As the moisture content increased from 65-70% to 85-90%, glucose consumption increased 20% and protein production increased 2.65

times. It should be noted that the first microorganism, *T. versicolor*, is a fungus and fungi prefer lower moisture contents. The second microorganism is a bacterium and, in general, bacteria prefer the higher moisture levels.

Aeration is also important during SSF. In addition to supplying oxygen and removing carbon dioxide, aeration is also required for heat and moisture transfer within a culture and with the surrounding environment (Mudgett, 1986). Since fungi require oxygen to grow, aeration is especially critical in SSF cultures. For example, during the decolourization of pulp effluent, *T. versicolor* immobilized near the surface of a liquid culture (within 2mm of the surface), was more productive than shake cultures (Martin and Manzanares, 1994). Furthermore, Reid and Seifert (1982) found that carbohydrate consumption and lignin degradation in SSF by *T. versicolor* was stimulated by higher levels of oxygen.

Different substrates also influence the growth and productivity of fungi. The addition of glucose during the decolourization of pulp effluent by *T. versicolor* doubled the amount of colour removed (Martin and Manzanares, 1994). Reid (1979) found that increasing the carbon content of the medium increased the degradation of lignin by *Phanerochaete chrysosporium*, however, increasing the nitrogen level had the opposite effect. Similar results were found by Yadav and Tripathi (1991) during the growth of *T. versicolor* on wheat straw and Katagiri *et al.* (1995) during the biobleaching of kraft pulp using the same fungus.

The importance of metal ions during SSF can be demonstrated by the addition of potassium ions to a fungal culture of *Rhizopus oligosporus* during SSF with lupin seed (Penaloza *et al.*, 1991). Due to the debittering process, lupin seeds, containing low levels of potassium, when used for fermentation, resulted in low biomass growth levels. However, upon addition of potassium, the amount of biomass produced was shown to be linearly dependent upon the amount of K^+ added to the medium, up to 10 mg/L of the

liquid phase, after which the biomass level remained constant. Further additions, up to 390 mg/L, did not increase biomass levels.

2.5.2. Biomass Determination in SSF

The determination of the amount of biomass within solid-state fermentation culture is very difficult due to the presence of the insoluble support material. One of the most common techniques is to relate the chitin content of mycelial biomass to the amount the dry matter in a liquid or agar culture (Swift, 1973; Kirkpatrick *et al.*, 1989; Ziomek *et al.*, 1991; Jones and Worrall, 1995). Assuming that the chitin content of the fungus remains constant when grown under SSF, the amount of biomass can be determined by measuring the chitin content of the culture.

Chitin is an unbranched polymer of N-acetyl-D-glucosamine and is a cell wall component of fungi. Upon acid hydrolysis, chitin yields glucosamine which can be measured by a variety of techniques including spectrophotometric or HPLC procedures (Aidoo *et al.*, 1981 and Ekblad and Nasholm, 1996). This method is open to criticism since the chitin content of the fungi can change with age of the mycelium and nutrient content and oxygen contents of the medium (Sharma *et al.*, 1977). However, it has been successfully used in many cases such as to study the growth of *T. versicolor* during pulp biobleaching (Kirkpatrick *et al.*, 1989; Ziomek *et al.*, 1991) and the decay of wood (Swift, 1973; Jones and Worrall, 1995).

Chapter 3: Mathematical Modeling

The production of biomass in the solid state process will be modeled in this project using a logistic model (Okazaki *et al.*, 1980). Using the parameters obtained from this model, equations will be given to model the production of PPO and decrease in sinapine content.

The kinetics and mathematical modeling of microbial systems in SSF is very important for successful design, scale-up, operation and optimization of solid state bioreactors. There are, however, several difficulties (Al-Asheh, 1993):

1. the systems are geometrically complex due to non-homogeneous spatial distribution of the components;
2. growth is limited to the surface of the particles, leading to concentration gradients and diffusion-related problems;
3. solid substrates are often heterogeneous, both in terms of structure and nutritional aspects;
4. the direct measurement of biomass concentration is not usually possible due to the presence of the support material so that indirect methods are usually used;
5. mixing of the system in order to obtain good representative samples for analysis are highly damaging to the fungal mycelia, which in turn lowers the growth rate of the microorganism.

3.1 Modeling of the Growth Kinetics in SSF

There are a variety of mathematical models proposed for growth in solid state cultures. The logistic model developed by Okazaki et al. (1980) will be examined here since it has been used to study the growth of the fungus *Apergillus carbonarius* on canola meal (Al-Asheh, 1993) and the fungus *Trichoderma reesei* on wheat bran (Smits *et al.*, 1998). This model was first used to describe the growth of koji mold on rice, but can also be extended to model enzyme and substrate concentrations. This model, which is given

by the following equation, considers the fungus in the exponential growth and stationary phases but does not consider the death phase:

$$\frac{dX}{dt} = \mu_m X(1 - \frac{X}{X_m}) \qquad (3\text{-}1)$$

where

X-biomass concentration (g/g SSC)
t-time (d)
μ_m-maximum specific growth rate (d^{-1})
X_m-maximum biomass concentration (g/g SSC)

The following initial condition applies:

at t=0, $X=X_o$

where X_o is the initial biomass concentration. On integration of Equation (3-1), the following expression is obtained:

$$X = \frac{X_m}{1 + \beta . e^{-\mu_m t}} \qquad (3\text{-}2)$$

where:

$$\beta = \frac{X_m}{X_o} - 1 \qquad (3\text{-}3)$$

These equations can be solved using an appropriate statistical software with X_m and μ_m as parameters.

3.2 Modeling PPO Production During SSF

The logistic law can be applied to the production of enzyme during the exponential phase by applying the logistic model of Okazaki et al. (1980). It is assumed that the production of enzyme is proportional to the biomass as follows:

$$\frac{dv}{dt} = k_v X \tag{3-4}$$

where

v-enzyme activity (nkat/g SSC)
k_v-rate constant for the production of enzyme (nkat/g SSC/ d)

and using the following condition:

at t=0, $v=v_0$

where v_0 is the initial enzyme activity after inoculation. Upon integration and substitution of Equation (3-3), the following equation is obtained and gives the relationship between v and t:

$$v = v_o + (\frac{k_v X_m}{\mu_m}) \ln(\frac{\beta + e^{\mu_m t}}{\beta + 1}) \tag{3-5}$$

3.3 Modeling of the Sinapine Decrease During SSF

The logistic law can also be used to model the decrease in sinapine content. The rate of decrease is assumed to be first order with sinapine concentration (S) and second order with biomass (X). is the same as defined in Equation (3-3):

$$\frac{dS}{dt} = -k_s S X^2 \tag{3-6}$$

where

S-sinapine concentration (g/kg dry SSC)
k_s-rate constant for sinapine decrease (g SSC^2/g X^2/d)

Upon integration, and using the initial condition that at t=0, $S=S_0$, the following equation can be derived:

$$\frac{S}{S_0} = \exp\left(\frac{k_s X_m^2}{\mu_m}\left(\ln\left(\frac{e^{-\mu_m t}(1+\beta)}{1+\beta.e^{-\mu_m t}}\right) + \frac{1}{1+\beta.e^{-\mu_m t}} - \frac{1}{1+\beta}\right)\right) \qquad (3\text{-}7)$$

A first order differential, equation (3-8), was also used to model the data. Upon integration, equation (3-9) is obtained:

$$\frac{dS}{dt} = -kt \qquad (3\text{-}8)$$

$$\frac{S}{S_0} = \exp(-kt) \qquad (3\text{-}9)$$

where

k - rate constant for sinapine decrease in first order equation (d^{-1})

These models were solved using the software package Scientist (Micromath).

Chapter 4: Materials and Methods

This chapter describes the experimental procedures used for the completion of this project.

4.1 Microorganism

The white-rot fungus *Trametes versicolor* ATCC 42530 was used in this study for both the submerged and solid state fermentation processes. This is the same microorganism studied by Lacki and Duvnjak (1996a,b,c) for the decrease in phenolic content of canola meal.

4.1.1 Agar Slants

The fungus was maintained on agar slants consisting of (%, w/v): 4.5 malt agar, 0.5 glucose and 0.5 yeast extract and distilled, de-ionized water. The medium was heated to dissolve the agar, after which approximately 5 mL was added to a 23 mL slant tube and covered. The tubes were then autoclaved at 121°C for 20 minutes and placed on a slight incline and allowed to cool overnight prior to inoculating with a piece of culture from a previously-prepared slant. The tubes were placed in an incubator at 30°C until the surface of the slant was covered with mycelium and afterwards stored at 4°C for up to 4 months before preparing new slants.

4.1.2 Inoculum Preparation

The liquid medium for submerged fermentation consisted of (%,w/v): 2 glucose, 0.5 yeast extract, 0.8 nutrient broth and 0.05 Vogel's trace minerals. The pH was adjusted to 5.50 using 2N sulfuric acid. One hundred eighty millilitres of medium were added to a 500 mL Erlenmeyer flask, later sealed with a foam plug and autoclaved for 20 minutes at 121°C. The medium, when cool, was inoculated with one piece of mycelium (ca. 0.25 cm^2) from a slant culture and placed on a rotary shaker at 150 rpm and 28°C for

8 days. The contents of several flasks were combined and homogenized for 30 seconds using a Sunbeam Osterizer 8 blender set on "liquefy". Unless otherwise noted, 5 mL were used to inoculate cultures for solid state or submerged fermentation in 125 or 250 mL Erlenmeyer flasks, while 10mL were used for solid state cultures in 500 mL Erlenmeyer flasks. The Vogel's mineral solution consisted of (%, w/v): 5 citric acid monohydrate, 5 zinc sulfate heptahydrate, 1 ferrous ammonium sulfate hexahydrate, 0.25 cupric sulfate pentahydrate, 0.05 manganous sulfate monohydrate, 0.05 boric acid and 0.05 sodium molybdate.

4.1.3 Fermentation Media

The medium used for submerged experiments was identical to that described previously, unless noted; however the tests were carried out by sterilizing 75 mL of medium in a 250 mL Erlenmeyer flask and using 5 mL of homogenized inoculum. The flasks were placed on a Lab Line orbital shaker at 150 rpm and 28°C. Submerged media containing meal were prepared by adding the desired amount of meal to the Erlenmeyer flask prior to the addition of 75 mL of liquid medium of pH 5.50. The final pH of the solution was recorded, although, the pH was not adjusted due to the buffering capacity of the meals.

The medium for solid-state fermentation consisted of canola meal and water, unless otherwise noted. Sufficient distilled, de-ionized water was added to canola meal to yield the desired moisture content after inoculation. For example, to obtain 60% moisture content in a 125 mL Erlenmeyer flask, 10g CM and 10g water were mixed and autoclaved at 121°C for 45 minutes. After adding 5 mL of inoculum, the correct moisture content is obtained. The moisture content was based on canola meal "as is", *i.e.*, the initial moisture content of approximately 4.6%, was not taken into consideration. If the medium was to be supplemented with Vogel's minerals solution, the minerals were added to the water phase prior to addition to the meal to ensure uniform distribution throughout

the medium. The concentration of Vogel's minerals solution given in the text is the concentration of the minerals in the liquid phase of the culture.

4.2 Fermentation Media Supplements

4.2.1 Effect of Inducers

The inducers tested in this project were 2,5-xylidene (also known as 2,5-dimethylaniline), sinapic acid (3,5-dimethoxy-4-hydroxy cinnamic acid), ferulic acid (4-hydroxy-3-methoxy cinnamic acid) and syringaldizine (4-hydroxy-3,5-dimethoxy benzaldehyde azine).

The 2,5-xylidene solution for induction was prepared according Rogulski *et al.*, (1991): 80 μL of a 0.2M 2,5-xylidene solution, prepared in 50% ethanol, was added to the flask containing 80 mL of inoculated medium after 3 days of growth. This gave a final 2,5-xylidene concentration of 0.2 mM per flask. There has been no published work concerning the use of SA as an inducer, so a concentration similar to that of 2,5-xylidene was used. Due to solubility difficulties, the maximum SA concentration obtainable in the stock solution was 0.05M. As a result, 320 μL were added to the flasks after 3 days to obtain a concentration of 0.2 mM. The ferulic acid induction was done as for 2,5-xylidene: 80 μL of a 0.2 mM solution in 50% ethanol was added to 80 mL of a 3 day culture. Since syringaldazine was not sufficiently soluble in 50% ethanol, 85% ethanol was used and a 1 mM solution was prepared. To a 3 day culture, 400 μL of syringaldazine solution were added, which gave a final concentration of 5μM. Only 400μL of syringaldazine solution were added since the effect of ethanol on *T. versicolor* is not known.

4.2.3 Effect of Meal Components

Other meal components, including sugars, proteins, sinapine (3,5-dimethoxy-4-hydroxy cinnamoylcholine), phenolic-free CM and CM broth equivalent to a 5% solution were also tested for PPO production.

Two additional types of carbon sources, sucrose and cellulose, were also examined for PPO production. Sucrose was added in a concentration of 3.5 g/L and cellulose at a concentration of 2.5 g/L. These were calculated to be the concentrations equivalent to a 5% CM medium. The protein-free, phenolic-free meal, as well as the CM proteins were prepared as discussed in section 4.2.5. The meals were added to medium as discussed in section 4.1.3. The CM proteins were added to the meal at a concentration of 6 or 12 mg/L. It was estimated that the concentration of soluble proteins in a 5% medium would be approximately 6 mg/L.

4.2.4 Aeration

Since different volumes of medium in the same size flask provide different rates of aeration (Al-Asheh, 1993), four different culture medium volumes, 50, 80, 110 and 140 mL in a 250 mL Erlenmeyer flask were used to provide different rates of aeration. The correct mass of CM was added so that, upon addition of medium and inoculum, the flask would still contain a 5% suspension of meal in medium. The amount of inoculum was also scaled so that its initial concentration would be the same in all flasks. The following amounts were used: 50 mL (46.9 mL medium, 2.5 g CM, 3.1 mL inoculum); 80 mL (75 mL medium, 4.5 g CM, 5 mL inoculum); 110 mL (103.1 mL medium, 5.5 g CM, 6.9 mL inoculum); 140 mL (131.25 mL medium, 7 g CM, 8.75 mL inoculum).

4.2.5 Oilseed Meals

Commercial canola and soybean meals were obtained from Ritchie Feed and Seed (Ottawa, Ont). These meals were used on an "as-is" basis and did not undergo any pre-treatment prior to experimentation. Since sunflower and safflower meal could not be obtained locally, these meals were prepared in the lab using seeds. Due to the difficulties in de-hulling, the meal consisted of both hulls and kernels.

Approximately 250 mL of seed were placed in an Osterizer 8 blender and ground on "grind" for 15 seconds then mixed. This was repeated two more times. The ground seeds were removed and a fresh 250 mL sample was placed in the blender. This procedure was repeated until sufficient residue was obtained. A method similar to Rizk (1991) was followed to prepare the meal. Eight hundred grams of ground seeds were added to 2 L of hexane and mixed for 48 hours. The meal was then gravity filtered and 2 L of fresh hexane were added and again mixed for 48 hours. The residual oil content at this point was found to be approximately 10%, so another 2 L of fresh hexane were added and the mixture allowed to stand for 48 hours prior to being vacuum filtered and dried at room temperature in a fume hood. The residual oil content was determined by extracting 25 g of meal with 250 mL of hexane for 24 hours, filtering and drying overnight. The mass loss was attributed to oil extracted from the meal. The residual oil contents of the sunflower and safflower meals were 1.91% and 0.46%, respectively.

The phenolic-free meal was obtained after extraction of CM with cold ethanol during the preparation of sinapine (see section 4.6.1.). This meal was then further extracted with boiling 2% HCl in methanol. The residual SAE content was 0.64 g/kg, compared to the original value of 22 g/kg. The protein-free meal was prepared following the methods of Gillberg and Tornell (1976) and Chen and Rohani (1992). CM was mixed with 0.2 M NaOH in a 1:20 suspension for 2 hours at room temperature. The pH was maintained at 11 by adding NaOH or HCl as required. The suspension was filtered twice through a coarse filter paper, concentrated by centrifugation, re-filtered and dried overnight at 80°C. The proteins were recovered by adjusting the pH of the supernatant to 4 using HCl, centrifuging, collecting on a filter paper and drying overnight at 80°C.

4.3 Enzyme Studies

4.3.1 Enzyme Preparations

The submerged enzyme preparation was prepared according to Lacki and Duvnjak (1996a). A submerged fermentation, using the media discussed previously, was followed

and when the enzyme concentration reached its maximum, several flasks were combined and the culture was centrifuged at 1960 g on a Beckman L7 ultracentrifuge. The filter sterilized (0.45μm) supernatant was then frozen using liquid nitrogen and stored in the freezer until needed.

The solid state enzyme preparation was prepared from a CM medium with moisture content of 60% moisture and 1 mL/L Vogel's minerals solution. The SSF culture was followed until the maximum enzyme concentration was obtained. Several flasks were then combined and a 20% (w/v) suspension of wet culture in distilled, de-ionized water was shaken for 1 h, prior to centrifuging. The supernatant was then filtered through a Whatmann #2 filter (it was not possible to filter through 0.45μm filter), frozen and stored in the freezer until needed.

4.3.2 Enzyme Activity

The enzyme activity was measured spectrophotmetrically using a Beckman DU-640 spectrophotometer. The reaction mixture, containing 2.7 mL citric acid buffer of pH 3.5 (1.344 g citric acid and 0.859 g dibasic sodium phosphate in 200 mL water), 100 μL of enzyme (or an appropriate dilution) and 200 μL of 2 mM ferulic acid, was combined in a cuvette and the loss of absorbance of ferulic acid was followed at 320 nm. The temperature was maintained at 25°C using a Beckman temperature controller.

Since it was observed that the rate of the enzymatic reaction depended upon the dilution of enzyme, the dilution was made such that the loss in absorbance was linear throughout the entire 10 minute duration of the test. This eliminated the need to estimate the end of the linear reaction and was found to give accurate, reproducible results. The dilutions were made in a 10 mL test tube, by adding 100 μL of concentrated enzyme preparation to the desired amount of water. Furthermore, a separate dilution was made for each analysis. For example, if one sample was to be analyzed 6 times, 6 test tubes

would be made since it is believed that this would avoid propagation of any initial dilution errors.

The rate of reaction was calculated according to Equation (4-1):

$$v = 10^6\left(\frac{A_{ini} - A_t}{\Delta t}\right)\frac{1}{\varepsilon}\frac{V_{sys}}{V_{enz}}\left(\frac{1}{60}\right)Dil \qquad (4\text{-}1)$$

where

v - rate of reaction (nkat/mL or nmol/s/mL)
A_{ini} - initial absorbance
A_t - final absorbance
t - length of spectrophotometric test (s)
ε - extinction coefficient (Mol^{-1} cm^{-1})
Dil - dilution factor
V_{sys} - volume in cuvette (3mL)
V_{enz} - volume of enzyme (mL)

The PPO activity during SSF was followed by measuring the enzymatic rate in the culture extract. The activity per gram culture was determined by multiplying the rate calculated using Equation (4-1) by the concentration of culture used for enzyme extraction. For example, if the enzyme was extracted using a 10% slurry (3 g wet culture and 30 mL water), then the activity would be v (nkat/mL)*(30 ml/3 g culture). When required, the moisture content of the culture was used to determine the activity per gram of dry culture.

4.4 Flooding Experiments

In an attempt to speed up the decrease of phenolic content in the SSF process, flooding experiments were performed. This involved removing a sample of the culture and preparing a suspension of culture in meal of either 20% or 40%. A typical solid state fermentation process containing 60% moisture and 10 mL/L Vogel's minerals solution was followed as in the normal procedure. After 3 days, part of the culture was used for flooding. A 20% suspension was prepared by placing 2 g of the culture in a round bottom boiling flask and adding 10 mL of water at 50°C. The 40% was prepared using 2

g of culture but only 5 mL of 50°C water. The system was allowed to react in an incubator at 50°C for the desired amount of time and the reaction stopped by addition of 1 mL of trichloroacetic acid. The samples were placed in an oven at 80°C until most of the water had been removed. The samples were then extracted with acidified methanol and the SAE, SIN and SA contents were determined as discussed in section 4.5.5. Since the residual phenolic contents of the culture were lower as the experiment progressed, larger

larger samples of the culture were needed in order to accurately measure the phenolic content of the sample. For the 5 and 7 day cultures, 3 g and 5 g of culture were used to make the required suspensions.

4.5. Analysis of Samples

4.5.1. Biomass in Submerged Fermentation

An aluminum weigh tin and Whatmann #2 filter paper were weighed. The entire contents of a 250 mL Erlenmeyer flask were filtered through the paper and the biomass rinsed with water. The filter paper and biomass were placed in the tin and dried overnight in an oven at 105°C. The dry weight was then recorded and the dry mass of the biomass was determined using a mass balance. Since the paper was found to lose mass during drying, several papers were treated in a similar manner, with the exception that no biomass was used, and this mass loss was added to the mass of the biomass. This difference was only significant near the beginning of the fermentation due to low levels of biomass.

4.5.2. Biomass in Solid State Fermentation

Due to the presence of the support material (canola meal), it was not possible to measure the biomass directly as was done for submerged fermentation. As a result, the

biomass was measured indirectly by determining the glucosamine content in a SSF sample. The procedure of Sakurai *et al.* (1977) was followed with the modification proposed by Jones and Worrall (1995) as well as being altered as needed for the equipment and materials (i.e. CM) in this laboratory. A sample of wet culture, approximately 2-4 g, was weighed and hydrolyzed overnight in a 30 mL test tube using approximately 2 mL of concentrated sulphuric acid, depending upon the sample size. The sample was then added to a 100 mL volumetric flask using 55 mL of water. The top of the flask was sealed using aluminum foil prior to autoclaving at 121°C for 1 h. After cooling, the pH was adjusted to 7.0 ± 0.2 using 4N NaOH initially, then 1N H_2SO_4 and 1N NaOH for final accuracy. The flask was cooled again (due to heat formation during neutralization) and filled to 100 mL mark with water.

Blix's (1948) method was used for glucosamine measurement. One mL of sample was combined with 1 mL of freshly prepared acetylacetone solution (1.5 mL acetylacetone in 50 mL of 1.25 M sodium carbonate) in a closed 30 mL test tube and its contents heated at 96°C for 1 hour. After cooling in an ice bath, 10 mL of ethanol were added followed by 1 mL of Erlich's reagent (1.6 g *p*-dimethylaminobenzaldehyde in 60 mL of 50:50 ethanol:concentrated HCl mixture). The tubes were allowed to remain undisturbed for 45 minutes to allow the precipitate to settle, and the pink colour of the liquid that formed was measured at 530 nm.

The amount of biomass was originally determined using a calibration curve obtained using pure biomass obtained from a submerged culture. However, since it was found that the amount of glucosamine measured was also a function of the sample size, the amount of biomass was then calculated using Equation (4-2) below. This equation was developed from mass balances using calibration curves for pure biomass and pure canola meal. It has been assumed that the total glucosamine measured is the sum of the

individual glucosamine contents of the meal and biomass and that each can be expressed as a linear function of the absorbance.

$$M_{bio} = \frac{Abs_{530} - .128M_{sample}}{1.184} \qquad (4\text{-}2)$$

where

M_{bio} - Mass of biomass in sample (g)
Abs_{530} - Absorbance at 530 nm
M_{sample} - dry mass of sample (g)

4.5.3. Sugar Analysis

For submerged and solid state fermentations, the soluble sugars were measured using the 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959). For submerged cultures, the filtered liquid phase was analyzed directly, however, if meal was present, the contents were filtered through a multi-purpose filter and the filtrate centrifuged at 5000 g for 15 minutes. The supernatant was then used for analysis. During solid state fermentation, it was necessary to extract the soluble components from the culture using a 10% (w/v) suspension of culture in water. The suspension was prepared by adding exactly 3 g of wet culture into a 40 mL centrifuge bottle followed by 30 mL of water. The contents were mixed on a Fisher shaking water bath model 127 for 1 hour on full speed. The shaker was operated dry and provided reciprocal shaking. The tubes were then centrifuged at 5000 g for 15 minutes and the supernatant used for analysis.

One mL of sample, diluted if the reducing sugar concentration was greater than 2 g/L, was combined with 3 mL DNS reagent in a closed 30 mL test tube. The mixture was boiled exactly 5 minutes then cooled exactly 1 minute in an ice bath. Twenty mL of water were then added and the absorbance at 600 nm was recorded as soon as possible. The blank was prepared using 1 mL of water treated as above. Since it was observed that

the absorbance increased with time, in order to be consistent, approximately 8 sugar samples were analyzed at one time and the length of each step had to be precise.

The DNS reagent was prepared as follows: 5 g of 3,5-dinitrosalicyclic acid was dissolved in 300 mL of water. In a separate flask, 1 g phenol, 0.25 g sodium bisulfite, 100 g sodium potassium tartrate and 5 g sodium hydroxide were dissolved in 200 mL water. The second solution was added to the first and the mixture was stored in a sealed, brown bottle covered with aluminum foil to avoid exposure to light

The reducing sugars were measured directly using the DNS method and compared to a standard curve prepared using glucose. The soluble, non-reducing sugars were first hydrolyzed, then measured using the DNS method with the same standard curve as for glucose. This curve was also verified using a standard curve prepared using sucrose. To hydrolyze the sample, 0.5 mL of a sugar sample, containing up to 10 g/L glucose equivalents, was boiled with 1 mL of 1N HCl in a closed tube for 15 minutes. After cooling, 1 mL of 1N NaOH was added. One mL of this mixture was then used for glucose measurement using the DNS procedure. It should be noted that 1 mole of sucrose will yield 1 mole of glucose and 1 mole of fructose, hence the absorbance will be double. However, since the molecular weight of sucrose is approximately double that of glucose (with the exception of one water molecule), the concentration, in g/L, is consistent.

4.5.4. pH

The pH of the submerged cultures was measured directly using a Fisher Accumet pH meter model 805 MP. For solid state cultures, it was not possible to measure the pH directly since there was no liquid phase. To overcome this, the pH was measured in the supernatant of the 10% suspension prepared as discussed in section 4.5.3.

4.5.5. Sinapic Acid Ester Content

The sinapic acid ester (SAE) content of the sample was determined by first extracting them from the CM or CM culture with acidified methanol followed by analysis using Austin's method (Austin and Wolfe, 1968) with the modification proposed by Mueller (Mueller *et al.*, 1978). The procedure used was identical to that described by Lacki and Duvnjak (1996c).

Approximately 0.5 g dry CM or 1-4 g of culture, depending upon its age and moisture content, were extracted using 25 mL of 2% 1N HCl in methanol in a 100 mL boiling flask, with condensers, in a water bath at 90°C, for 30 minutes. After cooling, the methanol was decanted into a 75 mL test tube and capped. Twenty-five mL of fresh acidified methanol were added to the boiling flask and the extraction procedure repeated. After decanting into the same test tube, approximately 12.5 mL of fresh acidified methanol were added and the flask boiled for 30 minutes. After decanting into the test tube, the volume of the combined extracts was measured and the SAE content determined as described below.

Two mL of acidified methanol were placed in a 3mL glass cuvette and the spectrophotometer blanked at 400 nm. Depending upon the concentration of phenolics in the extract, between 50-200 µL of the combined extracts were added and the initial absorbance measured ($A_{initial}$). One mL of 1N NaOH was then added and mixed and the absorbance (A_{final}) was immediately recorded at 400 nm. The concentration of SAE was calculated based on the differences in absorbance since sinapine produces a yellow colour in alkaline solutions. Sinapine bisulfite was used to prepare the standard curve.

$$SAE = \frac{(A_{initial} - \frac{2}{3}A_{final})}{\varepsilon}\left(\frac{2.0}{V_{ext}} + 1\right)\frac{V_{MeOH}}{M_{CM}}(1 + x) \qquad (4\text{-}3)$$

where

SAE - SAE content of the sample (g/kg)
$A_{initial}$ - initial absorbance
A_{final} - final absorbance
- slope of standard curve (0.03481 mL/ g)
v_{ext} - volume of the methanol extract (mL)
V_{MeOH} - total volume of methanol used for extraction (mL)
M_{CM} - mass of canola meal used for extraction (g)
x - moisture content of the sample (g/g)

The SIN and SA contents of the methanol extracts were measured using the procedure of Lacki and Duvnjak (1996c). The separation was done using a Waters HPLC consisting of a Waters 717 autosampler, a Waters 486 tunable absorbance detector, a Waters 410 solvent-delivery pump and a Waters 660 system controller. The temperature of the separation was 50°C and the wavelength was 328 nm. A C-18 Bondapak HPLC column (3.9 x 300mm) was used at a flowrate of 1 mL/min of solvent. The mobile phase consisted of solvent A (0.005 M di-n-butylamine and 0.005 M heptanesulfonic acid modified with 50% acetonitrile at pH 2.4) and solvent B (0.01 M di-n-butylamine and 0.01 M heptanesulfonic acid at pH 2.4) The composition of the mobile phase was changed linearly for 30 minutes from 2:8 to 7:3 solvent A to solvent B followed by 10 minutes of equilibration at the initial conditions.

4.5.6. Moisture Content

The moisture content of the sample was determined by drying at 105°C overnight. For solid state fermentation, approximately 25 g of wet culture were placed in an aluminum weighing dish and its mass recorded. Initially, the samples were dried to constant mass and the moisture content was calculated based on the mass loss, taking into consideration the weight of the dish. After several experiments, it became apparent that drying overnight was sufficient. As a result, the tin and sample were dried overnight in an oven at 105°C and the moisture content calculated as above.

4.6. Chemicals

The list of chemicals used for this project, as well as the suppliers can be seen in Table 4.1. Sinapine, since not commercially available, was prepared in the lab by extraction from canola meal.

4.6.1. Preparation of Sinapine Bisulfate

Sinapine was extracted from the canola meal as sinapine thiocyanate and purified as sinapine bisulfate according to Clandinin (1961). Five hundred grams of canola meal were de-fatted using 2 L of trichloroethylene overnight, with stirring and condensers at room temperature, prior to filtering and air drying in the fumehood. Approximately 60-80 g of de-fatted meal were extracted with 400 mL of ethanol for 8 hours in a soxhlet extractor. A new sample of de-fatted meal was used with fresh ethanol and the procedure repeated until all the meal was extracted. The ethanol extracts were combined and concentrated to a syrup in a rotary evaporator at 40°C, under vacuum. Distilled water was then added to the syrup to a volume of 500 mL prior to adding 100 mL of a 20% KSCN solution. The mixture was stored in the refrigerator, without disturbing for 48 hours, prior to collecting the sinapine by centrifuging at 1250 g for 20 minutes and decanting the supernatant. The wet crystals were dissolved in 200 mL of hot ethanol and the solution refrigerated for 24 hours. The sinapine thiocyanate crystals that formed were collected on a filter paper and dried overnight in a desiccator.

To transform the sinapine thiocyanate crystals into sinapine bisulfite, the crystals were dissolved in 110 mL of hot ethanol and 1 mL of concentrated sulfuric acid was slowly added. The solution was allowed to stand overnight in the refrigerator prior to collecting on a filter paper. The crystals were then dissolved in 85 mL of room temperature water and again filtered, under vacuum, to remove any particulates. One mL of sulfuric acid was again added and the crystals were allowed to form during 24 hours in

the refrigerator. The crystals were then collected on a filter paper and re-dissolved in a minimum amount of hot ethanol. After a final refrigeration, the crystals were collected and dried in a desiccator over $CaCl_2$. The purity of the sinapine was established visually and verified using the HPLC.

Table 4-1: List of chemicals used during the course of this research project[a]

A	Acetonitrile (EM Science); Acetylacetone (Sigma)
B	Boric acid (Fisher);
C	Cellulose (Sigma); Citric acid monohydrate (Sigma); Citric acid anhydrous (sigma); Cupric sulfate pentahydrate (BDH);
D	Dibasic sodium phosphate (Sigma); Di-n-butylamine (Sigma); 3,5-dinitrosalicyclic acid (Sigma); p-Dimethylaminobenzaldahyde (Sigma)
E	95% Ethanol (UO); Erlich's reagent (Sigma);
F	Ferrous ammonium sulfate hexahydrate (Sigma); Ferulic acid (Sigma);
G	Glucose (BDH); D(+)-Glucosamine hydrogen chloride (Sigma)
H	Heptanesulfonic acid (Sigma); Hexane (UO); Hydrochloric acid (BDH);
M	Malt agar (Difco); Manganous sulfate monohydrate (BDH); Methanol (EM Science)
N	Nutrient broth (Merck);
P	Phenol (BDH); Potassium thiocyanate (Sigma)
R	D(+)-Raffinose (Sigma)
S	Sinapic acid (Sigma); Sinapine (this work); Sodium bisulfite (BDH); Sodium Carbonate (Sigma); Sodium hydroxide (BDH); Sodium molybdate dihydrate (BDH); Sodium potassium tartrate (Sigma); Sucrose (Loblaws); Sulfuric acid (BDH); Stachyose (Sigma); Syringaldazine (Sigma)
T	Trichloroacetic acid (Sigma); Trichloroethylene (Fisher)
X	2,5-xylidene (Aldrich)
Y	Yeast extract (Difco)
Z	Zinc sulfate heptahydrate (Sigma)

[a] the name of the supplier is given in brackets: Aldrich- Aldrich Chemical Company Inc., Milwauke, WI., USA; BDH- BDH Chemicals Canada Ltd., Toronto, ON., Canada; Difco- Difco Laboratories, Detroit MI., USA; EM Science- EM Science Gibbstown, NJ., USA; Fisher- Fisher Scientific, Ottawa, ON., Canada; Sigma- SIGMA Chemical Company, St. Louis, MO., USA; UO- UO chemistry stores, Ottawa, ON., Canada

Chapter 5: Submerged Fermentation

The two types of fermentation examined in this thesis are submerged and solid state fermentation. The main goal of this chapter is to examine the effect of various parameters on polyphenoloxidase production. Chapter 6 will deal with the solid state process including the decrease in the phenolic content of canola meal.

5.1 Growth of Trametes versicolor in the Control Medium

The initial concentrations for the various media constituents were almost identical to those used by Lacki and Duvnjak (1996a) and Lacki (1997) since this was determined to be the optimum conditions for growth and enzyme production. The one exception was that the concentration of Vogel's minerals solution used in this project was 0.5 mL/L instead of 5 mL/L used in the previous study. The reason for this, as will be discussed later, was that the lower level was found to provide even greater levels of PPO.

Figure 5-1 illustrates a typical submerged process for PPO production by this fungus using the control conditions. This experiment has been performed in duplicate and the results averaged to provide error bars. At the first sampling time of approximately 2 days, there is already some biomass produced, so there does not appear to be a significant lag phase. The fungus is in the exponential growth mode up until 8 days, at which point the growth slows before reaching a maximum biomass concentration of approximately 6 g/L. Both the maximum level and the shape of the biomass curve are consistent with the results obtained by Lacki (1997).

The glucose concentration initially exhibits a slow decrease due to the low levels of biomass present. However, as the amount of biomass increases, so does the rate of glucose consumption. At 6 days the glucose level has reached its lower limit. The fact that the glucose concentration does not reach zero is most likely an artifact due to the golden colour of the medium. Since the glucose concentration is determined colormetrically, by reacting a

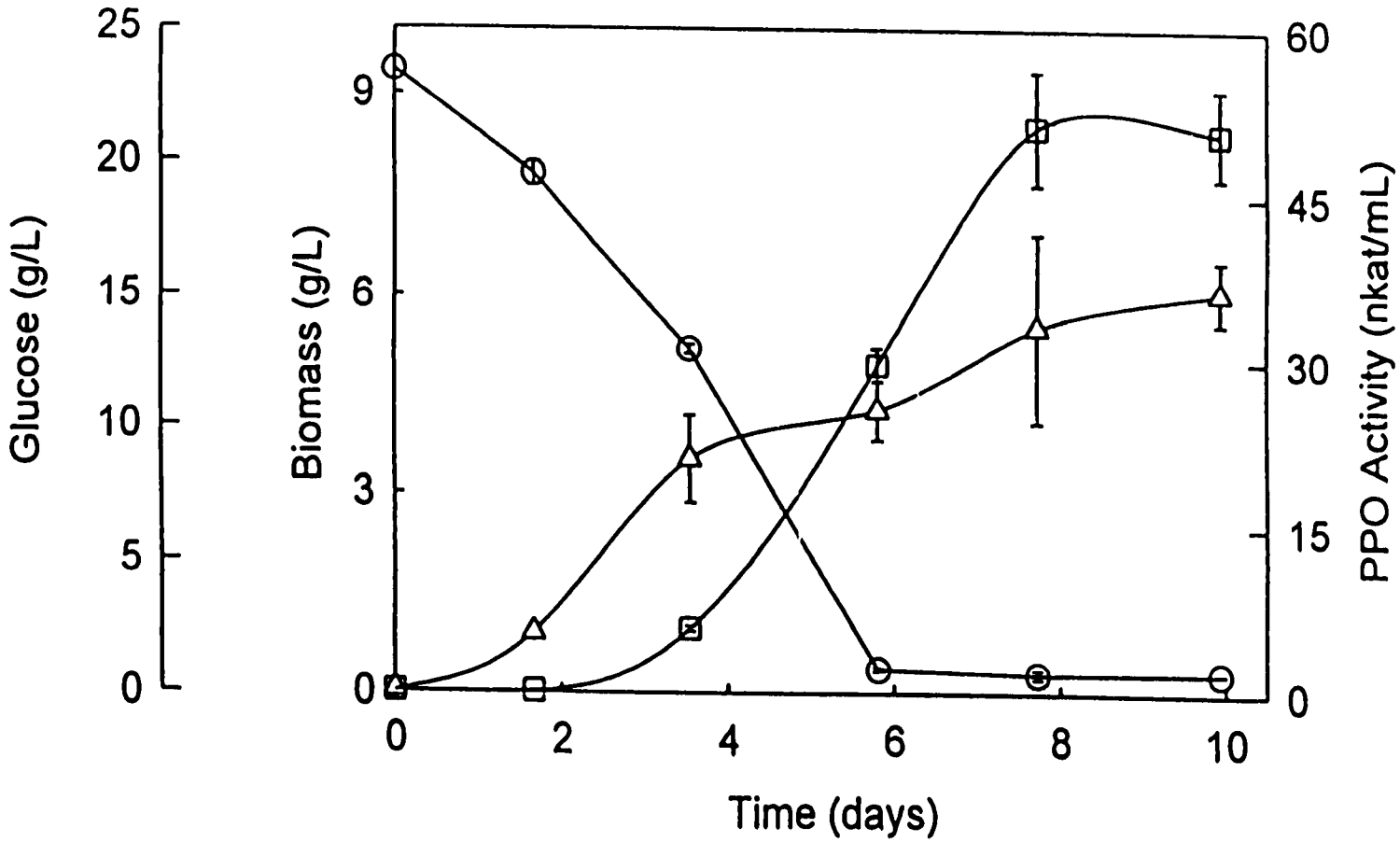

Figure 5-1: Production of PPO by *T. versicolor* in a submerged process in the control medium; (O)-glucose; (Δ)-biomass; (□)-PPO

sample of the medium and measuring the yellow-orange colour formed, it is likely that the golden colour of the fermentation medium interferes with the analysis. It can be assumed that after 6 days there is no glucose present.

The PPO activity does not begin to appear until 2-3 days. However there are initially very low levels due to transfer with the inoculum, however these are not detectable. After 3 days, the PPO production begins to increase quickly for another 5 days. During this time, the fungus is in the exponential growth phase since the biomass concentration is increasing exponentially. The activity of the culture reaches a plateau around 8 days. As a result, it is evident that PPO production by this culture, under the conditions studied, is parallel to biomass growth. This was also reported by Szklarz *et al.* (1989) for a newly isolated *T. versicolor* strain. In both cases, the maximum PPO activity coincided with maximum biomass levels. Furthermore, it should be noted that Szklarz *et al.* (1989) also compared this newly isolated fungus with the same strain that had been cultured in their laboratory. It was found that the cultured strain grew at a much faster rate than the newly isolated strain. This is most likely due to the fact that the cultured strains have been acclimatized to glucose and growth in a similar media while the newly isolated strain would most likely have been acclimatized to using more complex carbon sources, such as polysaccharides, and may require additional time to adapt to the synthetic media.

5.2 Effect of 2,5-Xylidene on the Growth of T. versicolor and the production of PPO in Submerged Cultures

2,5-xylidene is one of the best known inducers for laccase production in *T. versicolor*. Since this enzyme will also react with ferulic acid, the substrate used to measure PPO activity, it will be discussed as PPO, unless noted. Figure 5-2 shows the effect that this compound had on PPO. The production of this enzyme was induced on the third day by adding 80 μL of a 0.2M 2,5-xylidene solution in 50% ethanol, yielding a final 2,5-xylidene concentration of 0.2 mM in the medium (Fahraeus and Reinhammar, 1967). This

experiment was performed in duplicate and the average of two flasks, plus the standard deviation, is presented. For some data points, it appears that the error bars are not present, however, this is only due to very small standard deviations (i.e. smaller than the symbol width). It was decided to add the inducer on the third day since it is toxic to the biomass at their initially low levels. In addition, induction after three days is the procedure most often used by other researchers.

Upon addition of the inducer, the activity of the culture immediately increases to about 4 times that of the control, 42 nkat/mL vs 11 nkat/mL. The production curve continues to increase with the same shape of the control and achieves a maximum level of 85 nkat/ml, which is 1.6 times that of the control. A similar pattern was observed by Bollag and Leonowicz (1984) and Lacki (1997), however there were differences in the magnitudes of increase. In the first case, the difference was about 4.6 times, whereas in the second, the difference was only 2.5 times. Rogulski *et al.* (1990) also found a 3 times increase when inducing laccase in a 10 day culture with 2,5- xylidene, however the growth curves were not presented. Lacki (1997) observed PPO activities of 100 nkat/ml in the induced cultures and 40 nkat/mL in the control, which are similar to those obtained here. Unfortunately, it is impossible to compare the results of Bollag and Leonowicz (1984) with the activities obtained in this study since a different substrate, syringaldazine, was used for measuring enzymatic activity. It should be noted that the activity obtained in the control culture of Lacki (1997) was lower than that obtained in this study. This is most likely due to the method of enzyme assay and, as discussed in greater detail in section 4.3, is related to the dilution of the enzyme prior to analyzing spectrophotometrically.

Since the addition of 2,5-xylidene promotes the formation of laccase, as opposed to PPO, the initial fast increase in enzyme activity is due to laccase production. Since the initial increase in activity of 31 nkat/mL is almost the same as the final difference in activity (35 nkat/mL), and since, after 4 days, the activity increases parallel to the PPO activity of the control, it is likely that the inducer promotes the rapid formation of laccase, independent

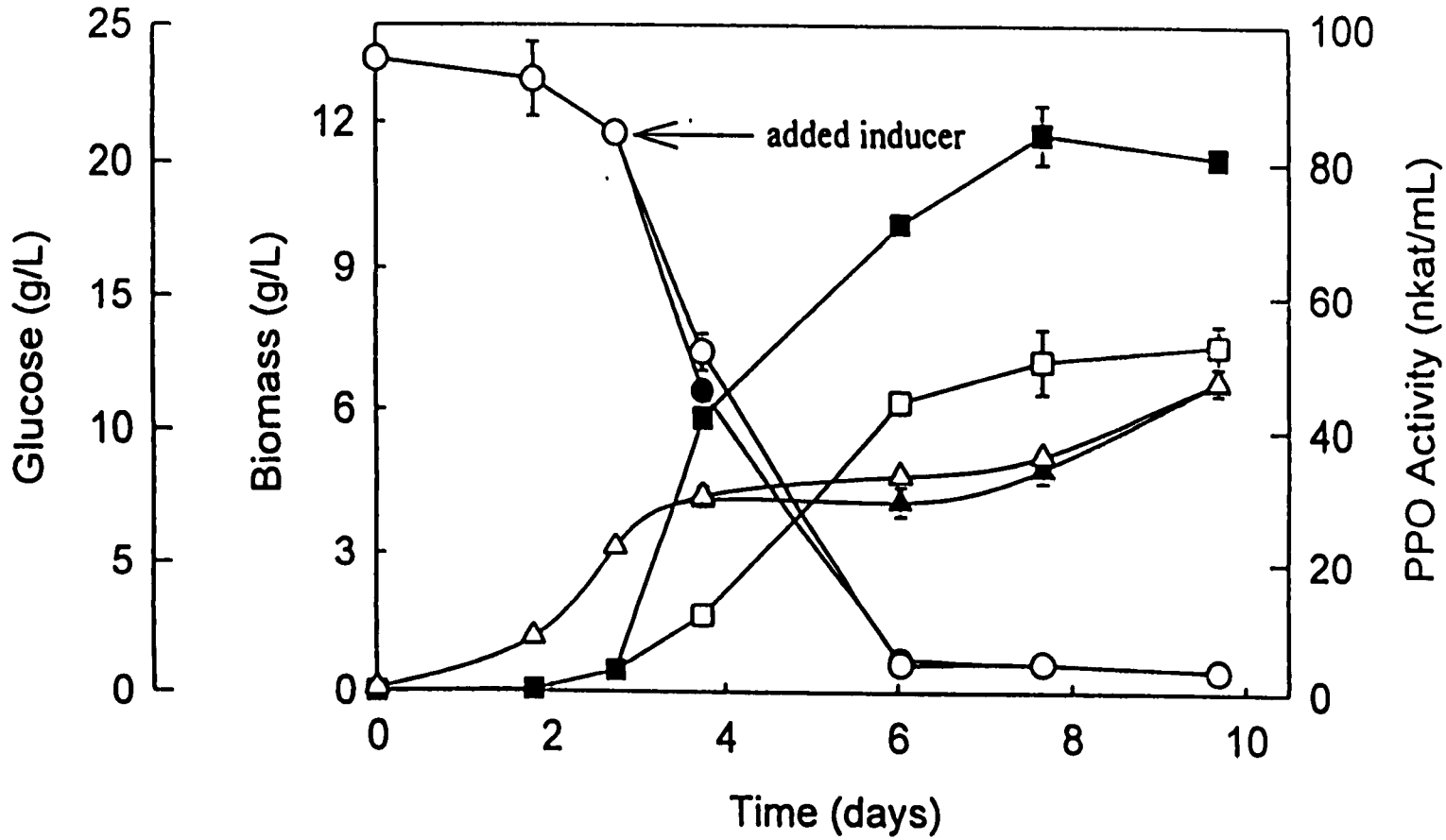

Figure 5-2: Effect of 2,5-xylidene on glucose consumption and biomass and PPO productions in a submerged process; open symbols denote control culture without inducer,closed denote cultures with 2,5-xylidene as inducer; (O)-glucose; (Δ)-biomass; (□)-PPO

of the production of PPO. This would imply that the inducer acts to promote formation of a different enzyme without affecting the constitutive PPO production. Bollag and Leonowicz (1984) detected one constitutive enzyme in the culture without inducer (molecular weight of 70000 Daltons) but two in the culture with inducer (70000 and 61000 Daltons), which would support this hypothesis. Unfortunately, since the induced culture of Bollag and Leonowicz (1984) was induced a second time, it was not possible to verify that the final differences in enzyme activity was equal to the initial increase in laccase activity due to induction.

It can be seen that the rate of glucose consumption increases slightly due to the addition of the inducer. Although this is contrary to Lacki (1997), this phenomenon was also seen in two additional experiments (Figure 5-8 and one additional experiment, data not shown). It has been shown by other researchers that the addition of glucose at the time of 2,5-xylidene induction increases the rate of production of laccase (Farhaeus and Reinhammar, 1967). Furthermore, Kirk *et al.* (1976) demonstrated the need for a carbohydrate source as a growth substrate for the degradation of lignin (i.e., production of lignolytic enzymes such as laccase and PPO). As a result of these findings, it is likely that the slight increase in glucose consumption is a consequence of the production of additional laccase activity.

There appears to be a slight decrease in the biomass concentration after adding the inducer, which could indicate that this compound, or the ethanol added with the7 inducer solution, is toxic to the culture. According to Fahraeus and Reinhammar (1967), 2,5-xylidene and other compounds of this type are toxic to fungi. It can then be concluded that the inducer, although immediately stimulating laccase production, was slightly inhibitory towards biomass growth. However, by the end of the fermentation, both cultures had the same amount of biomass. It should be noted that the biomass in the cultures with the inducer turned pink whereas the control cultures remained white. This pink taint to the biomass could indicate that the 2,5-xylidene was transformed into a compound which was

not further degraded. It is possible that this product, and not 2,5-xylidene, is the compound responsible for laccase production. This would be consistent with previous studies that found the optimum time for induction was from 3-10 days. Farhaeus and Reinhammar (1967) assumed that the reason for delaying the induction time was to allow for sufficient biomass to be present, due to toxicity effects. However, it is possible that the reason to delay addition of the inducer is due to the time required to synthesize the enzymes required for degradation of 2,5-xylidene into the component responsible for induction. In either case, it is evident that the addition of 2,5-xylidene induces the production of laccase.

5.3 Effect of 5% Canola Meal on the Growth of T. versicolor in Submerged Cultures

The use of canola meal has been shown to stimulate the production of cellulase and xylanase in submerged fermentation (Gattinger *et al.*, 1990). It was found that CM could be used in suspensions up to 8% before becoming too viscous (ibid). To examine the effect of CM on the production of PPO, a 5% (w/v) suspension was prepared by adding 4 g of CM to 75 mL of culture medium, sterilizing, then adding 5 mL of inoculum. Duplicate flasks were averaged and the results are presented in Figure 5-3.

The initial rates of formation of PPO are similar for both the control (0% CM) and that with 5% CM added. However, at 6 days, the production in the CM culture increases much more rapidly and to a greater extent. The CM culture has a maximum activity of 2.2 times that of the control. It was not entirely unexpected that the complex CM media would stimulate enzyme production. Fahraeus (1952) also observed a rapid increase in laccase production in a complex, natural extract medium. In that case, the culture containing malt extract also reached a maximum at 8 days. In a synthetic medium, with glucose as a carbon source, there was no activity until 9 days and the maximum occurred at 28 days (ibid). Furthermore, in that study, the maximum laccase activity was actually greater in the synthetic medium. However, it should be noted that the base media used were very different from one another. As a result, it is not known what influence adding the malt extract to the synthetic medium might have had on PPO production.

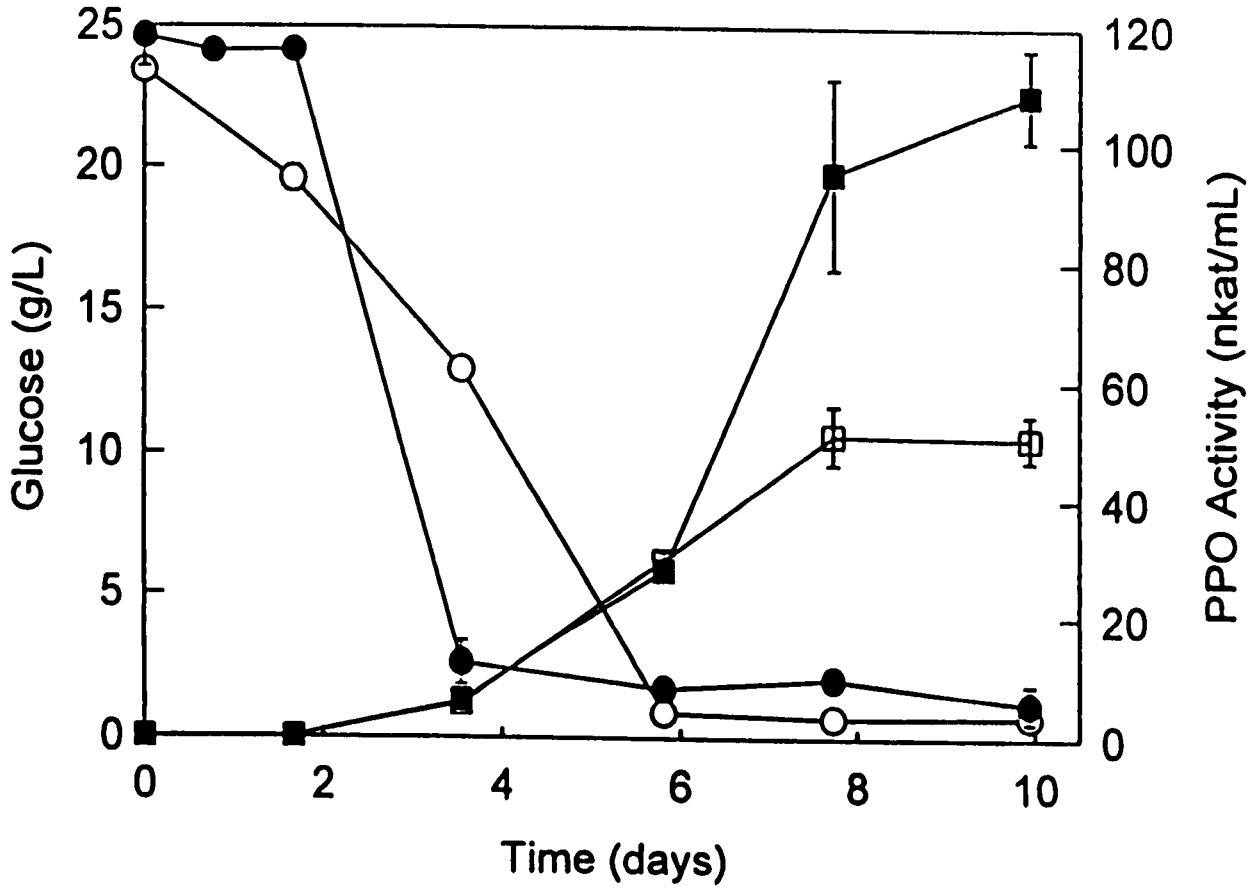

Figure 5-3: Effect of addition of 5% canola meal on glucose consumption and PPO production in a synthetic medium by a submerged process; open symbols denote control, closed denote 5% CM suspension; (O)-glucose; (□)-PPO

It would appear from Figure 5-3 that the glucose consumption in the CM media was initially much slower than in the control media, which could indicate an inhibitory influence of CM. However, this was not the case. The glucose concentration initially remains constant due to the fact that CM contains other non-reducing sugars, sucrose, raffinose and stachyose (Table 5-1), which are not detected by the method of analysis. *T. versicolor* produces the enzyme invertase (Lacki, 1997), which hydrolyzes sucrose into glucose and fructose. As a result, when the CM culture is inoculated, some of these enzymes are transferred into the CM medium and the invertase enzymes begin to hydrolyze these sugars and produce glucose and fructose, both of which are detected by the DNS method of sugar analysis. This means that while the fungus consumes both glucose and fructose, the overall level of reducing sugars remain approximately constant since more reducing sugars are being produced from the non-reducing sugars. Although the initial rate of consumption may be a bit slower initially, possibly due to lower aeration (Al-Asheh, 1993), the overall rate of glucose consumption is much higher in the CM system since it can be seen that the glucose is exhausted much earlier than the control. The final measured glucose levels are higher in the CM cultures than the control cultures. This is an artifact of the procedure due to the brown colour of the CM cultures, compared to the golden yellow colour of the CM-free media. The CM cultures would have more interference when measuring the glucose colourimetrically. It should be noted that the final glucose levels were not known exactly, but were presumed to be zero since glucose is a completely fermentable sugar.

In both systems, the PPO activity does not begin to increase dramatically until the glucose levels are much lower. This is consistent with the work of Rogulski *et al.* (1990), which suggested the optimum time for induction (for rapid increase in enzyme production) was after the majority, at least 70%, of the glucose had been consumed. At this point, there is still some sugar available for production of enzyme, but the growth of the fungus will not be inhibited by the high glucose levels.

Since there has been no evidence in the literature concerning the presence of 2,5-xylidene in CM, or the use of CM for PPO production, it was decided to examine various CM components in hopes of finding the compound(s) responsible for stimulating the

Table 5-1: Soluble sugars extracted from a 5% (w/v) suspension of CM in water

Sugar	Percent ± standard deviation
sucrose	6.97 ± 0.13
raffinose	0.85 ± 0.10
stachyose	2.57 ± 0.07

production of PPO. The enhanced production of PPO in the presence of CM has been considered as stimulation, not induction, since CM is most likely not specific for PPO production but has a variety of influences which lead to enhanced PPO activity.

5.4 Effect of Initial SA on the Growth of T. versicolor in Submerged Cultures

Since 2,5-xylidene is a substituted benzene, and it is known that phenolics can promote the production of laccase, it seems logical to first examine the effect of the most abundant phenolic acid in CM on PPO production. Figure 5-4 presents the process results for a medium containing an initial SA concentration of 0.2 mM. This value corresponds to the concentration of 2,5-xylidene that was used as an inducer in section 5.2. Again, the results are presented as average plus standard deviation of the two flasks for each medium.

When the inoculum, containing a very low level of PPO, was added to the culture, the medium turned bright red within 2 hours, due to the enzymatic transformation of SA into dehydroxydisinapic acid dilactone, DAD (Lacki, 1997). This colour lasted a few hours and completely disappeared by the following morning. This indicated a further transformation of the DAD. The enzymatic degradation of SA by PPO has been examined quite extensively by Lacki (1997) and will not be discussed here. The final colour of the

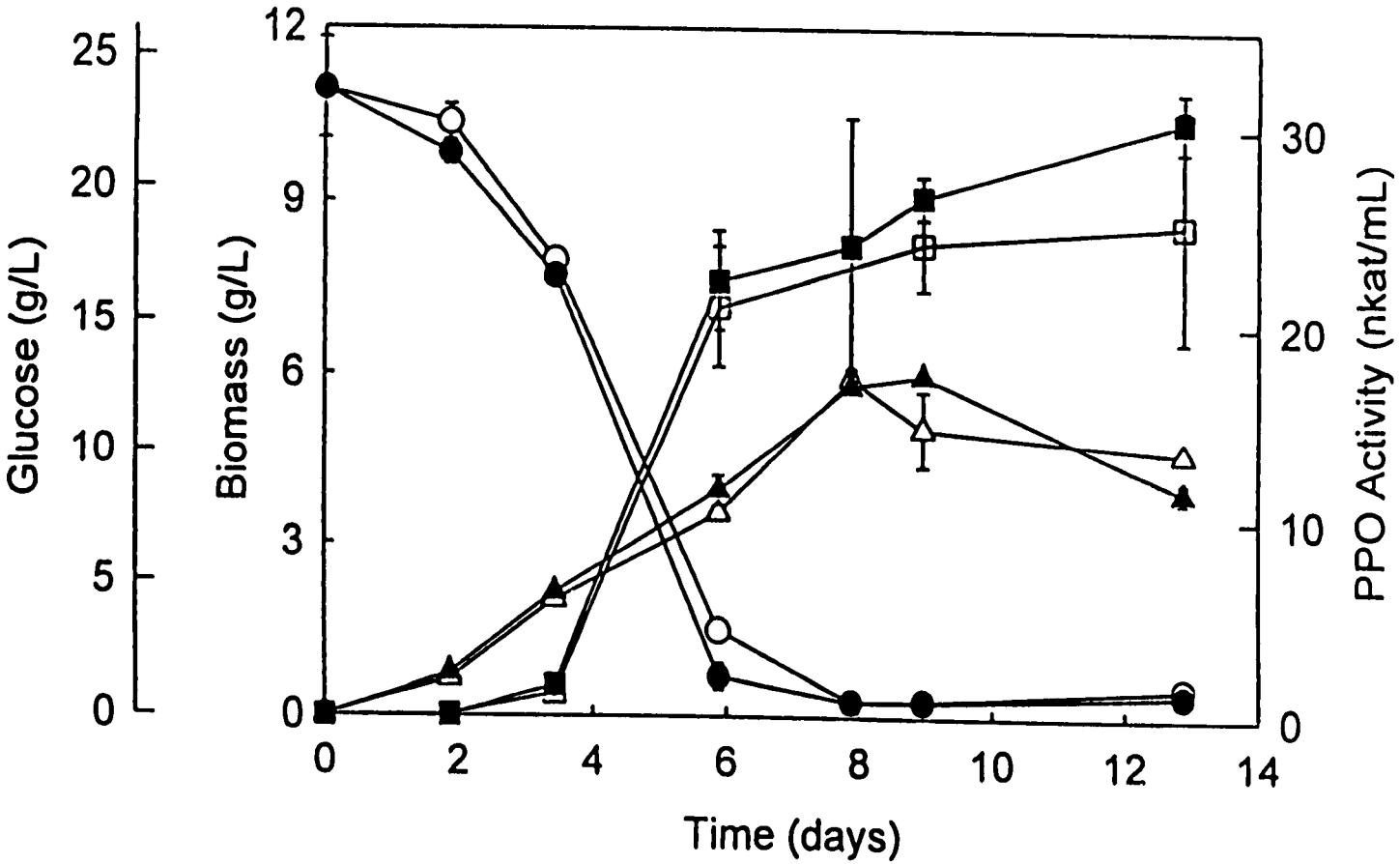

Figure 5-4: Effect of sinapic acid on biomass growth, PPO production and glucose consumption in a submerged process; open symbols denote control, closed denote SA cultures; (O)-glucose; (Δ)-biomass; (□)-PPO

biomass in both cultures was white, which differed from the pink colour for the cultures containing 2,5-xylidene.

It can be seen that the SA medium had a slightly faster rate of decrease in sugar concentration, indicating that SA or its degradation products may stimulate glucoseconsumption. At the same time, it is evident that the SA cultures also had a slightly higher biomass growth rate, although not to any great extent. This is consistent with the work of Buswell and Eriksson (1994) which found a 4% increase in *T. versicolor* biomass levels at a SA concentration of 1 mM on agar plates using glucose as the carbon source.

The rates of production of PPO are similar for both cultures, with the SA medium providing slightly higher levels of PPO. This could mean that one of the degradation products of SA can stimulate PPO production, although not to any significant extent compared to 2,5-xylidene. The enzyme activity is lower in this experiment than expected and is believed to be the result of the method used for enzyme measurement. As mentioned in section 5.2 and discussed in 4.3.2, the method initially used for the enzyme assay underestimates the enzyme activity. To correct for this in later experiments, higher dilutions were used when measuring the enzyme activity of these samples. This gave higher and more accurate values for the activity. However, since the method of analysis for both cultures was consistent, the results are acceptable for comparison.

5.5 Effect of SA as an Inducer on the Growth of T. versicolor in Submerged Cultures

Due to the fact that SA showed a slight, positive effect on PPO production when it was added in the beginning of the process, it was decided to examine if it would have a greater influence if added at a later stage. When the medium initially contained SA, it was transformed before the biomass began to grow, due to the PPO transferred to the culture during inoculation. However, when added as an inducer, it is added when the biomass is growing. Furthermore, it is likely that since the majority of the SA contained in CM is in

the form of sinapine, SA would be released much more slowly and would be present during the growth phase when using CM cultures.

Figure 5-5 shows the effect of SA added after 3 days of growth on PPO production and glucose consumption. The values presented are the averages and standard deviations of two flasks of each culture medium. Due to the low solubility of SA, a 0.05M solution in 50% ethanol was prepared. After addition of 320 μL to the culture, the SA concentration was 0.2 mM, as used for the induction with 2,5-xylidene. Within 15 minutes of addition, the culture medium had turned a bright red, however by the next morning it was also back to its normal, golden colour.

It can be seen that both types of cultures had the same rate of sugar consumption, however the SA cultures had higher PPO levels. With the exception of the data at 6 days, it is clear that the PPO activity is higher in the induced cultures than in the control culture. As a result, it can be stated that SA, as an inducer, had a slightly positive effect on PPO production, though, not nearly as great as 2,5-xylidene.

Upon addition of the inducer to the culture, biomass production initially decreased compared to the control, although, the final biomass levels were identical. This finding is consistent with the results observed during the induction with 2,5-xylidene. It is possible that either the compounds (SA and 2,5-xylidene) are toxic initially, or that the addition of ethanol initially inhibits biomass production.

5.6 Effect of Phenolics on the Growth of T. versicolor in Submerged Cultures

Several phenolics were examined for their ability to induce PPO production in *T. versicolor*. Sinapic acid and 2,5-xylidene were again used, however, ferulic acid and syringaldazine were also chosen since they have both been used for the induction of laccase (Leonowicz and Trojanowski, 1975; Skorobogat'Ko *et al.*, 1996). In addition, ferulic acid is contained in low levels in CM (Dabrowski and Sosulski, 1984) and syringaldazine has

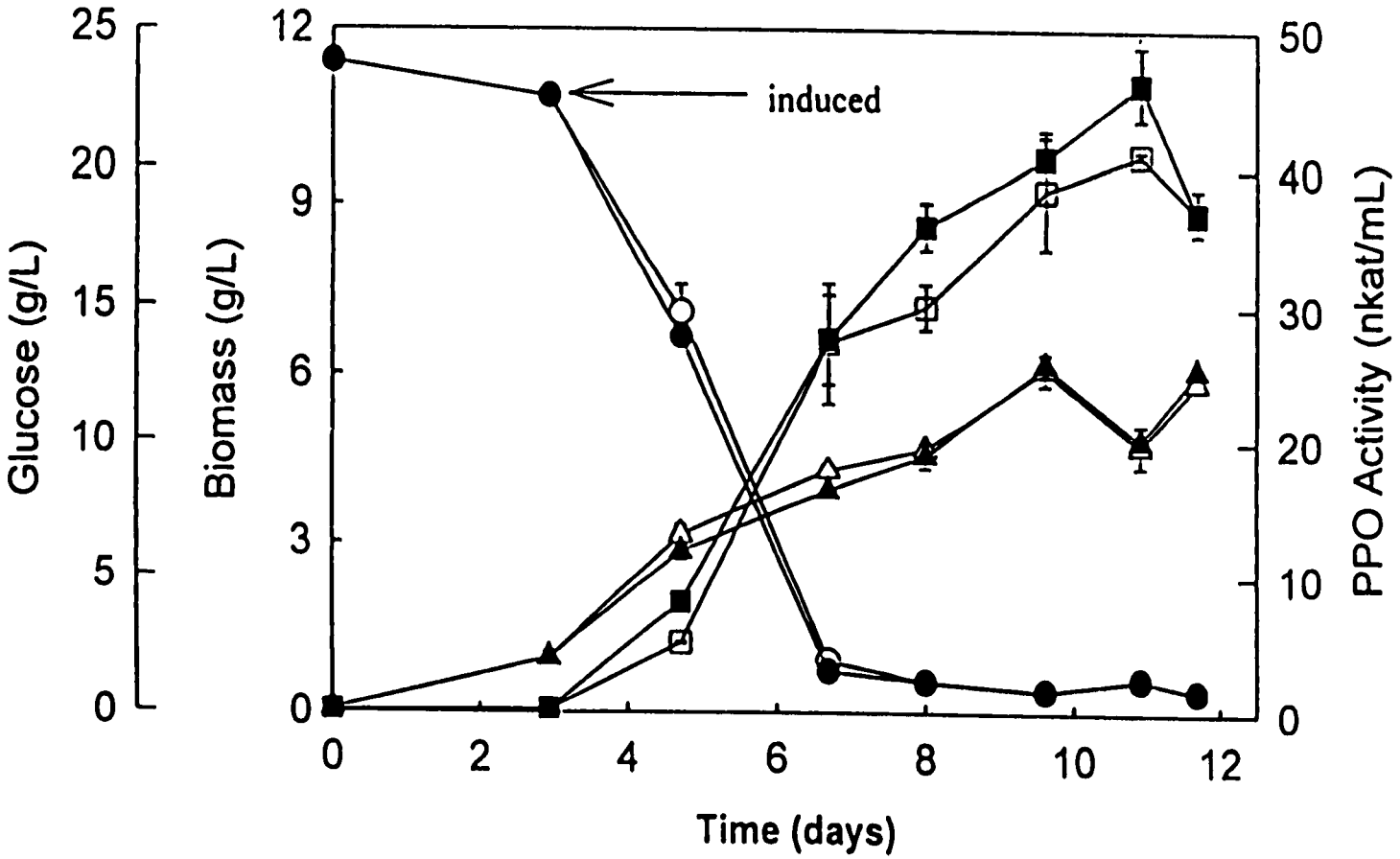

Figure 5-5: Effect of sinapic acid on biomass growth, PPO production and glucose consumption in a submerged process; open symbols denote control without inducer, closed denote cultures induced with SA; (O)-glucose; (Δ)-biomass; (□)-PPO

also been used as a substrate for measuring laccase activity (Harkin and Obst, 1973; Leonowicz and Grzynowicz, 1981)).

Upon addition of syringaldazine, the culture became red immediately; however contrary to what was observed for the SA and 2,5-xylidene cultures, the colour began fading after 2 minutes and soon returned to its original colour. Upon addition of the ferulic acid, the colour of the culture did not change. This is consistent with known information on these species: SA turns red due to the formation of DAD (Lacki, 1997). Syringaldazine becomes red due to the formation of a quinone moiety and is the basis for an enzymatic method to assay for laccase activity (Leonowicz and Grzynowicz, 1981). The decay of ferulic acid results in the loss of absorbance around 320 nm, however there are no further increases in absorbance in the visible wavelength (data not shown).

2,5-xylidene was again the best inducer, with a maximum activity of almost 3 times the control (Figure 5-6). This is higher than observed in section 5.2, however, the difference can be attributed to the more accurate PPO activity measurement. This value is consistent with the values of 2.5, 3 and 3 times obtained by Lacki (1997), Rogulski *et al.* (1990) and Bollag and Leonowicz (1984), respectively.

The next best inducer was SA and resulted in a maximum increase in PPO activity of 24% at 8 days. This is slightly higher than obtained earlier, but was again due to the enzyme assay method. Since this experiment was performed in single flasks, in order to test a greater number of phenolics, there may be some errors. For example, there is not a large difference in activity between the 10 or 12 day cultures. Furthermore, the value at 6 days seems low since the dip in activity is not consistent with the other phenolics or with what seen in the previous section.

The third best inducer tested was syringaldazine. Due to solubility difficulties, the final concentration of this compound was only 0.005mM, compared to 0.2 mM used for the other phenolics. Induction with this compound has been found to increase the laccase production in *Coriolus hirstutus* by 1000% (Skorobogat'Ko *et al.*, 1996). However, for the

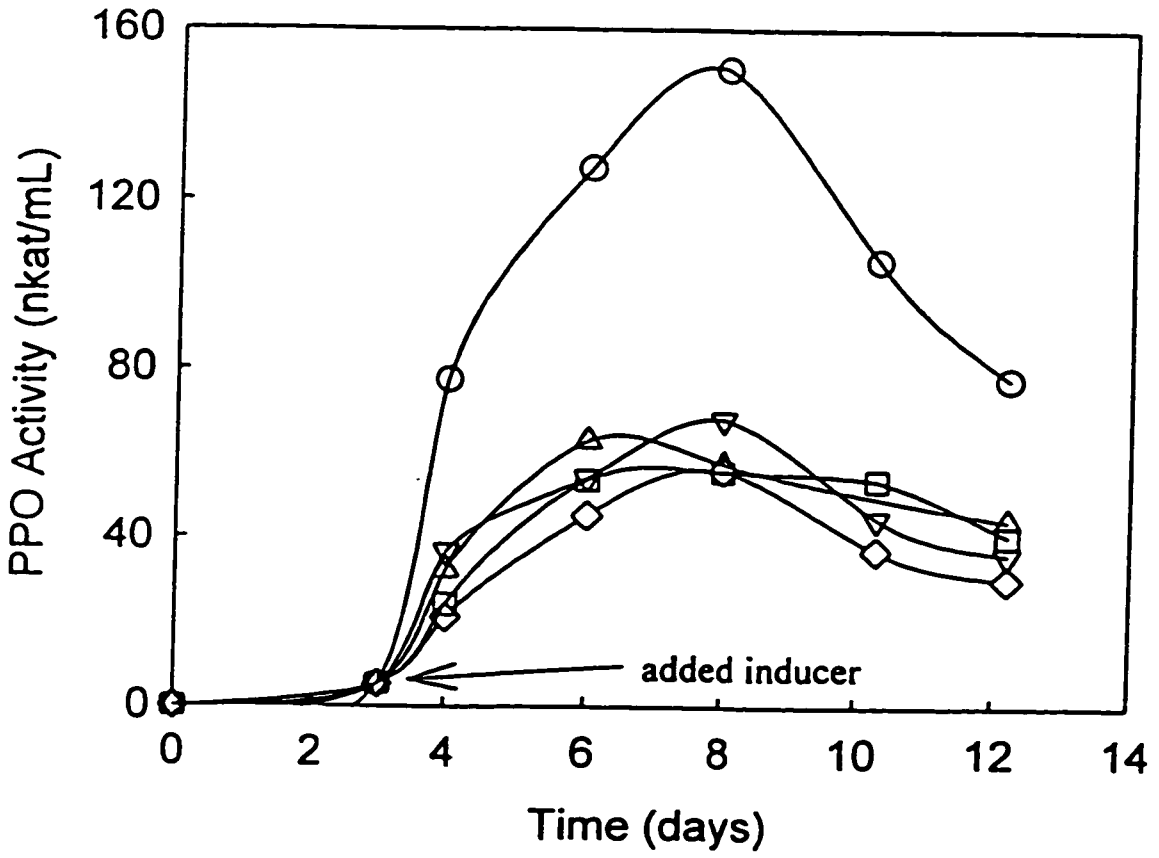

Figure 5-6: Effect of phenolics on the production of polyphenoloxidase (laccase) in a submerged process; (◊)-control; (○)-2,5-xylidene; (▽)-sinapic acid; (□)-ferulic acid; (△)-syringaldazine

T. versicolor strain tested in this study, which is also called *Coriolus versicolor*, such an inducing effect was not observed. This could be due to the rapid rate at which it is consumed or its low concentration. Syringaldazine is rapidly converted into a coloured quinone by laccase (Harkin and Obst, 1973) and judging from the colour changes observed in this reaction, it is transformed within 2 minutes. It is also possible that the level of this inducer was too low to have a great influence.

Finally, induction with ferulic acid resulted in a slight increase in PPO production, although this was much lower than expected. According to Leonowicz and Trojanowski (1975), induction of laccase in *T. versicolor* with 0.1 mM ferulic acid resulted in a "distinct increase" in laccase activity. In a separate study with *T. versicolor*, ferulic acid added to a concentration of 2.57 mM resulted in an increase in laccase production of 3.6 times and addition to a concentration of 5.15 mM ferulic acid resulted in an increase of 4.5 times (Asiegbu *et al.*, 1996).

Despite the fact that only 2,5-xylidene resulted in a very large increase in laccase production, it may be that these other phenolics were transformed too quickly to provide greater induction ability. It may be possible that the 2,5-xylidene is transformed into a compound that induces laccase, but is not quickly transformed by the fungus' enzymatic system, hence remains in the culture for a longer time. On the other hand, since SA, ferulic acid and syringaldazine are all rapidly degradable by PPO (Lacki, 1997), it is possible that these compounds are transformed quickly into non-inducing compounds. As a result, only small increases in activity would be seen. It is possible that higher concentrations, such as those used by Asiegbu *et al.* (1996), would counter this effect. However, this was not tested.

Figure 5-7 shows the growth curves for this experiment. Only the ferulic acid cultures showed a distinct increase in biomass levels. The media with the 2,5-xylidene and SA initially gave about the same levels of biomass as the control, however these cultures began to autolyze earlier than the control. Finally, the syringaldazine medium initially

inhibited biomass production. The reason for this is most likely due to the amounts of ethanol added to the cultures. Since 2,5-xylidene and ferulic acid are readily soluble in 50% ethanol solution, only 80 μL of inducer solutioin were needed to obtain the desired concentration of inducer, resulting in an addition of 40 μL of pure ethanol. This gave an ethanol concentration of 0.5 mL/L (0.4 mg/L). SA was not as soluble, so 320 μL of inducer solution were added, meaning that 4 times the amount of ethanol (2 mL/L) was added. This resulted in a very small decrease in biomass. However, for syringaldazine, an 85% ethanol solution was required and 400 μL were needed, resulting in the addition of 8.5 times more ethanol than the first two inducers (4.25 mL/L). Since syringaldazine (Harkin and Obst, 1973) and SA (Buswell and Eriksson, 1994) do not inhibit the growth of fungus, these findings may indicate that the ethanol is toxic to the fungus. Attempts were made to detect ethanol in the cultures near the end of the fermentation, however, the ethanol was either not present or was undetectable by the method used.

Figure 5-7 also shows that the maximum amount of biomass, which occured at 8 days, was higher in the ferulic and sinapic acid cultures. The syringaldazine and 2,5-xylidene cultures had about the same maximum amount of biomass as the control medium. The reasons for this are unclear at the present time. Since the ultimate glucose levels are identical for all cultures (Figure 5-8), there does not appear to be any additional sugar consumption in the ferulic acid culture. However, it may be that these phenolics simply stimulate biomass growth or cell autolysis, allowing some cultures to achieve higher biomass levels.

Keeping in mind that the syringaldazine, and to a small extent the SA cultures, had a lower initial biomass production, it is interesting that the rate of glucose consumption was much faster in these two cultures than the others. It may be a result of the fungus also using the sugar for the production of enzymes required to degrade the ethanol, although this was not examined.

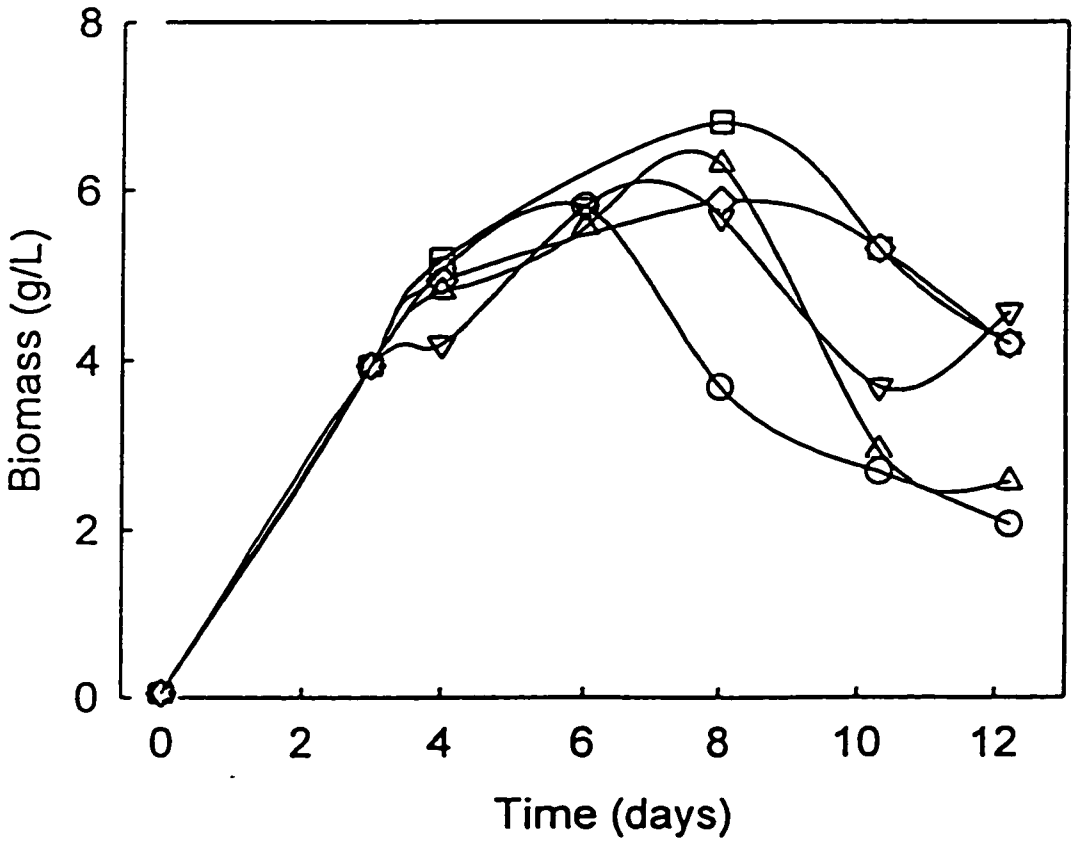

Figure 5-7: Effect of phenolics on biomass concentrations in a submerged process; (◊)-control; (○)-2,5-xylidene; (△)-sinapic acid; (□)-ferulic acid; (▽)-syringaldazine

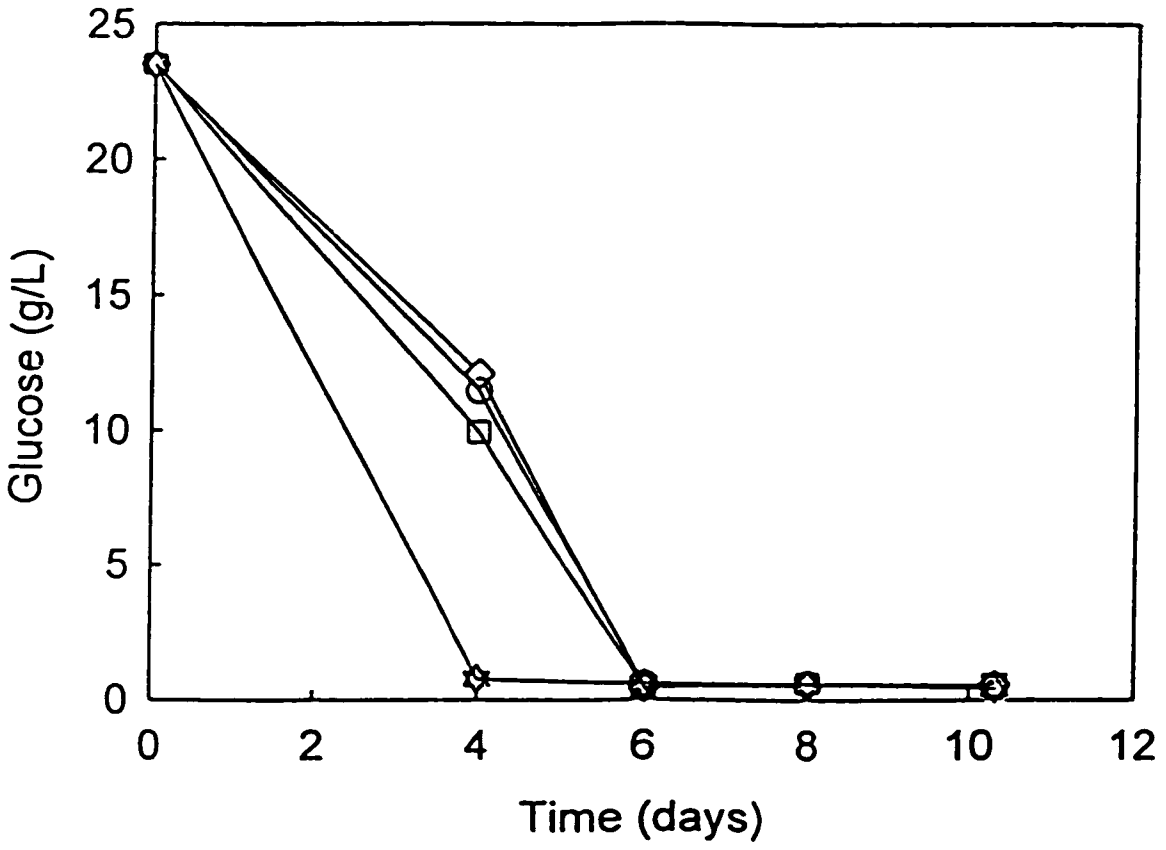

Figure 5-8: Effect of phenolics on glucose consumption in a submerged process; (◊)-control; (○)-2,5-xylidene; (△)-sinapic acid; (□)-ferulic acid; (▽)-syringaldazine

5.7 Effect of Other Meal Components on the Production of PPO

Since the phenolics tested did not seem to provide as great an increase in PPO production as anticipated, it was decided to test some additional canola meal constituents for PPO production. These included sinapine, since it is the major phenolic in CM, proteins from CM, since the nitrogen content of the medium can affect PPO production (Kirkpatrick *et al.* 1989) and two carbon sources, sucrose and cellulose, since the type of carbon source can also affect PPO production (Srinivasan *et al.*, 1995). The results can be seen in Table 5-2. The cultures, each containing the control medium plus the component listed in the table, were grown approximately 7 days. Two to five flasks for each component were analyzed separately and the results averaged. The results are presented in terms of percentage of PPO activity of the control for that experiment since two different sets of fermentations were combined and the results presented below. The concentration of the constituents used were based on the levels which would have been found in a 5% CM suspension. For example, the amount of sucrose obtained in a 5% CM solution is 3.5 g/L, hence this value of sucrose was added to the broth and compared to the control without sucrose.

Table 5-2: Effect of meal components on PPO production in submerged fermentation

Meal Component	number of flasks	percentage of control ± standard deviation
control	3[a],5[b]	100%
0.75 mg/mL sinapine	2[a]	107.8% ± 12.1%
6 mg/mL CM proteins	2[a]	124.3% ± 6.0%
12 mg/mL CM proteins	2[a]	119.4% ±5.5%
3.5 g/L sucrose	5[b]	102.9% ± 5.6%
2.5 g/L cellulose	5[b]	90.3% ± 5.4%
soluble components from 5% CM mixture	5[b]	290.5% ± 20.2%
5% CM	4[b]	309.0% ± 4.9%

[a] is from one set of experiments, [b] from another

The addition of sinapine to the medium had no influence on PPO production. The lack of PPO stimulation was unexpected for 2 reasons: (1) it was found earlier that SA slightly stimulated PPO production and (2) it was believed that, since SIN is the choline ester of SA, it would first be hydrolyzed into SA then degraded. However, the reason for the lack of induction may be due to the fact that SIN is degraded by a different mechanism than SA (Lacki, 1997). As a result, the intermediate product(s) from SA degradation, which stimulated PPO production, may not have been formed during SIN degradation. As a result, it can be concluded that the SIN content of the meal is not responsible for the large increase in PPO production when CM is added to the culture.

The CM used contains approximately 37% proteins. Lacki (1997) found that a 10% (w/v) CM suspension at pH 5 had a soluble protein concentration of 12 mg/mL. As a result, a value of 6 mg/mL was estimated to be the protein concentration of a 5% CM suspension. Furthermore, assuming that all 37% CM protein is soluble, the total concentration of proteins in a 5% suspension would be 18.5 mg/mL. However, due to an insufficient quantity of extracted protein, the higher nitrogen medium contained 12 mg/ml. As it can be seen, the proteins stimulated PPO production, however not to as great an extent as the 5% CM medium. It has been found that a high nitrogen medium stimulated the production of laccase in a culture of *Phanerochaete chrysosporium* that did not normally produce laccase (Srinivasan *et al.*, 1995). On the other hand, during the brightening of kraft pulp, Kirkpatrick *et al.* (1989) found that pulp was enzymatically whitened to a greater extent, i.e. more enzyme production, in a low nitrogen medium using *T. versicolor*.

The results found here do not necessarily exclude the protein fraction from being responsible for PPO stimulation in CM cultures. The CM proteins were prepared by solubilizing the CM proteins at pH 11, separating from the meal and precipitating at pH 4. It is possible that, for example, the amino acid(s) or protein(s) responsible for PPO stimulation are soluble at pH 5.5 (during culture conditions) but not at pH 11. This would mean that these compounds would not have been extracted during the protein isolation

experiment and would not have been available during this test. Furthermore, it is also possible that the extracted proteins were denatured during this procedure and may have lost the ability to stimulate PPO production by *T. versicolor*.

It can be seen that sucrose had no influence on PPO production. This was expected since the fungus possesses invertase, the enzyme responsible for the hydrolysis of sucrose into glucose and fructose. Furthermore, the additional glucose was not expected to stimulate enzyme production since the effect of glucose on PPO production had been previously examined by Lacki (1997). In that case, it was shown that there was no difference in maximum PPO activity at glucose concentrations of 6.5 to 47.5 g/L.

The addition of cellulose decreased PPO production, which was initially unexpected since Srinivasan *et al.* (1995) and Eggert *et al.* (1996) both demonstrated a requirement for cellulose during laccase production by fungi. However, the reason was evident once the culture media were prepared: the cellulose did not dissolve. The cellulose particles in the broth may have interfered with oxygen transfer, thus decreasing the rate of biomass formation and hence PPO production. Another possible explantion is that the strain of *T. versicolor* used in this laboratory has lost its ability to degrade cellulose. The fungus has been shown to grow in a medium containing cellulose as a carbon source, prepared in a similar way as here (Kirk *et al.*, 1976), as well as producing cellulase (Westermark and Erikson, 1974). It is possible that the cultured fungus used in our lab, which has not been exposed to cellulose has lost its ability to produce cellulase. This can be considered to be in agreement with the work of Szklarz *et al.* (1989), which found that a cultured strain of *T. versicolor* had lost some potential for enzyme (cellulase) production.

The effect of the soluble CM components on PPO production was examined. A 5% CM suspension in water was mixed thoroughly before sterilizing at 121°C for 15 minutes. Once cool, the suspension was filtered and centrifuged. The control medium was then prepared by adding the required components (glucose, etc.) to the supernatant broth. This was then re-sterilized and inoculated at the same time as the other cultures. The results

indicate that, although the average values are slightly lower than the 5% CM media, due to the standard deviations, it is likely that there is not a significant difference between the two cultures. This means that it is a soluble component(s) of CM that is responsible for the increased PPO production. Furthermore, since the majority of the compounds tested are soluble, there may be some interactions, but this was not tested.

5.8 Effect of Vogel's Minerals on the Growth of T. versicolor in Submerged Cultures

It was shown in the previous section that it is a soluble component of CM responsible for the stimulation of PPO production. However, since the phenolics, carbohydrates and proteins tested did not provide as great a stimulation for PPO production as CM, it was decided to test other soluble components. Since CM is also very abundant in minerals, it seemed logical to test the effect of minerals on PPO production. Vogel's minerals solution was used since it is the source of minerals in the control medium. The addition of Vogel's minerals provides Zn^{+2}, Fe^{+2}, Cu^{+2}, Mn^{+2}, MoO^{-}_{4}, as well as boric and citric acids, to the medium.

Figure 5-9 shows the effect of Vogel's minerals on the production of PPO for mineral concentrations of 0, 0.5, 1.0, 5.0 and 10 mL/L. The control culture used throughout this project contains 0.5 mL/L of these minerals. It can be seen that the presence of Vogel's minerals has a negative effect on PPO production in this test. This was completely unexpected since: (1) microorganisms require minerals for growth and (2) Vogel's minerals showed a positive effect on PPO production in the study of Lacki (1997). However, one of the differences between this work and that of Lacki (1997) was the presence of Vogel's minerals in the inoculum during this project. Since there was 0.5 mL/L minerals in the inoculum mixture, a small quantity of the culture medium (5 mL) was transferred into the cultures along with the initial biomass. This means that although no Vogel's minerals were initially added to the 0 mL/L flask, there were trace amounts present due to inoculation. In the study by Lacki (1997), the inoculum did not contain Vogel's minerals and so there

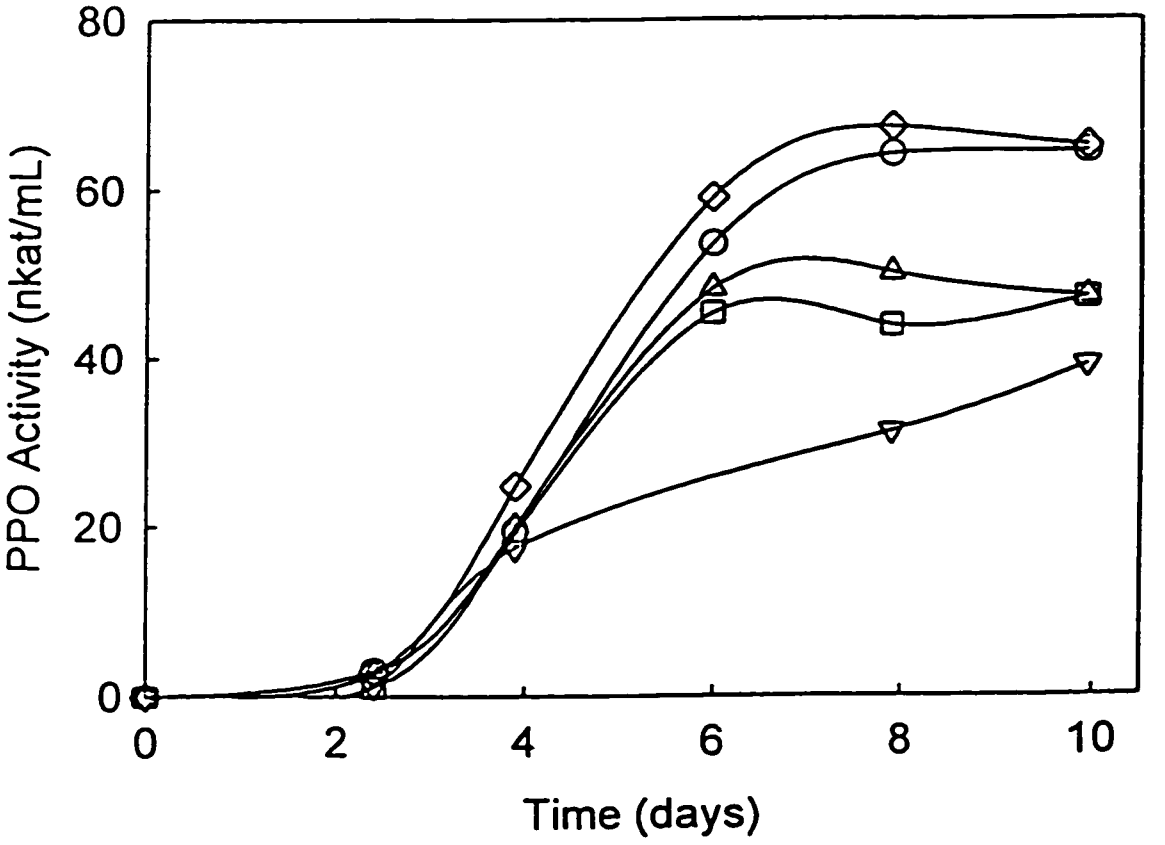

Figure 5-9: Effect of Vogel's minerals concentration on PPO production in a submerged process; (◊)-0 mL/L; (○)-0.5 mL/L; (△)-1.0 mL/L; (□)-5 mL/L; (▽)-10 mL/L

would be no metal ions present in the culture medium. These two pieces of information indicate that trace amounts of Vogel's minerals are required for growth and PPO production. This is in agreement with Salas *et al.* (1996) who found that: (1) laccase production by the fungus *Ceriporiopsis subvermispora* was severely repressed without the presence of Mn^{+2} or Cu^{+2} and (2) increasing the amount of these minerals up to 10 times the required level did not further increase laccase production. However, this was not in agreement with Dittmer *et al.*, 1997, who found that Cu^{+2} can be used to induce laccase in *Phanerochaete chrysosporium.*

The rate of sugar consumption follows the same order as for enzyme production (Figure 5-10). This is also in agreement with the biomass production rates (Figure 5-11). The lower Vogel's minerals concentrations were the least inhibitory to biomass production and had the fastest rate of glucose consumption. As the concentration increased, the rate of glucose utilization and biomass production decreased. It can be seen that 10 mL/L almost completely suppressed growth and resulted in 66% lower biomass levels than 0 or 0.5 mL/L as well as very little glucose consumption.

5.9 Effect of Aeration on the Growth of T. versicolor in Submerged Cultures

Another important parameter during aerobic fermentations is the amount of oxygen provided to the culture. To examine the effect of aeration in this experiment, it was decided to test different volumes of medium in 250 mL Erlenmeyer flasks. For submerged cultures on a rotary shaker, lower volumes of medium in a flask result in higher rates of oxygen due to both better mixing and larger interfacial surface areas between the gas and liquid phases per unit of medium. The culture volume normally used is 80 mL. In order to keep inoculum concentrations in the medium constant, the amount of initial biomass to be added was scaled according to the amount of medium.

The two smaller volumes have the fastest initial increase in enzyme activity, indicating that oxygen helps to stimulate enzyme production (Figure 5-12). This is in

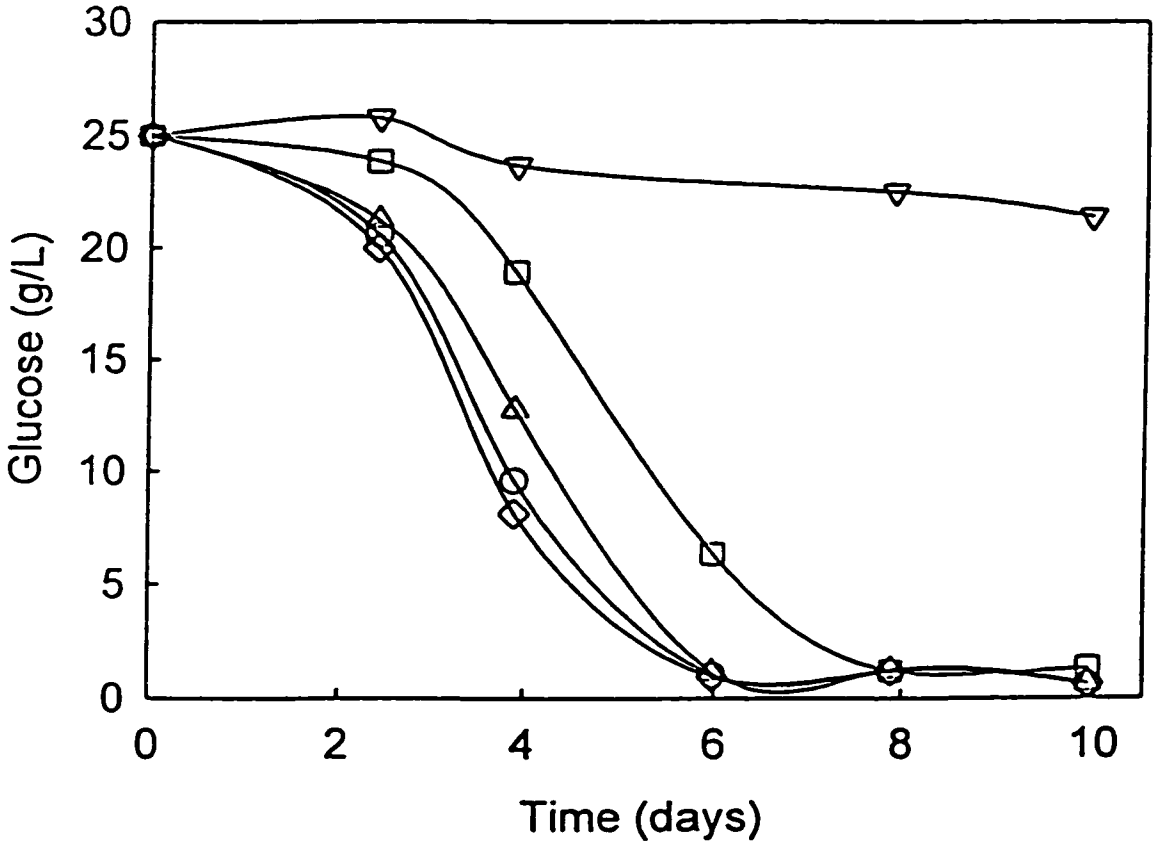

Figure 5-10: Effect of Vogel's minerals concentration on glucose consumption in a submerged process; (◊)-0 mL/L; (○)-0.5 mL/L; (△)-1.0 mL/L; (□)-5 mL/L; (▽)-10 mL/L

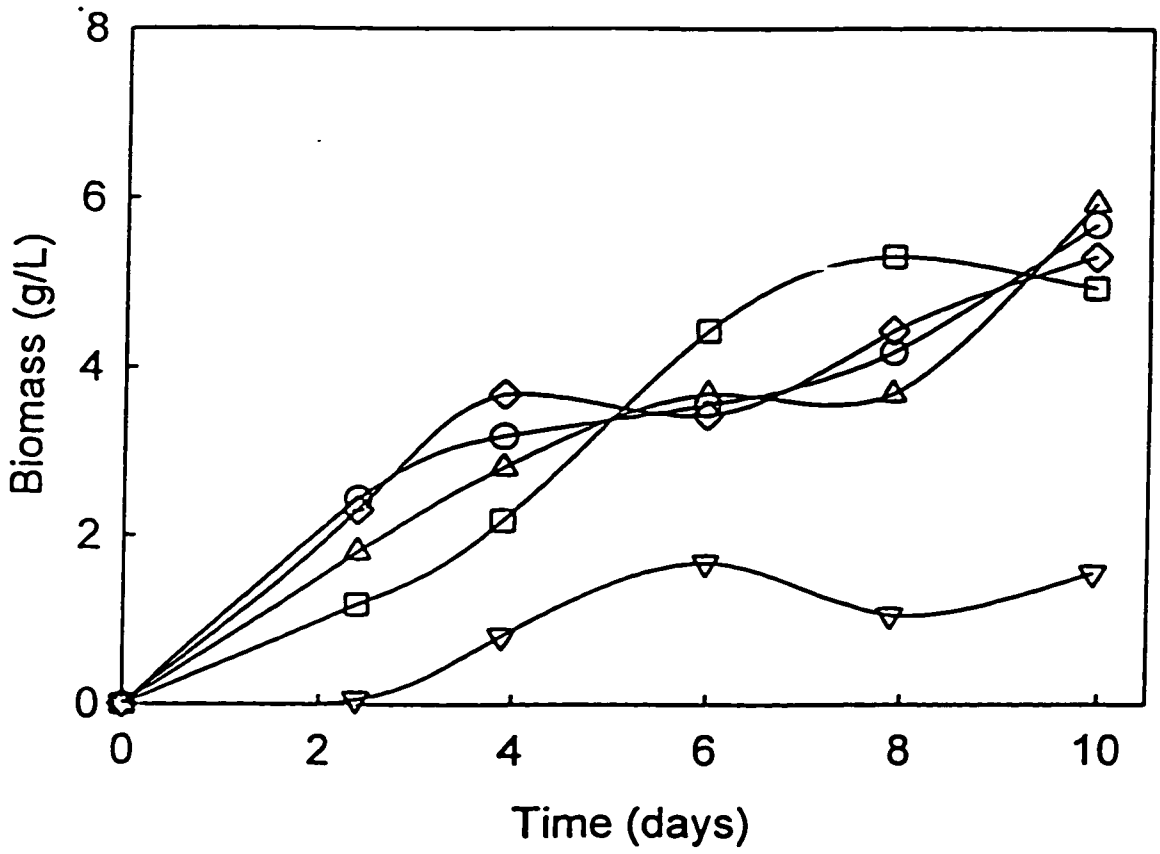

Figure 5-11: Effect of Vogel's minerals concentration on biomass production in a submerged process; (◊)-0 mL/L; (○)-0.5mL/L; (△)-1 mL/L; (□)-5 mL/L; (▽)-10 mL/L

agreement with Reid and Seiffert (1982) who found that higher oxygen concentrations stimulated both carbohydrate consumption and lignin degradation in several fungi including *T. versicolor*. Furthermore, faster growth rates have been observed with *T. versicolor* grown in the presence of higher levels of dissolved oxygen (Ziomek *et al.*, 1991).

However, it may also be possible that the oxygen did not affect enzyme production *per se*, but that it helped increase the rate of biomass production, which in turn led to higher initial levels of PPO. Biomass was not followed in this experiment due to the difficulties in its separation from the CM.

The 110 mL culture showed the next fastest rate of enzyme production. As would be expected, it was much slower than the lower volumes. There was still some mixing of the contents with the larger volumes, and hence some resulting aeration and the fungus was able to produce some PPO, although at a slower rate. The 140 mL culture resulted in the lowest amount of this enzyme produced.

The enzyme activity of the 50 mL culture, despite increasing very rapidly, did not reach as high a level as the 80 or 110 mL cultures. As was shown by Reid and Seiffert (1982), cultures receiving more oxygen grew faster than the control, but also began to autolyze earlier. With this in mind, the results show (Figure 5-12) that the 50 mL culture reached its maximum level sooner than the other cultures, at 6 days, but began to autolyze soon after. This entails the release of proteases which would degrade the PPO and would result in lower PPO activities. This is in agreement with the work of Dosoretz *et al.* (1990) which found that oxygenation of submerged cultures increased extracellular protease activity in *Phanerochaete chrysosporium.*

Figure 5-13 shows the glucose concentration as a function of time. It would appear that the lower volumes had the slowest consumption rates, however this is not true. As discussed earlier, CM contains sucrose, which is not detected in the glucose analysis but is slowly hydrolyzed into glucose by the fungus. As a result, the glucose concentration measured at 2 days is the result of both decrease due to consumption and increase due to

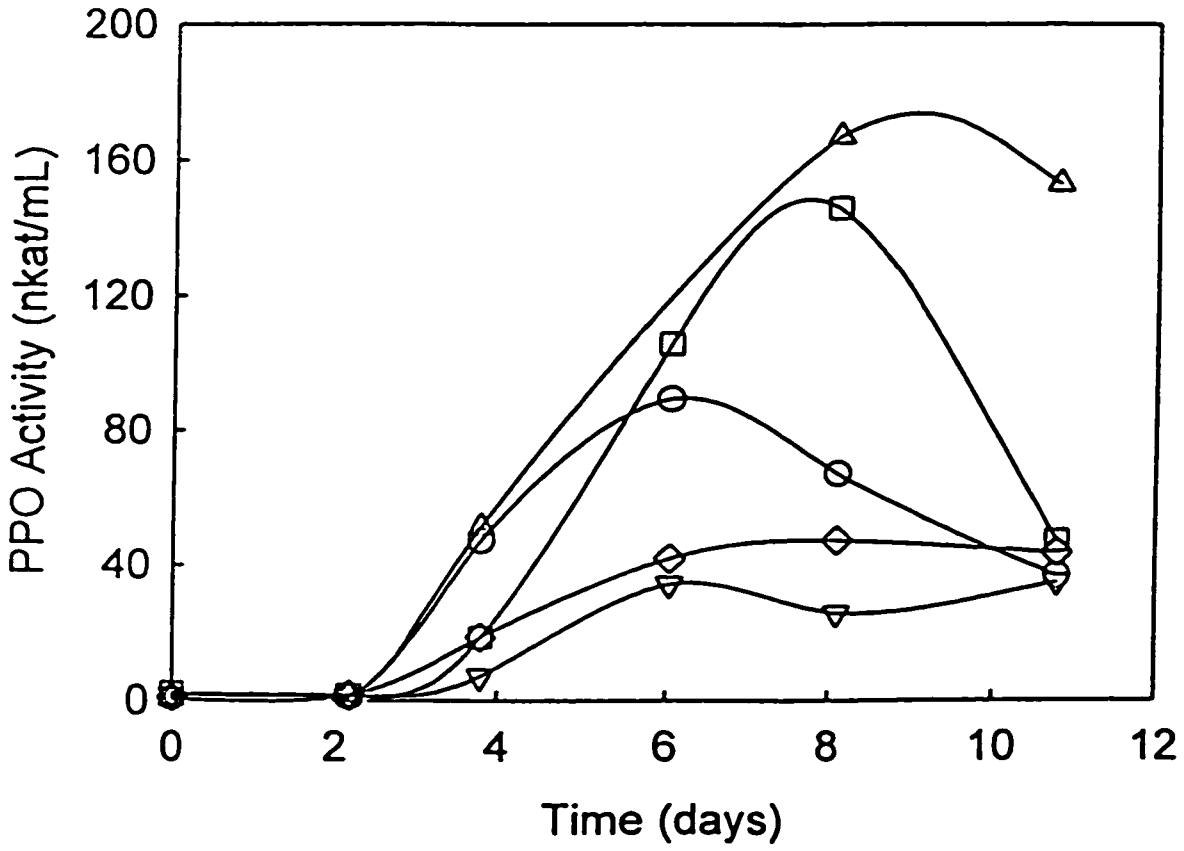

Figure 5-12: Effect of aeration on PPO production in a submerged process in the medium with 5% CM and without 5% CM; (◊)-80 mL, no CM; (O)50 mL; (Δ)-80 mL; (□)110 mL; (▽)-140 mL

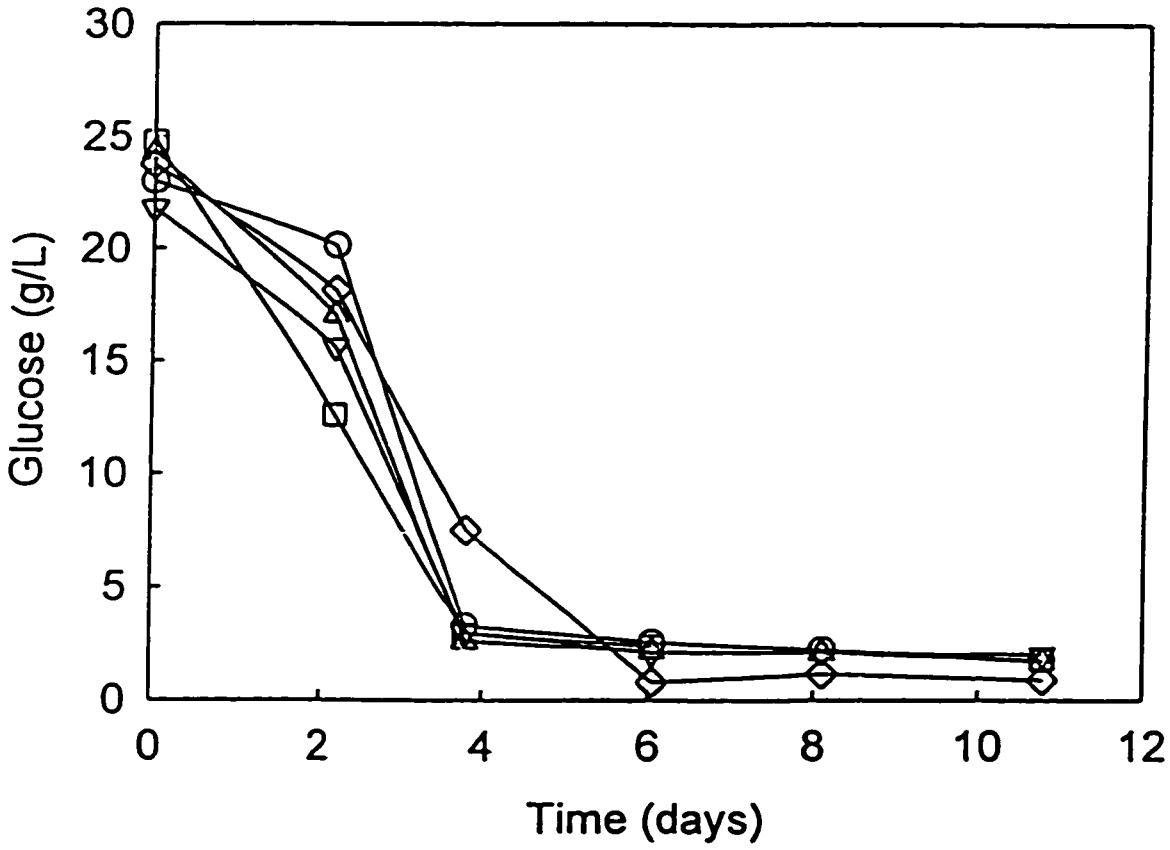

Figure 5-13: Effect of aeration on glucose consumption in a submerged process in the medium with 5% CM and without 5% CM; (◊)-80 mL, no CM; (○)-50 mL; (Δ)-80 mL; (□)-110 mL; (▽)-140 mL

hydrolysis of sucrose. This means that, despite the fact that it appears as though the glucose level has only dropped slightly, it is more likely that the 50 mL culture has hydrolyzed the most sucrose. The 140 mL culture, which has a faster rate of decrease, most likely began consuming glucose without hydrolyzing a significant amount of sucrose. The medium without CM had the slowest rate of glucose consumption but had a residual glucose level lower than any of the CM cultures. As discussed earlier, this is due to the colourometric interference of the medium when measuring glucose.

The concentration of SAE in the meal during the fermentation has been given in Figure 5-14. The initial SAE values of between 6-7 g/kg is lower than the 22 g/kg in the meal due to extraction of the SAE into the fermentation media. There is a very rapid decrease in SAE concentration over the first 3 hours due to inoculation with a large PPO concentration in the inoculum. The inoculum contained approximately 40 nkat/mL and so there was an initial PPO activity of 2.5 nkat/mL in the fermentation media. The SAE content decreased as the volume of media increased. However, taking into consideration the initial SAE levels, the amount of decrease is approximately constant.

5.10 Effect of Canola Meal Concentration on PPO Production

It was found earlier that a 5% CM suspension stimulated PPO production to a much larger extent than the control or 2,5-xylidene media. Furthermore, since this stimulation was found to be due to the soluble fraction of the meal, it seemed reasonable to examine the influence of the CM concentration on PPO production. Meal concentrations of 1%, 3% and 5% were used. Higher concentrations were not used since the suspension was too viscous at 8% (Gattinger *et al.*, 1990). In addition, a medium was included using a meal with reduced levels of SAE (approximately 6 g/kg). Finally, 5 mL of a 5% CM medium was aseptically added to some control cultures containing 0% CM. This was used to determine if the compound responsible for enhancing PPO production

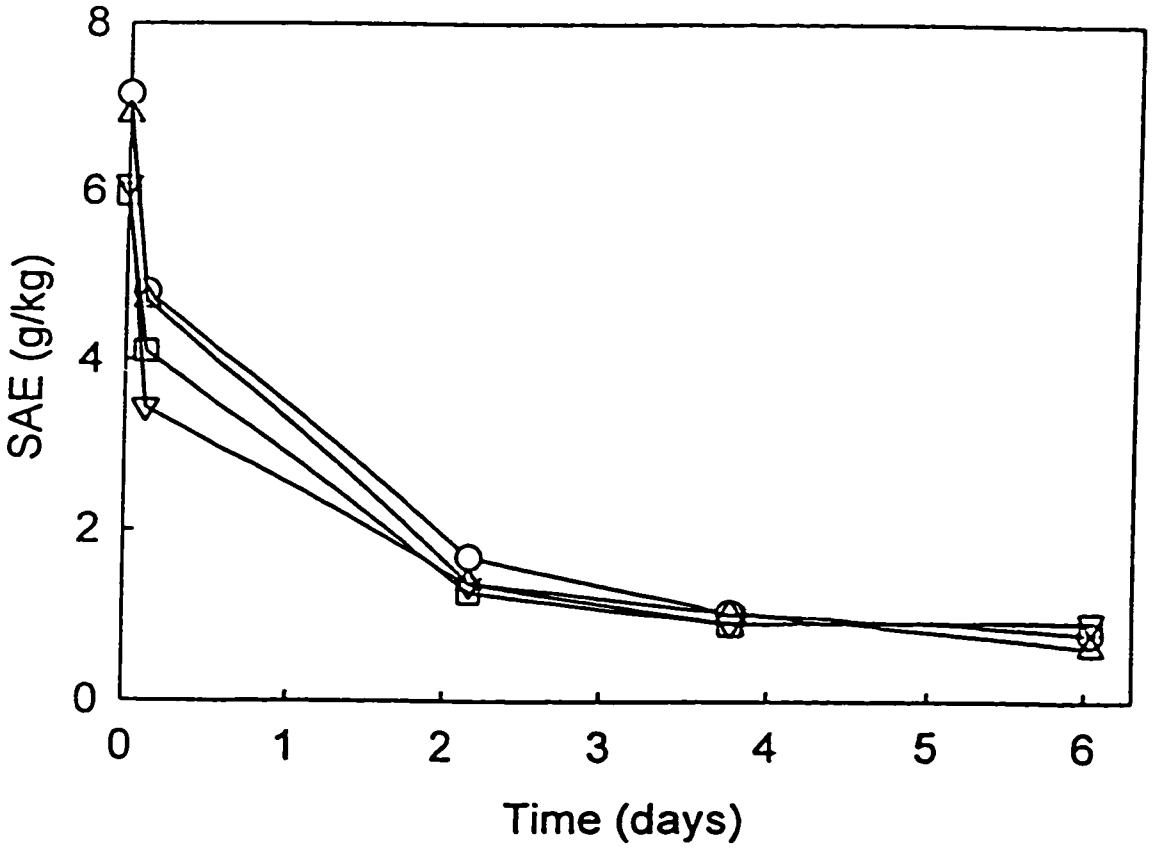

Figure 5-14: Effect of aeration on SAE decrease in a submerged process in a medium with 5% CM; (○)-50 mL; (△)-80 mL; (□)-110 mL; (▽)-140 mL

was required during the initial growth phase or could be added once growth was nearly complete (*i.e.*, stationary phase).

Figure 5-15 shows the influence of these parameters on PPO production. It can be seen that among the meal media, the lower CM concentrations had a more rapid initial increase in PPO activity. As was demonstrated in section 5.9 and as shown by other researchers (Reid and Seiffert 1982; Ziomek *et al.*, 1991), oxygen stimulates theproduction of PPO. Since the lower CM concentrations were less viscous, it is likely that oxygen transport was better in the medium with lower concentrations of CM. As a result, the cultures with the lower CM concentrations could grow faster and hence PPO activity would increase the most rapidly.

On the other hand, the higher CM concentrations had higher final PPO levels. The maximum levels for the 0%, 1%, 3% and 5% CM concentrations were 47,113, 152 and 165 nkat/mL, respectively. Since the 1% medium had over 2 times the activity of the 0% medium, this indicates that the PPO-stimulating compound(s) found in CM are required in small quantities. In addition, since there are not large differences between the 3% and 5% optima, this may indicate that once this small level of compound(s) is present, a further increase will not have a significant effect on PPO production. It should be noted that the 6 day, 1% medium could be low, which would still be consistent with only trace quantities being required for PPO stimulation. However, it may also be possible that the 6 day sample is correct and that the 8 day culture is high, which would be consistent with the data to be presented in the next two sections.

The 5% reduced phenolics meal was prepared by extracting CM with 2% HCl in methanol, as discussed in the materials and methods section. The SAE concentration in the meal was 6 g/kg instead of the normal value of 22 g/kg. Unfortunately, due to space limitations on the shaker, samples were only taken for the last three days. However, it is still evident that the PPO levels in this culture were similar to the 5% regular CM media. This provided further validation that it is not the soluble SAE that are responsible for

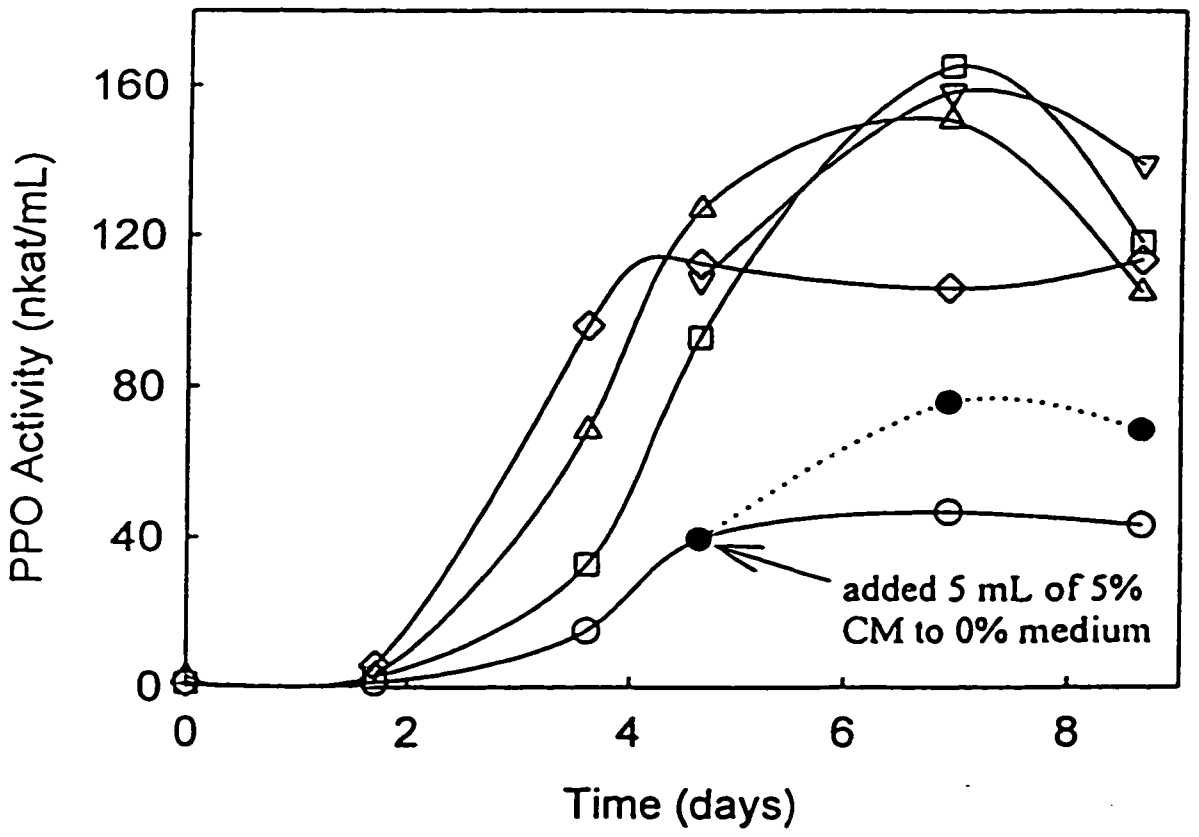

Figure 5-15: Effect of CM concentration on PPO production in a submerged process; (○)-no CM; (●)-0% CM + 5mL of 5% CM medium; (◊)-1% CM; (△)-3% CM; (□)-5% CM; (▽)-5% CM containing 6 g/kg SAE

stimulating PPO production. Another separate experiment (data not shown) using CM containing SAE in a concentration of 0.64 g/kg showed that the PPO activities were similar in 5% suspensions of this meal, or with 5% regular CM.

The control medium containing no CM, when supplemented with 5 mL of the 5% regular CM culture, had increased levels of PPO (Figure 5-15). The 5 mL of medium transferred into the CM-free culture contained CM, culture medium as well as biomass and enzymes. The final CM concentration in the medium supplemented with the 5 mL of CM medium was 0.3%. The PPO activity in the culture with the added CM media would be expected to be a maximum of 54 nkat/mL, due to the addition of 5 mL of the 165 nkat/mL culture to 80 mL of the 47 nkat/mL culture, if there were no further increase in production. Since the activity reaches a level of 75 nkat/mL, it is evident that there is some stimulation of PPO production in the CM-free culture after the addition of the 5 mL of CM medium. The activity in this culture may be lower than the other CM media due to the late addition in the growth phase, the lower concentration of CM, the low levels of glucose at the time of addition or that the inducing compound(s) may have been already partially consumed.

Figure 5-16 shows the glucose concentration as a function of time. The CM-free culture to which the 5 mL of CM medium was added is not shown since, at the time of induction, at 4.5 days, the glucose has been exhausted. Prior to this time, the glucose concentration would be identical to the 0% media. As was seen earlier, the CM cultures, with the exception of the 1% suspension, had a slower initial decrease in glucose than the control, CM-free media. However the glucose ultimately reaches the residual level fastest in the CM cultures. The apparent slow initial utilization of glucose is due to the hydrolysis of the non-reducing sugars into reducing sugars.

5.11 Effect of Soybean Meal Concentration on PPO Production

Since CM stimulated PPO production, it was decided to examine the effect of soybean meal (SBM) on the production of this enzyme. The medium with SBM

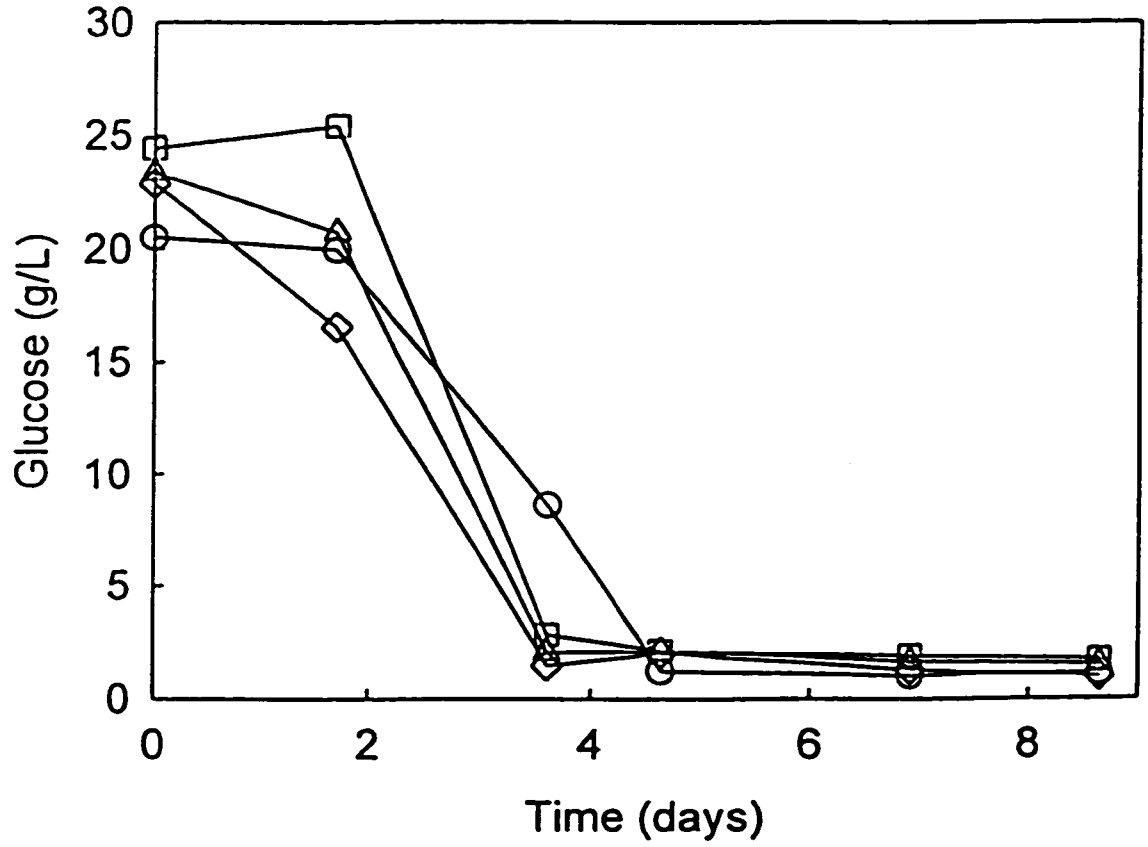

Figure 5-16: Effect of CM concentration on glucose consumption in a submerged process; (O)-no CM; (◊)-1% CM; (Δ)-3% CM; (□)-5% CM;

concentrations of 1%, 3%, 5% and 10% were used. The control culture contained 0% SBM. It can be seen that 5% SBM produced the greatest PPO activity, however, as was observed for CM, the 1% and 3% SBM both had faster initial rates (Figure 5-17). In this case, the 3% SBM was faster than the 1% SBM. This can be explained since the SBM had a more uniform particle size (data not shown) and was more dense (Table 5-3) than the CM. The SBM consisted of dense particles with a small amount of fine particles, whereas the CM had much higher amounts of fine material. Since the SBM particles remained near the bottom of the culture flask, it is possible that they did not interfere with oxygen transport as much as the cloudy and more viscous CM media. As a result, if there is greater oxygen transfer, there can be greater PPO production. The 10% SBM produced the lowest amount of PPO. This can be attributed to a more viscous medium and less oxygen transfer. This is in agreement with the glucose utilization curve, which shows that an extra 2 days were required to use all of the glucose in the 10% SBM medium (Figure 5-18).

Table 5-3: Bulk Density of Oilseed Meals

Meal Variety	Bulk density ± standard deviation (g/mL)
Canola	0.582 ± 0.013
soybean	0.664 ± 0.012
sunflower[a]	0.219 ± 0.011
sunflower[b]	0.272 ± 0.007

[a] is loosely packed sunflower meal
[b] is tightly packed sunflower meal

5.12 Effect of Sunflower Meal Concentration on PPO Production

Sunflower meal, SFM, is another important oilseed meal and was also used for comparison of PPO production. It was again neccesary to obtain the optimum concentration of SFM. The same concentrations as SBM were tested. The SFM was

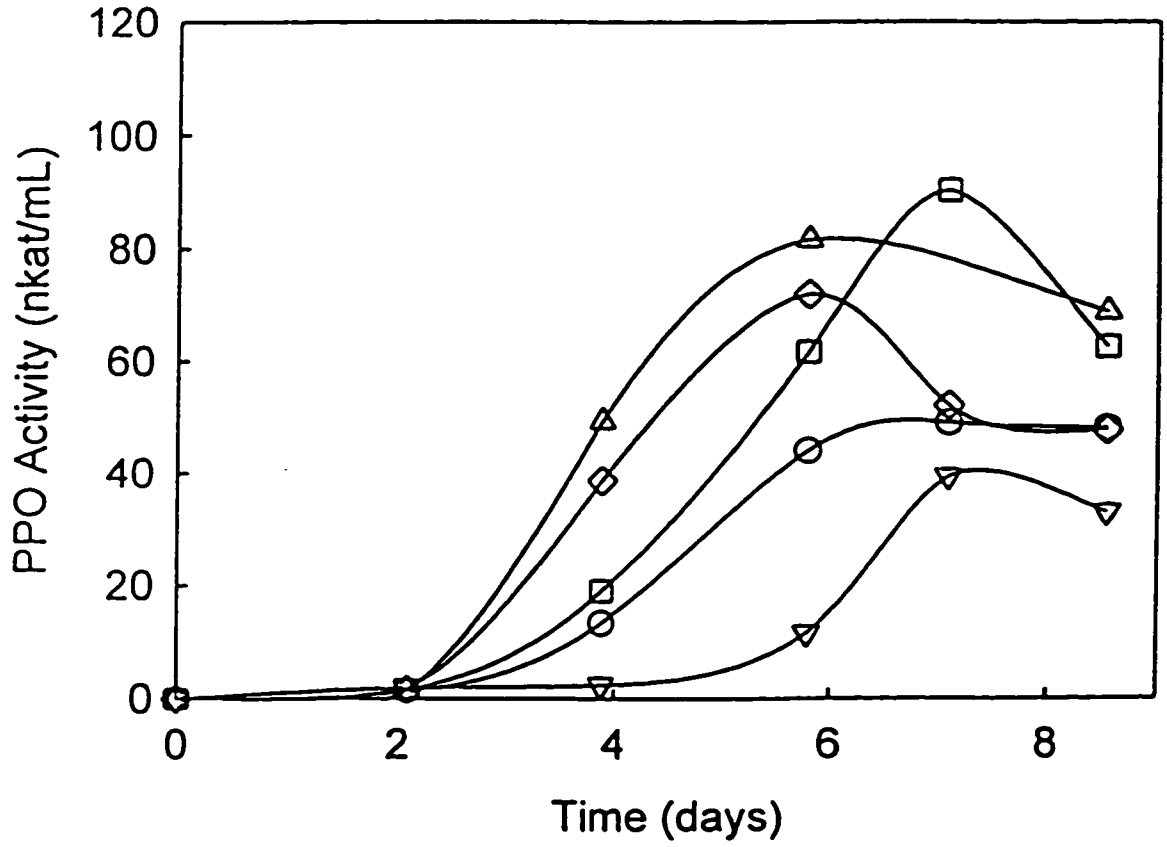

Figure 5-17: Effect of SBM concentration on PPO production in a submerged process; (○)-0% SBM; (◊)-1% SBM; (△)-3% SBM; (□)-5% SBM; (▽)-10% SBM

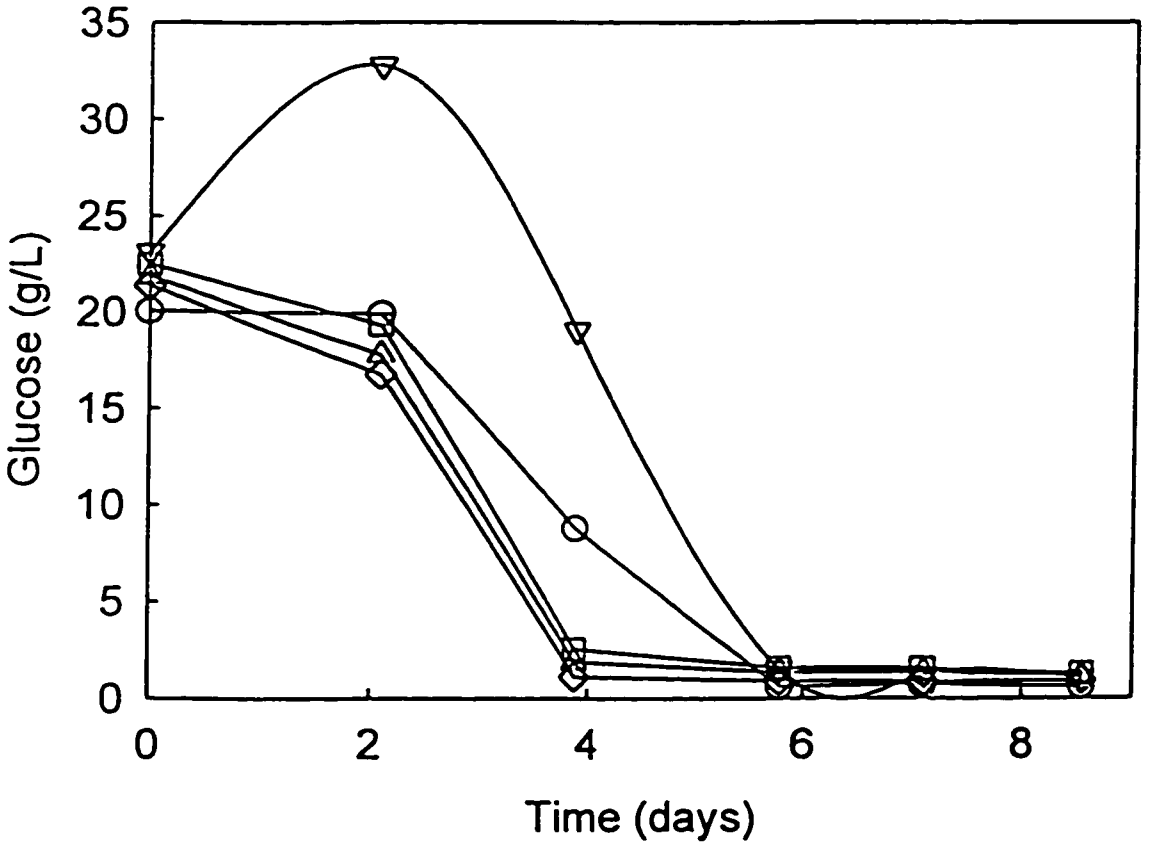

Figure 5-18: Effect of SBM concentration on glucose consumption in a submerged process; (○)-0% SBM; (◊)-1% SBM; (△)-3% SBM; (□)-5% SBM; (▽)-10% SBM

much less dense than SBM or CM (Table 5-3). As a result, a smaller mass of SFM occupies the same volume as a larger mass of SBM or CM. This means that the 5% SFM culture would be much more viscous than the 5% SBM or 5% CM media. In addition, the SFM contained larger amounts of fine particulate material. As a result of the increased viscosity and decreased aeration in the 5% SFM culture, it is apparent why the 3% SFM was the optimum concentration for PPO production (Figure 5-19). The final data point for the 3% SFM culture has not been shown due to contamination.

As was observed for the SBM study, the 10% SFM had the lowest amount of enzyme, even less than the control, 0% media. The 1% culture also had a slower increase in PPO production than the 3% SFM, which indicates that perhaps the stimulating compound(s) are not supplied in high enough concentrations at 1% meal. The 5% SFM culture has a slow initial increase but reaches a level greater than the 1% SFM, but not higher than the 3% SFM. The maximum PPO activity observed in these tests was 85 nkat/mL.

The rate of glucose consumption was greatest for the 3% and 1%, respectively, followed by the 5% SFM cultures (Figure 5-20). As was observed for the SBM, the 10% SFM culture took longer to exhaust its glucose supply. The residual sugar level is not as low as the others due to the interference from the colour of the media. In fact, it can be seen that the 0% and 1% are at the same residual level and then the final concentration increases with SFM concentration.

5.13 Effect of Oilseed Meal Concentration on PPO Production

Having determined the optimum concentrations for the CM, SBM and SFM, it was desired to compare the media with the optimum concentrations of each of these 3 meals simultaneously to determine the best oilseed meal for PPO production. It can be seen that the 5% CM medium had the maximum PPO activity, followed by the 5% SBM then 3% SFM (Figure 5-21). The maximum activities were 140 nkat/mL, 118 nkat/mL

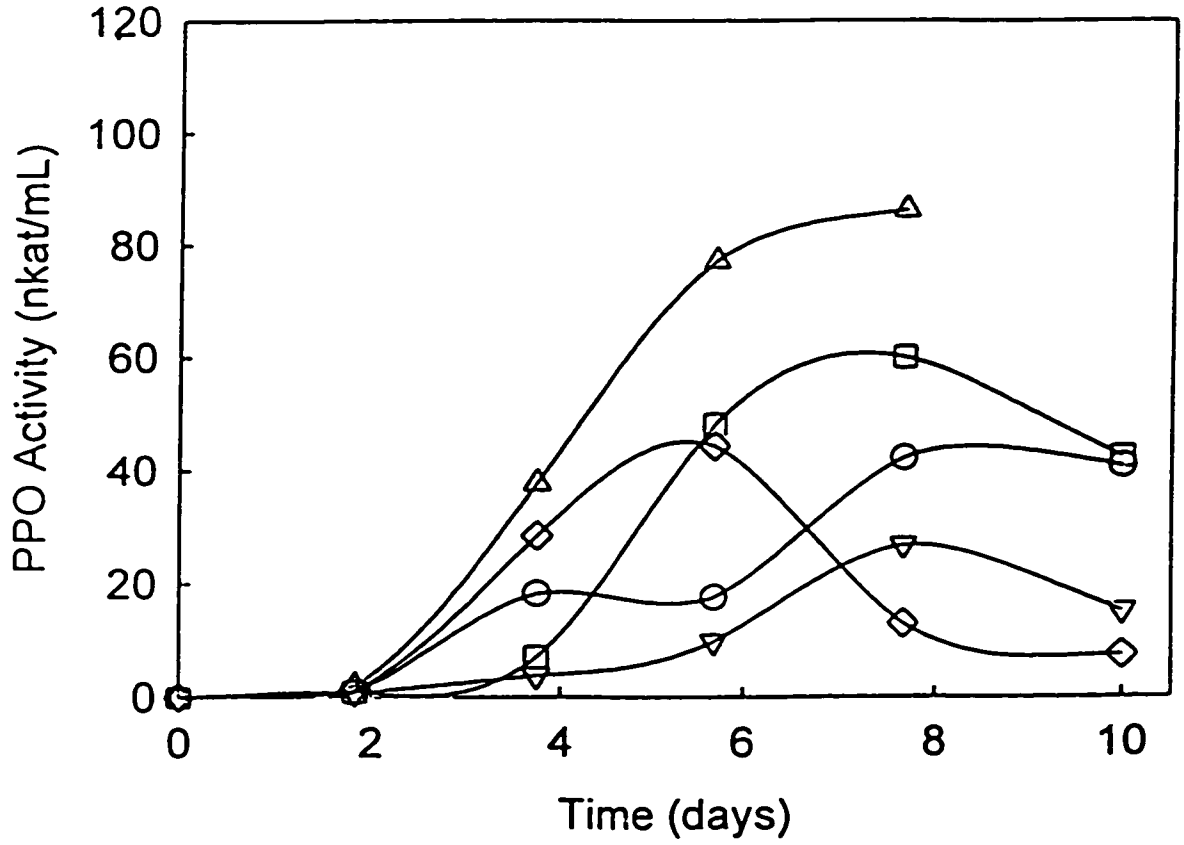

Figure 5-19: Effect of SFM concentration on PPO production in a submerged process; (O)-0% SFM; (◊)-1% SFM; (Δ)-3% SFM; (□)-5% SFM; (▽)-10% SFM

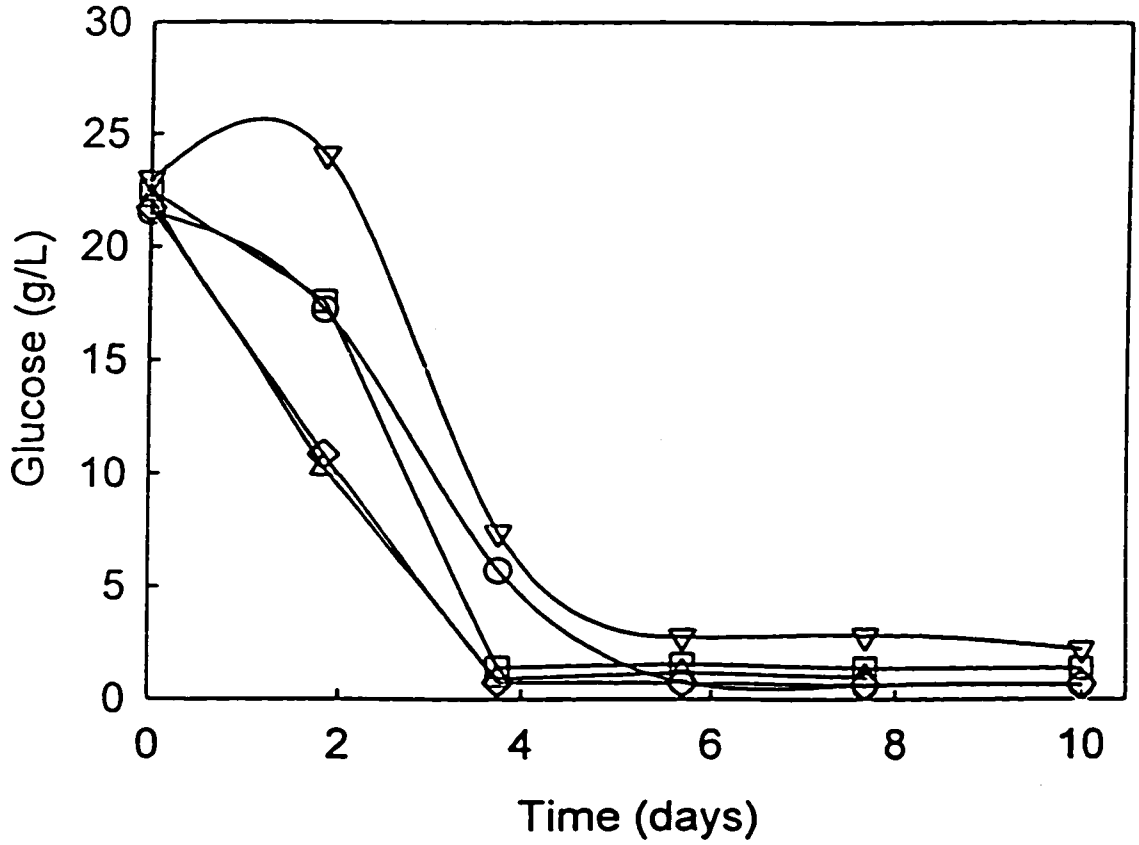

Figure 5-20: Effect of SFM concentration on glucose consumption in a submerged process; (○)-0% SFM; (◊)-1% SFM; (△)-3% SFM; (□)-5% SFM; (▽)-10% SFM

and 102 nkat/mL for the three cultures, respectively. The control media had a maximum activity of 60 nkat/mL.

Glucose consumption was initially fastest with the SFM. However, by 4 days, all oilseed meals had reached the same final value (Figure 5-22)

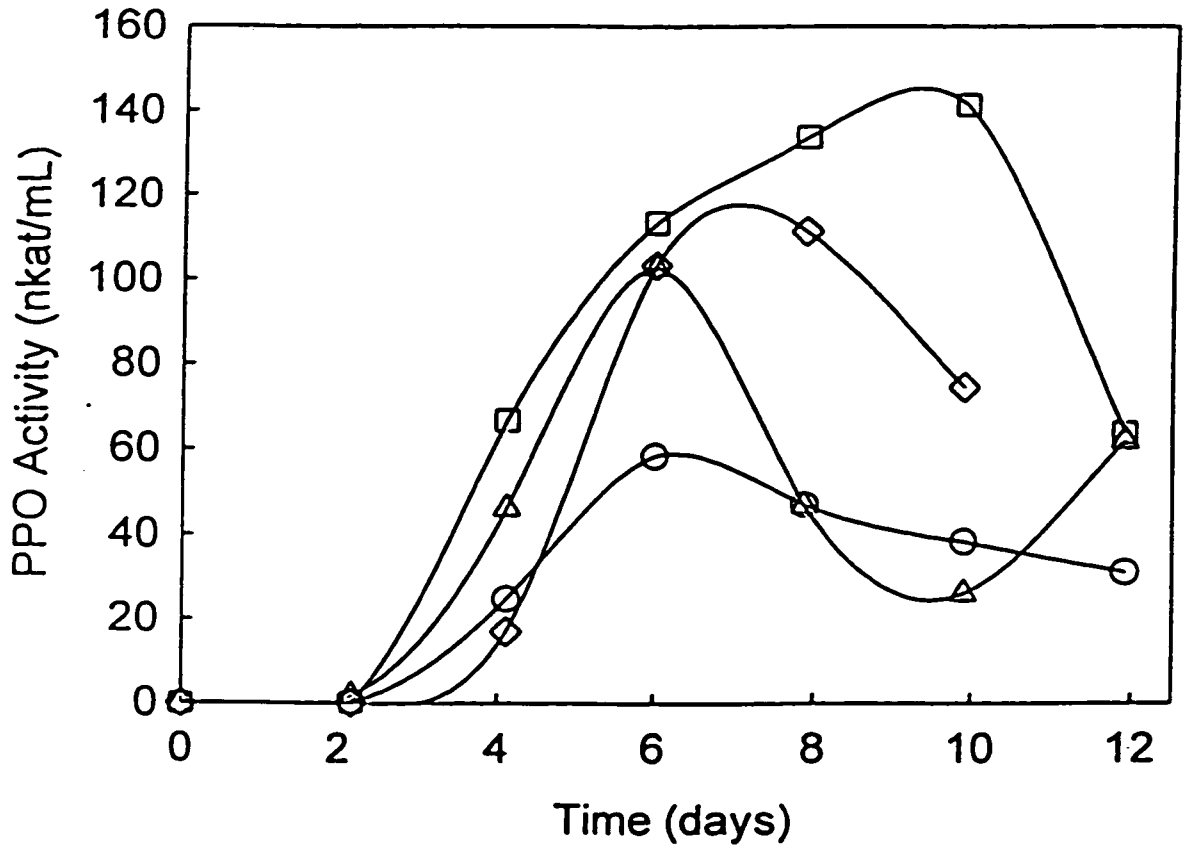

Figure 5-21: Comparison of three oilseed meals for PPO production in a submerged process; (O)-no meal; (◊)-5% SBM; (Δ)-3% SFM; (□)-5% CM

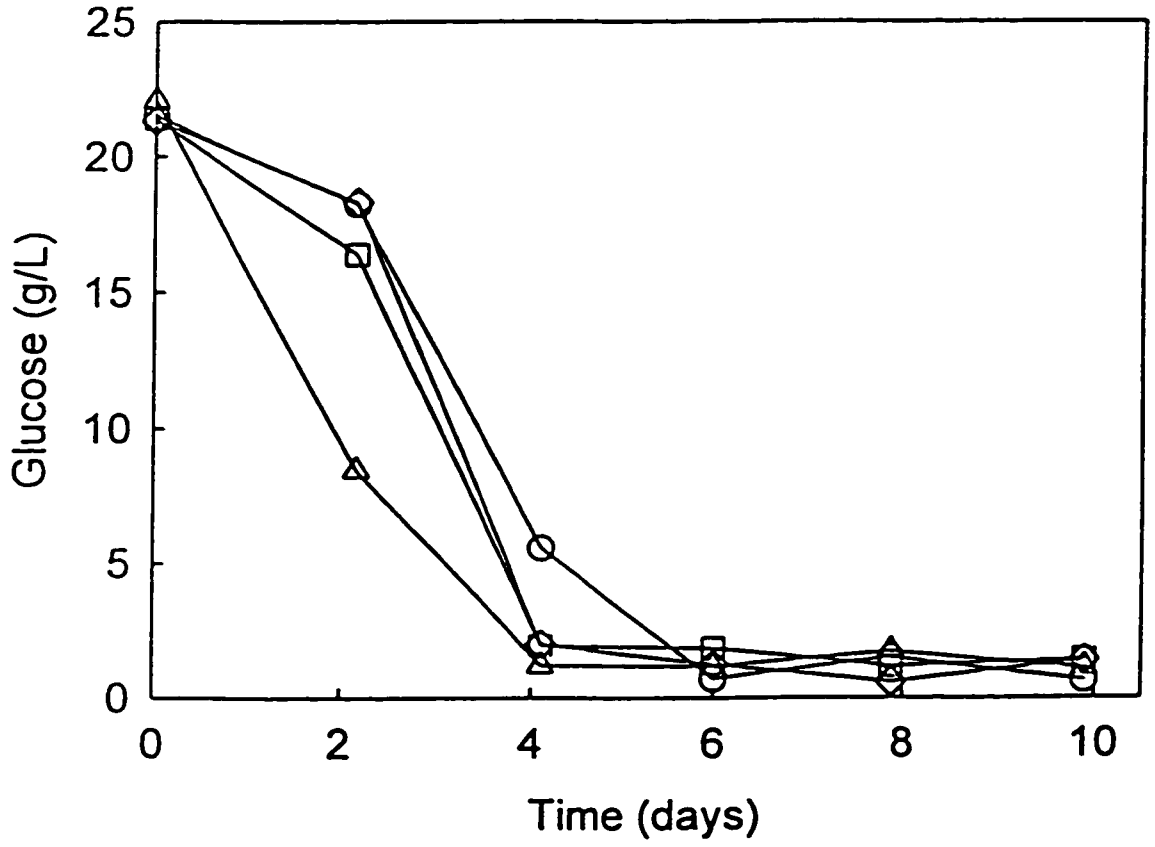

Figure 5-22: Comparison of three oilseed meals for glucose consumption in a submerged process; (○)-no meal; (◊)-5% SBM; (Δ)-3% SFM; (□)-5% CM

Chapter 6: Solid State Fermentation

A solid state fermentation (SSF) process was used to produce polyphenoloxidase (PPO) and decrease the phenolic content of CM. Since the growth of *T. versicolor* on CM in a solid state process has not been previously studied, several important SSF parameters were considered.

6.1 Typical SSF Process with Trametes versicolor on CM

Figure 6-1 illustrates the growth of the fungus in a 60% moisture content CM mixture containing 10 mL/L Vogel's minerals in the liquid phase. As discussed in section 4.1.3, the medium was prepared by adding the minerals to the water and then adding this solution into the required amount of CM. This ensured a thorough distribution of the minerals. No additional nutrients were added since CM is a complex and rich raw material. As can be seen, since there is enzyme production and SAE decrease, it can be assumed that this medium is adequate for a control medium. Furthermore, to avoid any decrease in SAE due to PPO present in the inoculum, the biomass pellets were washed several times with sterile water prior to inoculating the media.

The biomass appears to increase very rapidly initially, which would indicate a short lag phase. It has been suggested that there is a slow initial increase in biomass concentration in the colonization phase observed for *T. versicolor* during SSF on straw (Valsmeda et al., 1991). The slow initial increase in biomass concentration would be needed to repair mycelia damage due to homogenization, to become acclimatized to the new conditions and to begin to synthesize the enzymes required by the microbe. This slow increase in biomass concentration was not observed in these experiments, most likely due to the different substrates used for growth. CM contains about 7-8% sucrose and other low molecular weight sugars which are easily consumed by the fungus for

growth. On the other hand, it is likely that the straw would have more polysaccharides, so that the initial growth would be much slower since these complex molecules would have to be first broken down before being consumed. Visual observations from this experiment showed that after 1 day, there was very little growth. There was a little more on the second day and by about the third day there was significant growth. At this point, the amount of biomass coverage appeared to be constant, but was difficult to verify these small differences visually. After about 10 days, the biomass appeared to be entering the death phase and a decrease in the amount of mycelia can be seen. This was accompanied by a collapse in the mycelia structure and the production of a sticky, viscous substance.

One of the problems associated with biomass measurement in SSF is that it can not be measured directly due to the presence of the support material. As a result, the biomass concentration must be measured indirectly. Chitin, a component of fungal cell walls similar in structure to cellulose, can be hydrolyzed into glucosamine. This can be measured spectrophotometrically after reaction with Erlich's reagent. This is a very lengthy procedure and, as discussed in section 4.5.2, requires approximately 8 hours to perform. A calibration curve using biomass collected from submerged media was initially used, however it was found that the sample size (i.e. amount of CM) also influenced the apparent biomass concentration. As a result, the equation used to calculate the amount of biomass took into consideration the sample size and combined calibration curves for both pure biomass and pure CM.

The glucosamine content for an 8 day submerged culture of *T. versicolor* was determined to be 2.1%. This value is consistent with those measured for *T. versicolor* presented in the literature (Table 6-1). The glucosamine content is known to change with age and growth conditions and may be different in submerged and solid state fermentation (Jones and Worrall, 1995). For this report, it has been assumed that the chitin content of the fungus is constant throughout the entire growth cycle. This assumption has been previously used to study the growth of *T. versicolor* during the

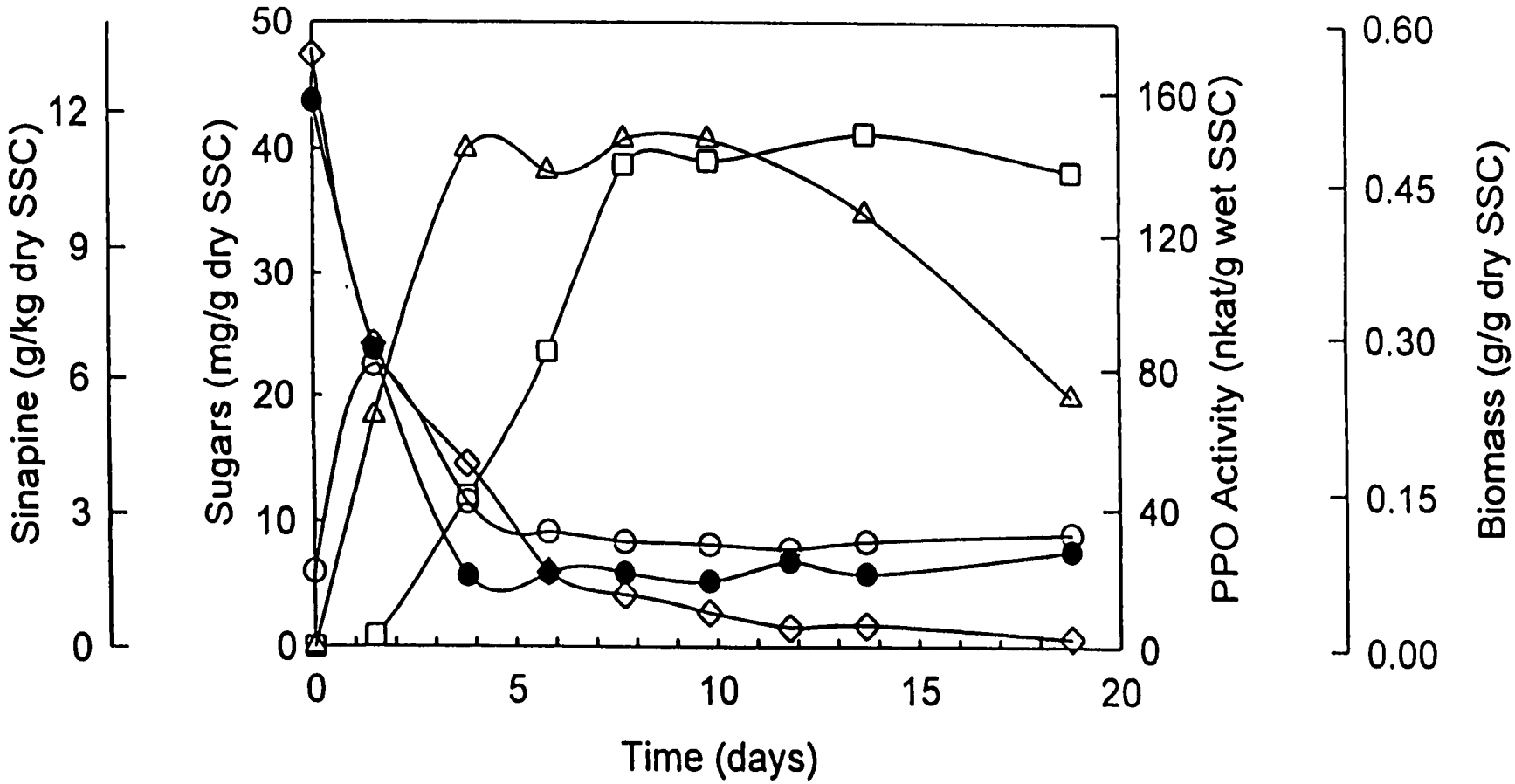

Figure 6-1: Typical SSF of *T. versicolor* on CM medium with 10 mL/L Vogel's minerals and 60% moisture content. (◊)-sinapine; (○)-glucose; (●)-non-reducing sugars; (△)-biomass; (□)-PPO

brightening of kraft pulp (Kirkpartick et al., 1989). It was noted in that study that the results must be "interpreted with a degree of caution" (*ibid*). As a result, the solid state biomass concentrations presented throughout this project should also be used qualitatively to provide an indication of the growth patterns.

Table 6-1: Reported values of glucosamine content of *T. versicolor*

Author(s)	Glucosamine content (%)	medium
Swift (1973)	1.16%	SSF with sawdust
Kirkpatrick *et al.* (1989)	3.07%	submerged with 0.5% kraft pulp
Jones and Worrall (1995)	1.58-2.28% 0.33% 0.53%	SSF with birch [a] malt extract broth malt extract agar

[a] depended on age of mycelium

The maximum amount of biomass observed in the present study is approximately 0.5 gram biomass per gram of dry culture. During the SSF growth of *T. versicolor* on sawdust, Swift (1973) observed a maximum biomass concentration of 0.58 g/g SSC. Jones and Worrall (1995) determined a value of 0.31 g/g SSC for *T. versicolor* on birch wood. As a result, the SSF biomass concentrations found in this project can be considered to be consistent with literature values. It should be noted that this value depends entirely upon the assumption that the glucosamine contents of the biomass from the submerged and solid state cultures are the same.

The PPO does not appear until 4 days, then begins to increase rapidly afterwards. For SSF with *T. versicolor* on wheat straw, laccase did not appear until 5 days and increased linearly afterwards until 40 days (Valmesda et al., 1991). However, for growth on sugarcane bagasse, the maximum laccase activity occurred after 5 days and slowly decreased afterwards (Pal et al., 1995). The appearance of PPO at 4 days during SSF with CM can be considered consistent with the above results. In addition, the maximum activity of 10-12 days also coincided approximately with that found during the submerged process.

It should be noted that the PPO activity could not be measured directly. As discussed in section 4.3.3, a 10% SSC (w/v) suspension (3g wet SSF culture was added to 30 mL water) was shaken for 1 hour. After centrifugation, the clear supernatant was used to measure enzyme activity and sugar content. During the measurement of the enzyme activity early in the process, at low PPO concentrations, when the extracted enzyme was added to the buffer solution without dilution, the solution became cloudy and the loss in absorbance of ferulic acid could not be measured. However, by 4 days, the PPO was sufficiently concentrated so that a 10 times dilution of the enzyme solution could be used and the precipitate avoided. As a result, it cannot be concluded that there was no PPO activity before 4 days, just that the activity was not detectable until this time. During a study by Katagiri *et al.* (1995), laccase could not be measured in an extracted solution during fungal SSF biobleaching of pulp. However, when incorporating the pulp into the enzyme assay, laccase activity was observed. This indicated that the extracellular enzymes were adsorbed onto the pulp. Taking this into consideration, it is also possible that, before 4 days, there are low levels of PPO produced, but it is entrained within the CM and cannot be extracted. At later times, 4 days, there is a sufficient amount of PPO so that some can be extracted.

The major form of soluble sugars in CM are the non-reducing carbohydrates. Bell (1993) indicates that CM contains 2-3% starch, 7-8% sucrose and approximately 5% cellulose. The soluble sugars from the CM used in this project were analyzed using HPLC (Table 5-1) and the major sugar was sucrose (7%) followed by stachyose (2.6%) and raffinose (0.85%). Since glucose is a reducing sugar and these three are non-reducing sugars, the two types of sugars can be measured separately. Furthermore, the non-reducing sugars can be transformed into reducing sugars using enzymes such as invertase, which catalyzes the hydrolysis of sucrose into glucose and fructose. While there appears to be some glucose initially present, this is more likely due to the colourometric interference of the extracted solution. During analysis, the reducing sugars, glucose and

fructose, were measured then the sample was hydrolyzed to determine the total amount of soluble sugars.

Figure 6-1 shows that the glucose concentration initially increases with time. Since the fungus uses glucose for growth during submerged fermentation, upon first glance it would be expected that the glucose concentration would also decrease during SSF. However, since the majority of the CM sugars are in the non-reducing form (e.g. sucrose), these are first hydrolyzed by the fungus into glucose and then used for growth. As a result, as the sucrose is converted into glucose, the glucose concentration increases and the sucrose concentration decreases. It would be expected that there would be a much slower initial decrease in sucrose consumption, due to the lag phase. However, this was not observed, possibly due to the transfer of invertase with the inoculum. The fungus, which can produce invertase (Lacki, 1997), grows in the form of pellets in submerged cultures. Yet, despite the fact that the pellets are thoroughly washed with water prior to homogenization, the broth entrapped within the pellets cannot be removed. This small amount of culture broth is released during homogenization and the enzymes are transferred to the culture during inoculation. As a result, there are most likely low levels of invertase initially present and this decreases the sucrose concentration.

The final observation concerning the sugars is that the residual level of the sugars does not reach zero. The reasons for this are the same as in the submerged cultures, namely interference due to colour of the extracted medium. The initial and final glucose levels are identical, which indicates that the 5 g/g SSC glucose initially present is due to the colour artifact as opposed to initial glucose present.

The decrease in sinapine (SIN) content during a typical fermentation is also shown in Figure 6-1. SIN decreases rapidly over the first 6 days, from a level of approximately 13 g/kg to 0.2 g/kg, a reduction of 98.5%. A residual level of 0.03 g/kg (99.8% decrease) is obtained after 18 days of fermentation. There are two phases in the SIN decrease: the first is enzyme controlled and the second is diffusion controlled. For approximately the

first 6 days, there are large quantities of soluble SIN present, and the enzymes, once produced, can rapidly degrade this SIN. After this time, the process becomes diffusion controlled since the SIN must diffuse from within the particle to the bulk liquid before being able to react with the enzyme.

It should be noted that this is the minimum decrease of the sinapine content. As was done for Lacki (1997), the peak adjacent to SIN on the HPLC chromatograph, iso-ferulic choline, was included in the SIN calculation. This compound is not degraded by *T. versicolor*. During early stages of fermentation, it is very easy to distinguish the two peaks, due to the large differences in height. Once the SIN content has been decreased, there are no longer two separate peaks, but one small, broad peak. Since it was not possible to distinguish between the two peaks, the peaks were combined. This would overestimate the SIN content and hence underestimate the SIN decrease.

6.2 Effect of Temperature on PPO Production, Sugar Levels and Phenolics in SSF

Since the temperature of the culture will affect the growth of the fungus, it was decided to examine this parameter during SSF with *T. versicolor*. If the temperature is too low, growth is often slow, whereas if the temperature is too high, the fungus cannot tolerate the heat and will not be able to grow. Figure 6-2 shows the PPO production for temperatures ranging from 24-40°C. This experiment was performed in triplicate using cultures containing 10 mL/L Vogel's minerals and 60% moisture. Three flasks, each 10 days old, were individually analyzed and the results averaged. The results are presented on both a wet and dry basis. The wet basis can be considered to be the "as is" culture concentration whereas the dry basis can be approximately considered as PPO production per mass of meal. As can be seen, the PPO production is, within error, the same for temperatures of 24-32°C. There are very low levels of PPO at 36°C and no activity at 40°C. It is not known why the errors in PPO activity are so large in this experiment. The low enzymatic activity at 36°C is in agreement with visual observations of very little

growth in the culture. Smits *et al.* (1998) also observed a similar phenomenon during the SSF fermentation of wheat bran using the fungus *Trichoderma reesei*. In that case, the growth rate increased linearly with temperature until 36°C, after which there was a sharp decrease in growth rate with no growth observed at 40°C.

Temperatures between 24 and 32°C do not have a great influence over the final concentration of phenolics in this experiment (Figure 6-3). The final SIN and SA contents are virtually the same and the SAE content is only sightly lower at the higher temperature. It is not surprising that this occurs since it was shown that all have similar enzyme concentrations. There are decreases of 98%, 96% and 87% for the SIN, SA and SAE concentrations, respectively.

On the other hand, the phenolic contents of the higher temperatures are higher. This is due to the lower enzyme production. There is still some decrease compared to untreated CM (the initial values for SIN and SAE are 13 and 22 g/kg with an initial SA concentration of approximately 200 mg/kg). This can be attributed to transfer of small quantities of PPO with the inoculum as well as some decrease due to autoclaving.

The final glucose levels are seen in Figure 6-4. The final level of 9 mg/g wet SSC (20 mg/g dry SSC) is approximately the same as seen in Figure 6-1. This most likely corresponds to the background level of sugar due to the coloured culture media. The slight differences in glucose levels can be attributed to experimental error.

These figures illustrate that the temperature, provided it is below the temperature that limits growth, does not have a great influence over the final PPO activity and level of phenolics. However, since biomass concentration was not followed, it is difficult to discuss it further.

6.3Effect of Moisture Content on PPO Production, Sugar Levels and Phenolics in SSF

There is a moisture content that is optimal for microbial growth. If the amount of water is below this, growth cannot be supported whereas higher moisture contents

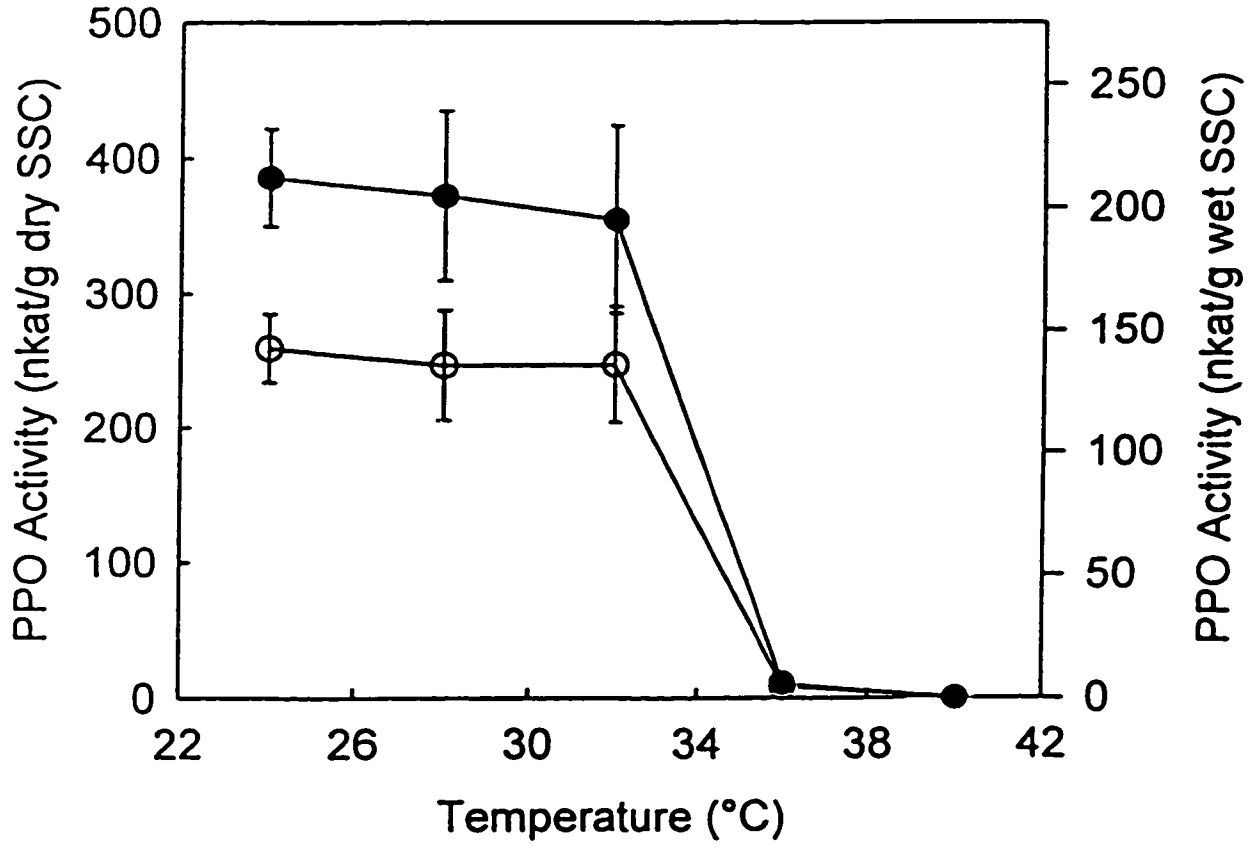

Figure 6-2: Effect of temperature on the production of PPO in SSF with 60% moisture, 10 mL/L Vogel's minerals after 10 days of growth. Open symbols represent PPO on wet basis while closed represent PPO on dry basis

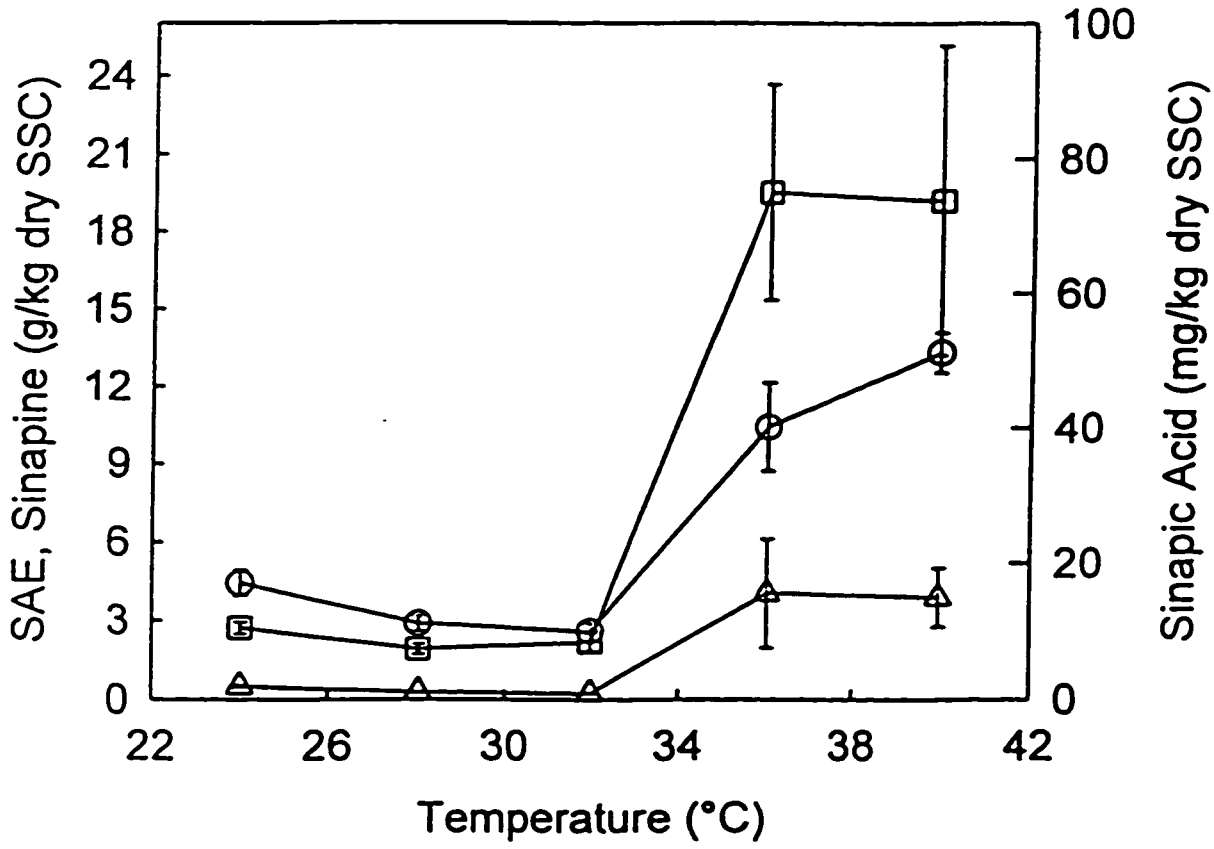

Figure 6-3: Effect of temperature on the residual level of CM phenolics during SSF with 60% moisture, 10 mL/L Vogel's minerals after 10 days of growth; (O)-SAE; (Δ)-sinapine; (□)-sinapic acid

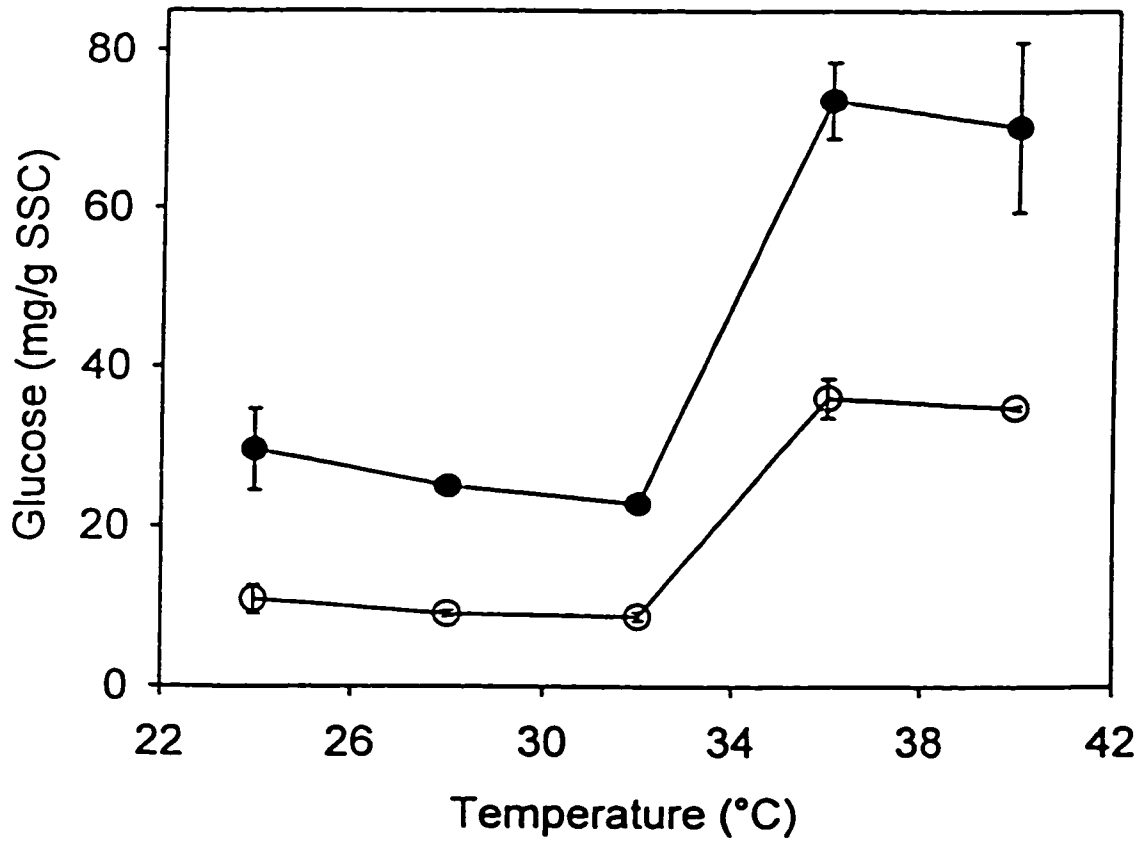

Figure 6-4: Effect of temperature on the residual glucose concentration in SSF with 60% moisture, 10 mL/L Vogel's minerals after 10 days of growth. Open symbols denote wet basis, closed denote dry basis

interfere with gas transfer to the microorganism, which also limits growth. In order to assess the optimum moisture content for *T. versicolor* on CM, three 14 day cultures were used to measure biomass, residual sugar, PPO activity and phenolic content. The results for each moisture content were averaged and the values, as well as the standard deviations are presented in Figures 6-5 to 6-8.

The effect of moisture content on biomass production can be seen in Figure 6-5. The biomass concentration has been represented on both a dry and wet basis. The maximum moisture content used was 80% since higher aqueous concentrations made the culture a slurry instead of a solid phase. At 30% moisture, there was very little growth visually observed and this is in agreement with Figure 6-5.

It can be seen that the maximum amount of biomass on a wet basis is at 40% moisture and this decreases until 80% (Figure 6-5). On the other hand, the amount of biomass per gram of dry culture is approximately constant from 50%-65%. This shows that the maximum growth occurs at a moisture content of 50-65%. This is in agreement with the optimum moisture contents (55% and 53%, respectively) for the growth of the fungi *T. versicolor* on wheat straw and *Aspergillus carbonarius* on CM (Yadav and Tripathi, 1991; Al-Asheh, 1993).

Figure 6-6 shows the effect of moisture content on residual sugar content. It can be seen that all moisture contents above 40% decreased both the reducing (i.e. glucose-type) and non-reducing (i.e. sucrose-type) sugars to approximately the same levels.

Despite the fact that there was some growth at 40% moisture, the conditions were unfavourable for PPO production (Figure 6-7). Similarly, there was very little enzyme production at 50% moisture and the amount increased with moisture content. It was expected that, as for biomass, there would be an optimum moisture content for PPO production and that this would be around 50-60%. In fact, it was quite unexpected that the PPO production would increase with moisture content up to 80%. It was initially

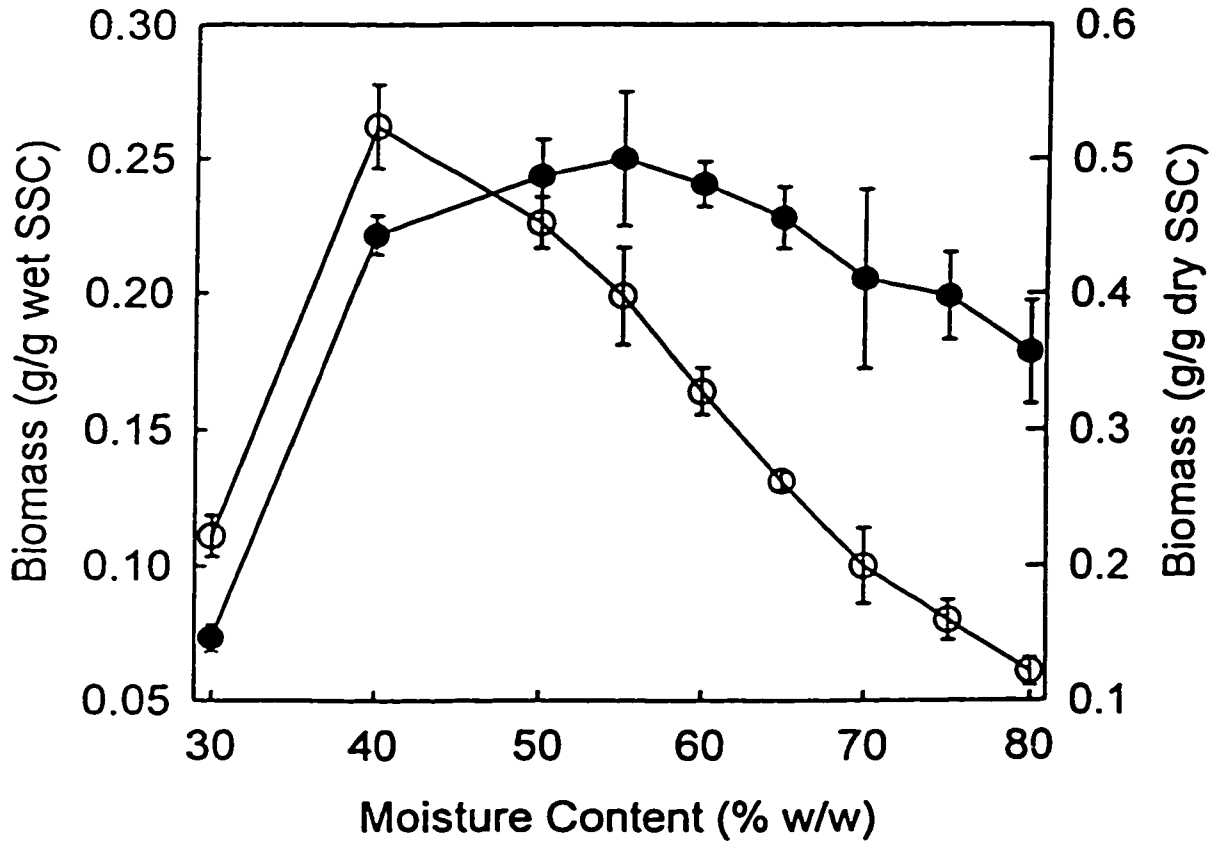

Figure 6-5: Effect of moisture content on biomass production in SSF after 10 days. Open symbols denote wet basis, closed denote dry basis

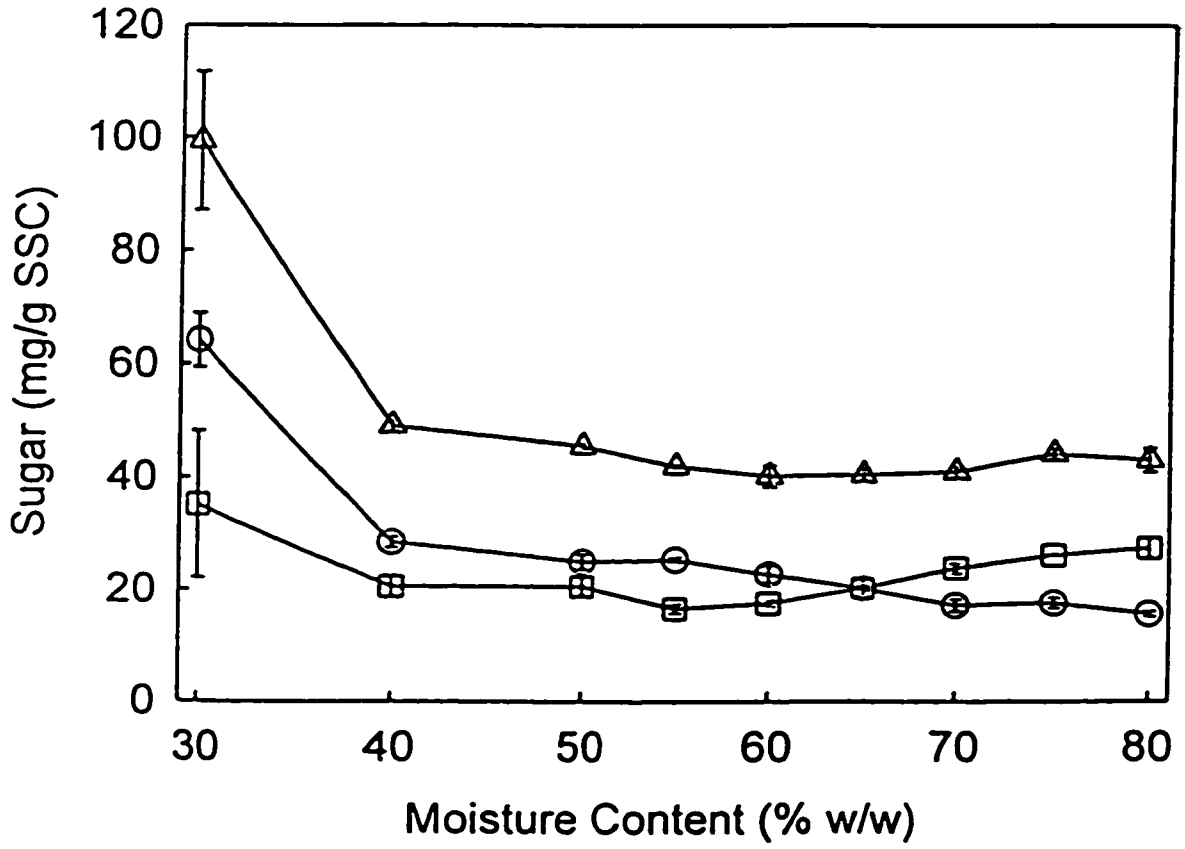

Figure 6-6: Effect of moisture content on the residual sugar concentration in SSF (mg/g dry SSC): (O)-glucose ; (□)-non-reducing sugars; (Δ)-total soluble sugars

believed that such high levels would interfere with oxygen transfer as well as being furthest from the fungus' "natural" habitat; however this was not observed. Since the submerged fermentations with CM gave high enzyme production, the results are explainable. The reason may be that the water facilitates the diffusion of substances that stimulate PPO production from the CM to the fungus. Alternatively, the water could help to diffuse the PPO from the area surrounding the fungus, causing the biomass to produce larger quantities of the enzyme. It is likely that the large increases in PPO production due to moisture would be due to a combination of these two phenomena. It can be concluded from this experiment, that in terms of PPO production, increasing the moisture content from 60% to 80% results in almost a 4 times increase in culture productivity and an almost 7 times per unit CM.

The effect of moisture content on residual phenolic content of the cultures can be seen in Figure 6-8. As expected, there is very little decrease on SA, SIN and SAE at 30% moisture. The decrease that does occur is due to autoclaving (Lacki, 1997) and the decrease due to enzymes transferred with the inoculum. Although there was apparently no PPO present at 40% moisture, there is still a large decrease in phenolic content. This is most likely due to having a very low PPO content that was not detectable at these low levels due to spectrophotometer interference. Since there has been a long contact time, 10d, even with the low PPO concentration, the phenolic content decreases. However, due to the low moisture content, there is likely very little diffusion of SAE from the meal to be contacted with the PPO. As a result, it is likely that there are some areas near the fungus containing zero phenolics and other areas, having high phenolic content, that have not been in contact with the PPO. At 50% moisture, there are slightly higher enzyme concentrations and a little more water, hence there is more diffusion of the SAE and PPO, but the same reasoning as 40% applies. At 65% and higher, there is a great deal more enzyme and plenty of water so that all phenolics are decreased to levels greater than 95%. It can be concluded that in SSF with moisture contents above 60-65% the phenolic

content can be decreased to acceptable levels in terms of livestock feed. This is important since the meal is often sold at low moisture levels to decrease transportation costs and reduce risks of contamination. As a result, higher culture moisture contents would mean higher operating costs involved for drying.

6.4 Effect of Vogel's Minerals on PPO Production, Sugar Levels and Phenolics in SSF

As discussed earlier, minerals are important for microbial growth and enzyme production. Furthermore, PPO also contains copper as a central atom (Fahraeus and Reinhammar, 1967). For these reasons, it was decided to test the effect of supplementing the solid state culture with Vogel's minerals on PPO production and SAE decrease. It was found that the addition of Vogel's minerals to the submerged, CM-free medium resulted in a decrease in PPO production and was attributed to sufficient mineral transfer with the inoculum. Although CM contains some minerals, it is possible that supplementing CM with minerals could further stimulate PPO production. The Vogel's minerals solution was added to the liquid phase of a 60% moisture culture in concentrations of 0, 1, 10 or 50 mL/L. Two flasks for each time and each concentration of Vogel's minerals were analyzed and the averages and standard deviations plotted. The control medium used for the previous tests contained 60% moisture and 10 mL/L Vogel's minerals.

Figure 6-9 illustrates the influence of the Vogel's minerals concentration on PPO production. Contrary to submerged fermentation, the addition of Vogel's minerals stimulated PPO production. It should be noted that there is not a large difference for concentrations of 1mL/L and 10 mL/L, however, the trend is still evident. The PPO activity of the 50 mL/L culture at 10 days is likely lower due to an experimental error, for example, not adding the correct amount of inoculum.

The decreases in the SIN and SA content during the fermentation are shown in Figures 6-10 and 6-11, respectively. Although the results are quite similar, it can be seen

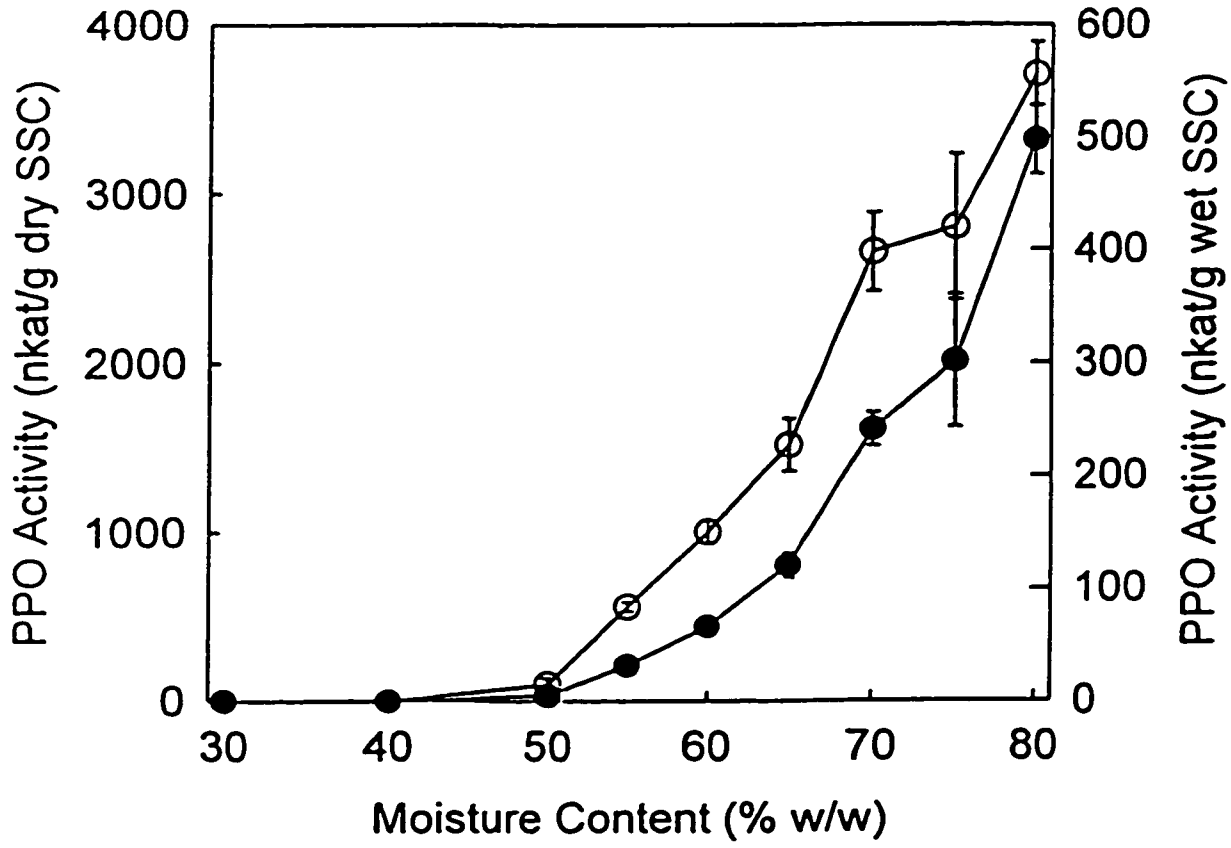

Figure 6-7: Effect of moisture content on the final PPO activity in SSF at 30°C with 10 mL/L Vogel's minerals. Open symbols represent PPO on wet basis, closed on dry basis

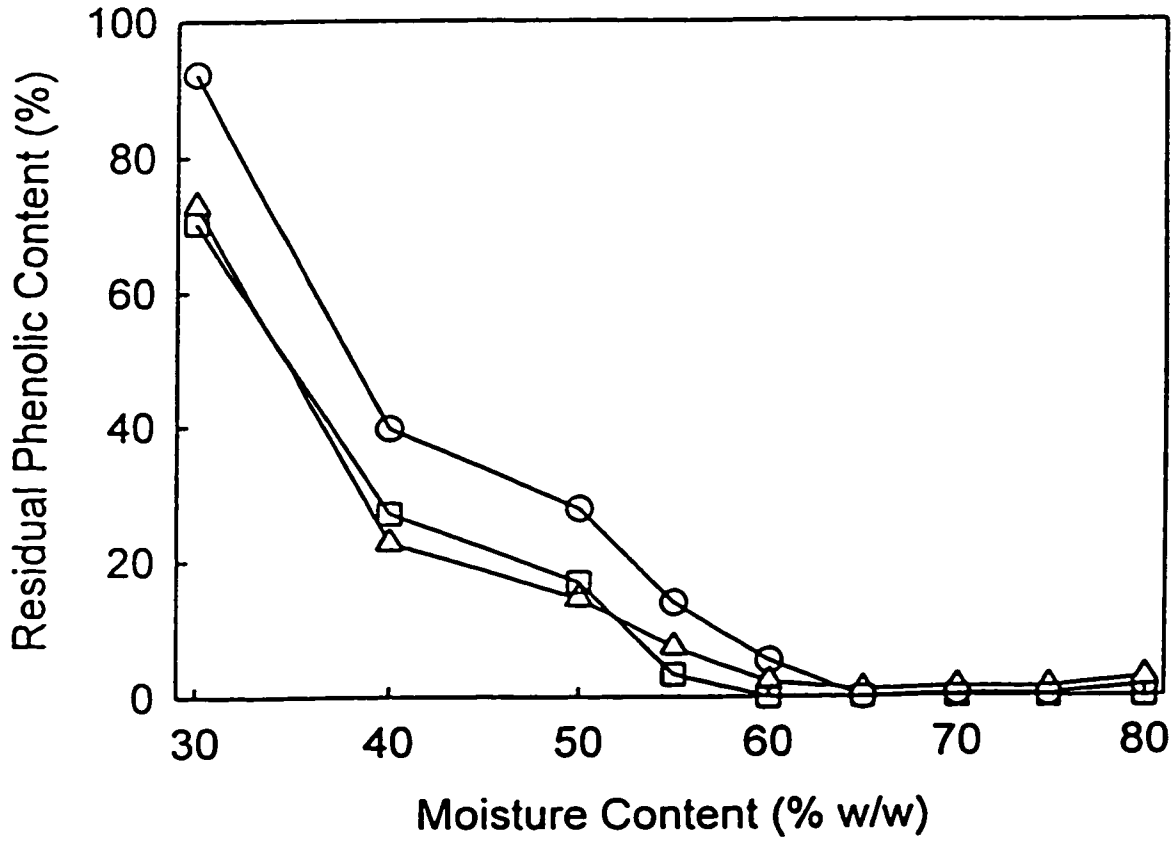

Figure 6-8: Effect of moisture content on the residual level of CM phenolics at the end of a 10 days SSF; (O)-SAE; (Δ)-sinapine; (□)-sinapic acid

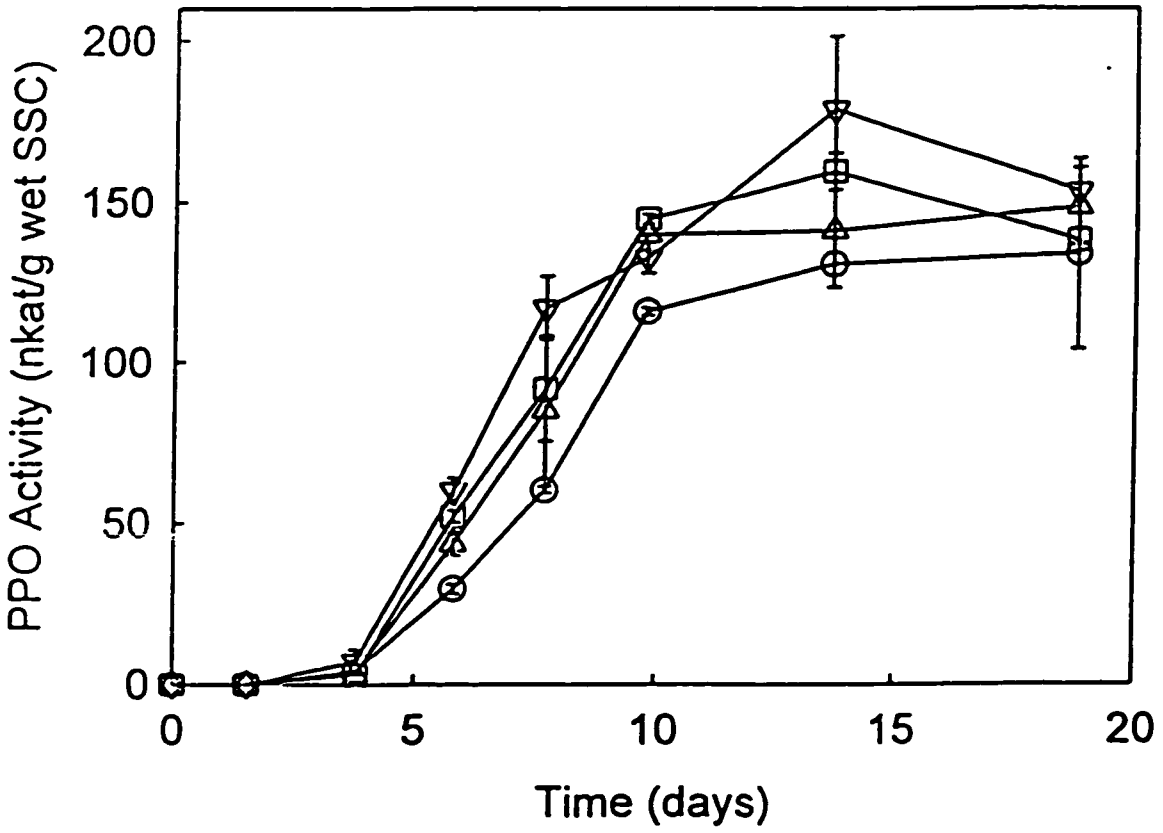

Figure 6-9: Effect of Vogel's minerals on PPO production in SSC; Vogel's minerals concentration (mL/L of the liquid used for the SSC): (O)-0; (□)-1; (Δ)-10; (∇)-50

that the 50 mL/L Vogel's minerals concentration shows the fastest decreases in the phenolic content due to the higher PPO levels. There are no differences in the final concentrations of phenolics, due to each culture having sufficient enzyme to catalyze the reactions. In all cases for both SA and SIN, the decrease in phenolic content was greater than 97%.

6.5 Effect of Inoculum Size on PPO Production, Sugar Levels and Phenolics in SSF

The amount of inoculum can often affect the performance of a microbial process. When there is a large initial biomass concentration, the fungus can reach its maximum concentration more rapidly; However, since there would be a larger percentage of older cells, often the productivity suffers. It was decided to test amount of inoculum during a solid state process using *T. versicolor* at a moisture content of 60%. Two flasks for each data point were analyzed separately and the results averaged.

The effect of inoculum size on PPO production can be seen in Figure 6-12. Within experimental error, all inoculum concentrations have the same initial increases in PPO production. It is also evident that, after 8 days, the lower inoculum size does indeed have the highest PPO levels. It has been shown previously in this study that PPO is produced during the growth phase of the fungus. Furthermore, it is also likely that the cultures will all reach similar final biomass concentrations, since all have same initial concentration of nutrients. As a result, the lower inoculum concentrations will have a higher percentage of newer (*i.e.*, growing) biomass and thus more PPO would be produced. It was shown that, for glutamic acid production, the larger inoculum size had initially faster production, however the smaller inoculum size eventually resulted in a higher productivity (Nampoothri and Pandy, 1996). During the decrease in phytic acid content of CM, Al-Asheh (1993) showed that larger inoculum sizes resulted in faster decreases. However, as long as the inoculum size was greater than the minimum required size, a 100% decrease in the phytic acid content was found between 43 and 53 hours.

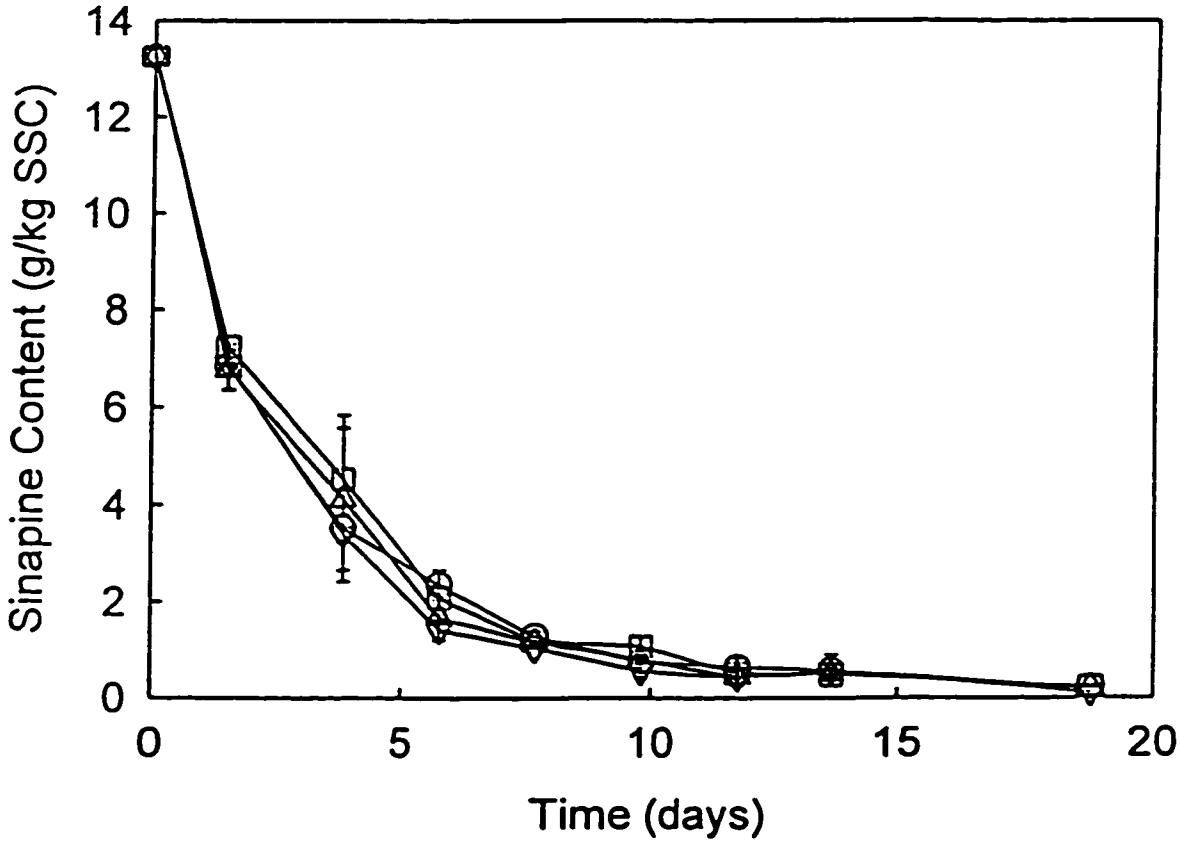

Figure 6-10: Effect of Vogel's minerals on sinapine content decrease in SSF; Vogel's minerals concentration (mL/L of the liquid used for the SSC): (○)-0; (□)-1; (△)-10; (▽)-50

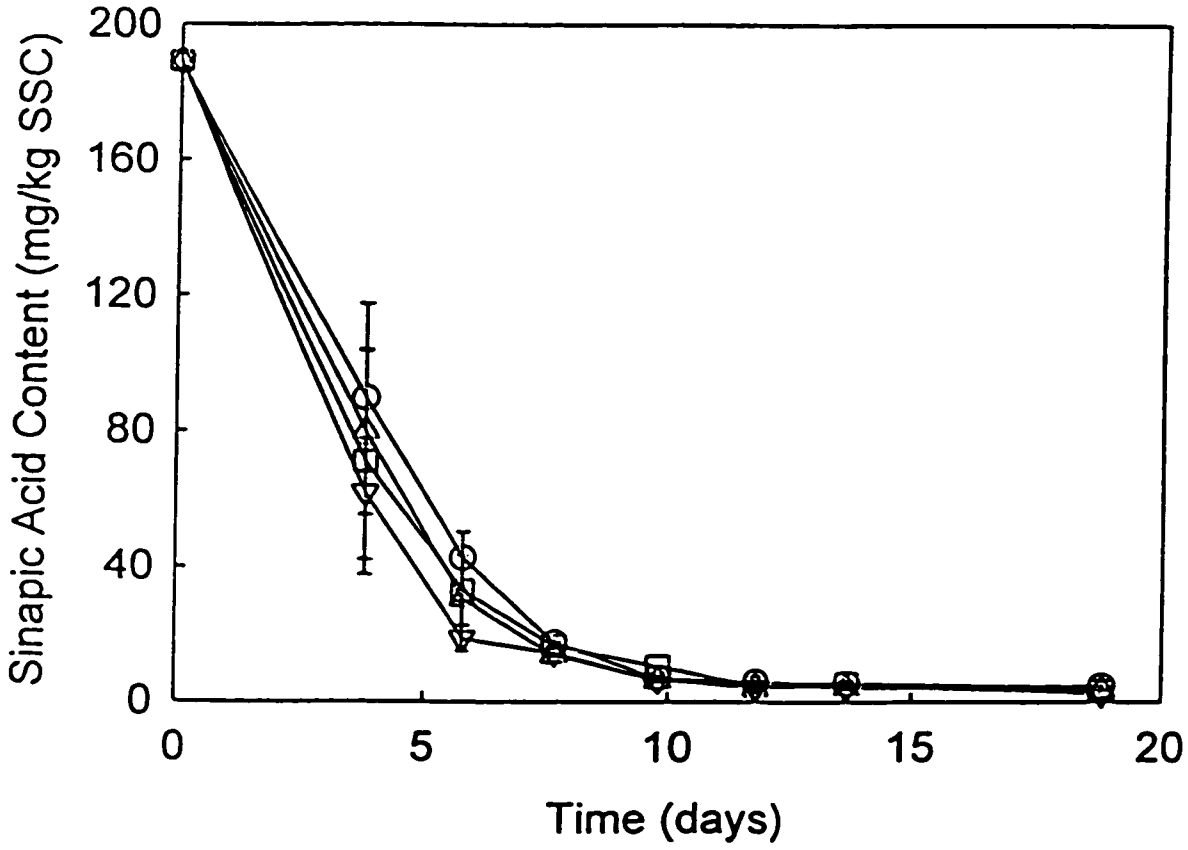

Figure 6-11: Effect of Vogel's minerals on sinapic acid content decrease in SSF; Vogel's minerals concentration (mL/L of the liquid used for the SSC): (○)-0; (□)-1; (△)-10; (▽)-50

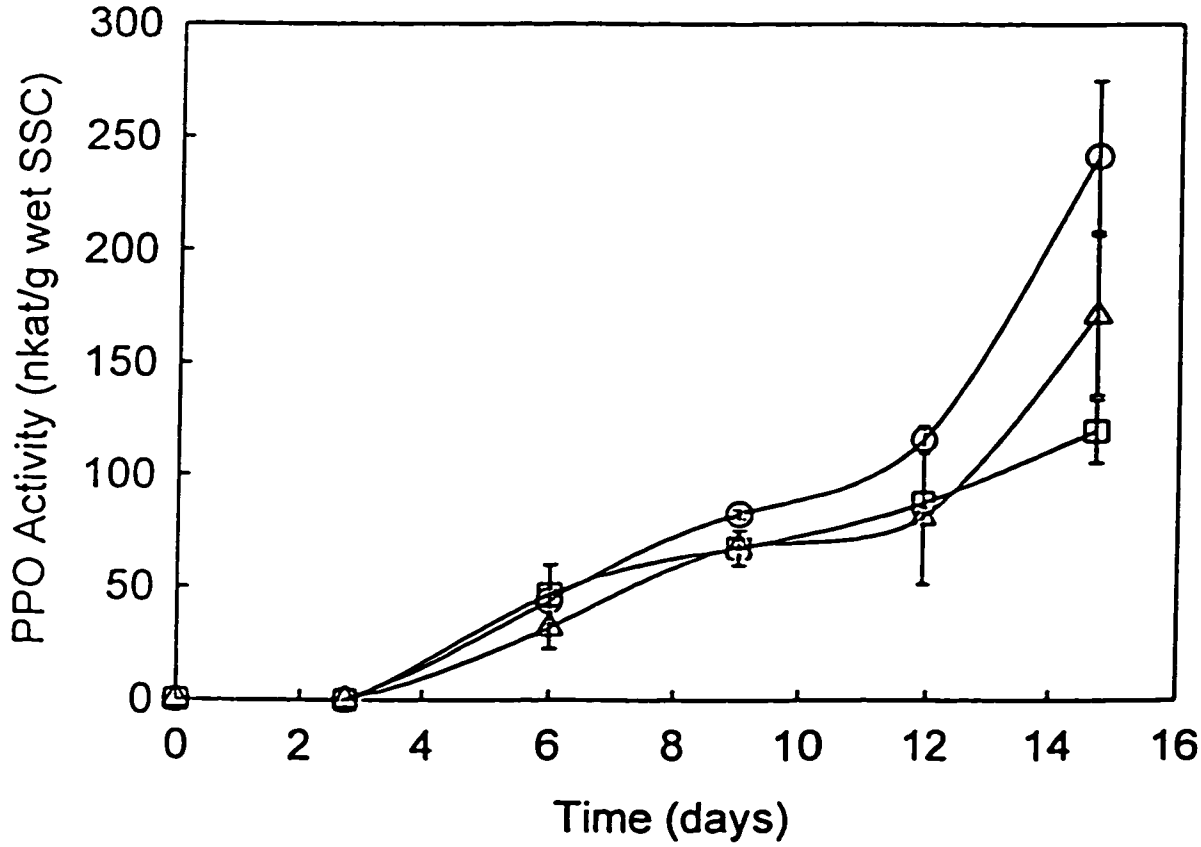

Figure 6-12: Effect of inoculum size on PPO production during SSF on CM; Inoculum added: (○)-2.5 mL; (□)-5 mL; (△)-10mL

In terms of phenolic content, it can be seen that the largest inoculum size has the fastest initial decreases in SAE, SIN and SA (Figures 6-12, 6-13 and 6-14). The reason for this has nothing to do with actual growth, but is a result of the PPO in the inoculum. With an increase in inoculum size, more PPO was introduced initially into the solid medium and that resulted in the fastest initial SAE decrease. In addition, with a larger amount of inoculum, the enzyme was better distributed throughout the meal. The decreases in SAE, SIN and SA are 95%, 98% and 97% respectively for all inoculum sizes after 15 days.

6.6 Effect of Homogenization on PPO Production, Sugar Levels and Phenolics in SSF

Homogenization is an important parameter during fermentation processes with mold. Unlike bacteria and yeast, which usually exist as individual cells, cells of molds grow in the form of mycelia. As a result, molds very often tend to grow in pellets or clumps. When grown in submerged media with agitation, *T. versicolor* will form pellets, usually less than 1cm in diameter. In order to ensure adequate distribution of the inoculum, the pellets must be dispersed. This is done using a blender which shears the pellets apart under high speed mixing and provides numerous centres for growth. Unfortunately, this process will disrupt some of the cells and may cause slower initial growth, as was observed with the SSF growth *of Aspergillus carbonarius* on CM (Al-Asheh, 1993). Furthermore, the cultures homogenized for longer times, although initially grow slower, often are more effective due to having a greater number of growth sites. The liquid inoculum for the control cultures during SSF was homogenized for 30 seconds since this was found to be optimum for submerged growth (Lacki, 1997).

The effect of homogenization time of the inoculum on the production of PPO and decrease in phenolic content is shown in Figures 6-16 to 6-19. The PPO activity is first detected at about 3 days and increases linearly until 15 days (Figure 6-16). It can be seen that homogenizing for 30 seconds appears to be initially best, but by 12 days all

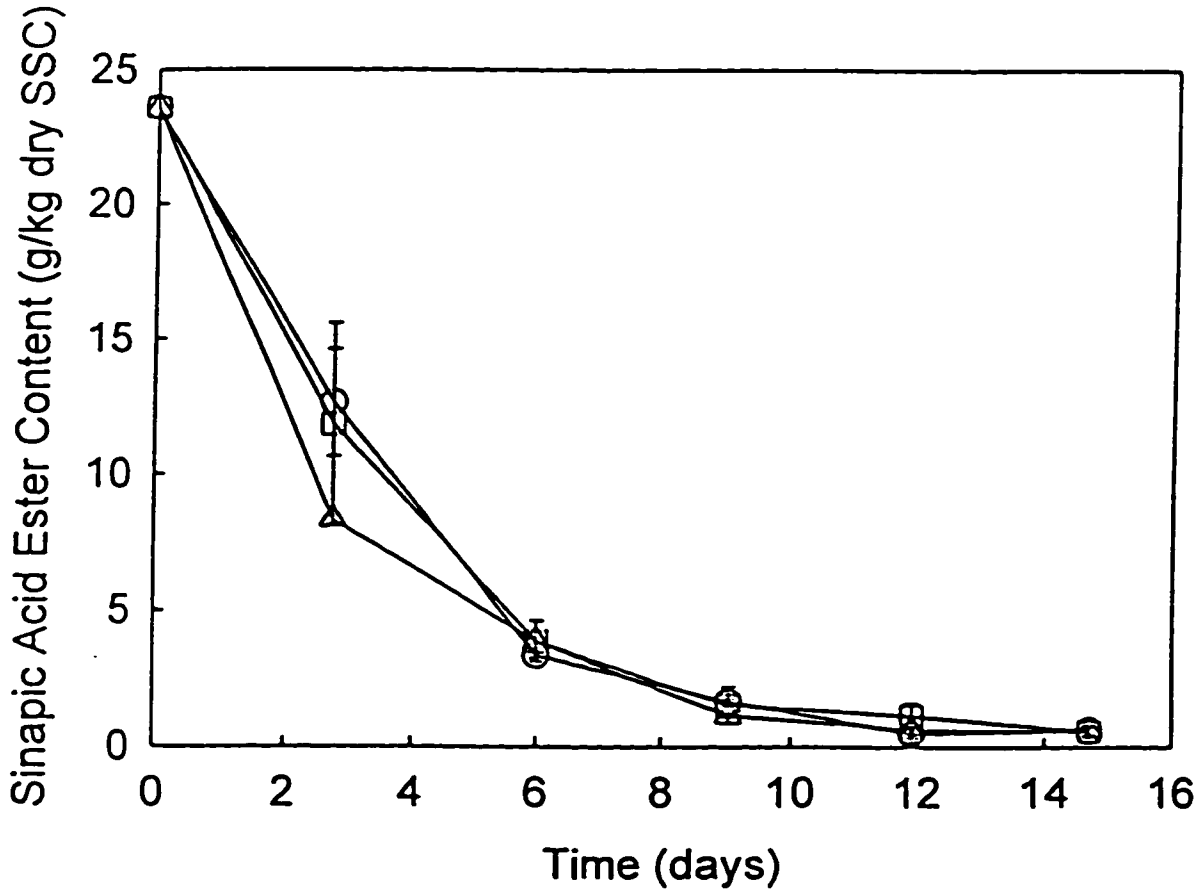

Figure 6-13: Effect of inoculum concentration on SAE content decrease in CM during SSF. Inoculum added: (○)-2.5 mL; (□)-5 mL; (Δ)-10 mL

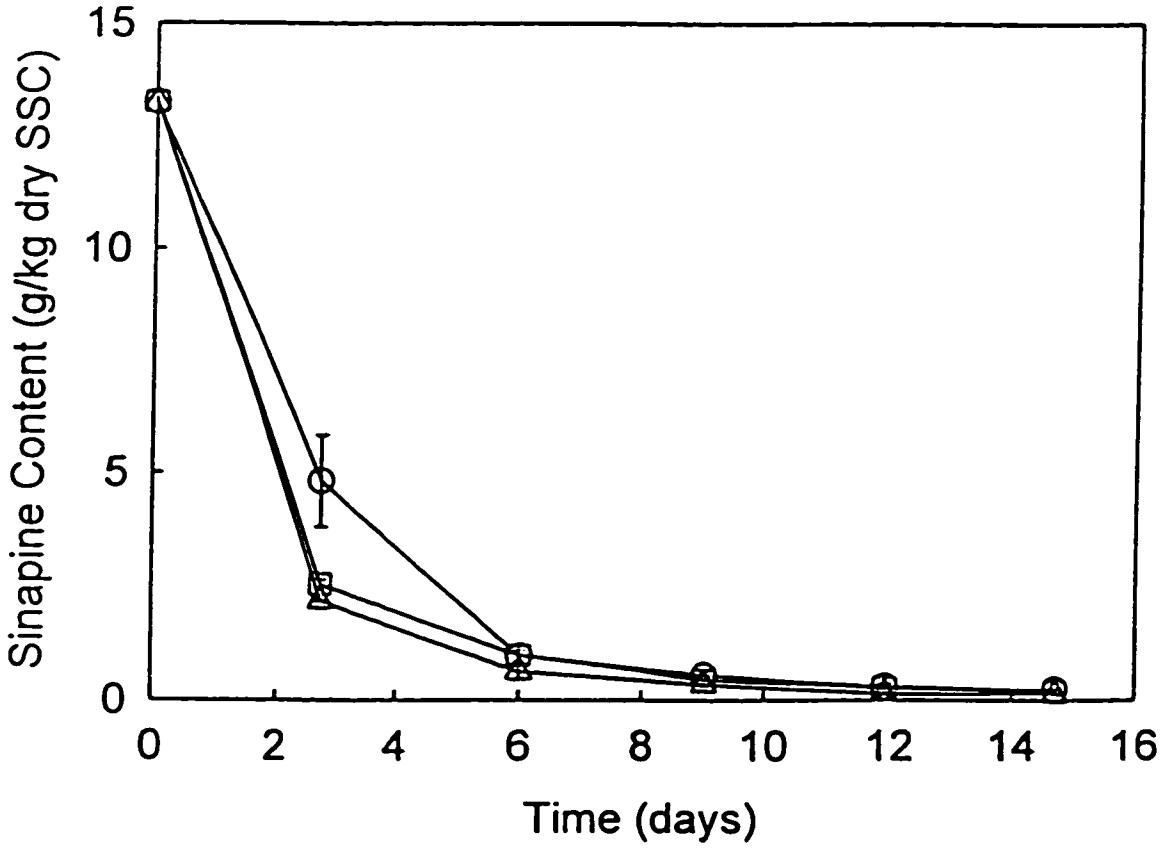

Figure 6-14: Effect of inoculum concentration on sinapine content decrease in CM during SSF; Inoculum added: (○)-2.5 mL; (□)-5 mL; (Δ)-10 mL inoculum

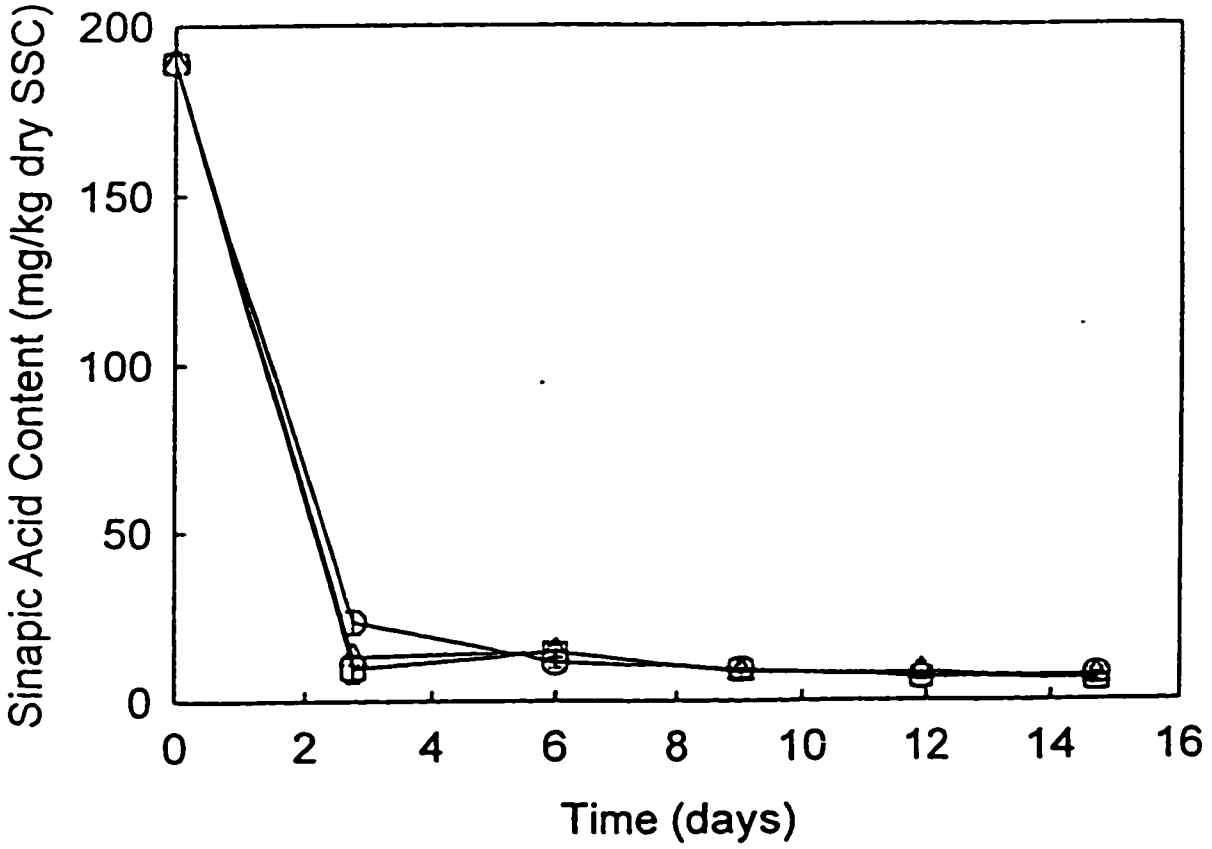

Figure 6-15: Effect of inoculum concentration on sinapic acid content decrease in CM during SSF; Inoculum added: (○)-2.5 mL; (□)-5 mL; (Δ)-10 mL

homogenization times have the same PPO activity. As a result, it can be stated that this fungus is a very hearty microorganism and as a result homogenization is not an important parameter for PPO production.

The initial decrease in phenolic content appears to be fastest with the longer homogenization times (Figures 6-17, 6-18 and 6-19), however this could be due to releasing more enzyme from the biomass during homogenization. At low homogenization times, the mycelia are not disrupted as much as at longer times and would not release as much enzyme as if the biomass were completely disrupted. Since, after 3 days, all cultures have the same level of phenolics, once again, homogenization does not seem to be overly important for phenolic content decrease. The SAE, SIN and SA levels in all cultures decrease to the same final levels, regardless of length of homogenization: 95%, 98% and 92%, respectively.

6.7 Effect of Oilseed Meal Type on PPO Production During SSF

Since the results have shown that CM is a good substrate for the production of PPO, it was decided to compare CM, soybean meal (SBM), sunflower meal (SFM) and safflower meal (SWM) in a solid state process for the production of PPO. This preliminary experiment was performed using four, 13 day old cultures of SBM, SFM and SWM. Only 3 CM cultures were used due to a very low PPO level in one culture. Since the cultures had differing moisture contents, the data have been presented on both a wet and dry basis.

It can be seen that the CM cultures had the highest levels of PPO, followed by SWM and SFM, with very little production in the SBM cultures (Table 6-1). Although visual inspection showed similar biomass growth in all cultures, and since the SBM added to submerged media enhanced PPO production, it is unclear why the level of PPO was very low in the solid state process. In a separate experiment using only one SBM culture, a PPO activity of 24 nkat/g dry SSC at a moisture content of 66% was obtained.

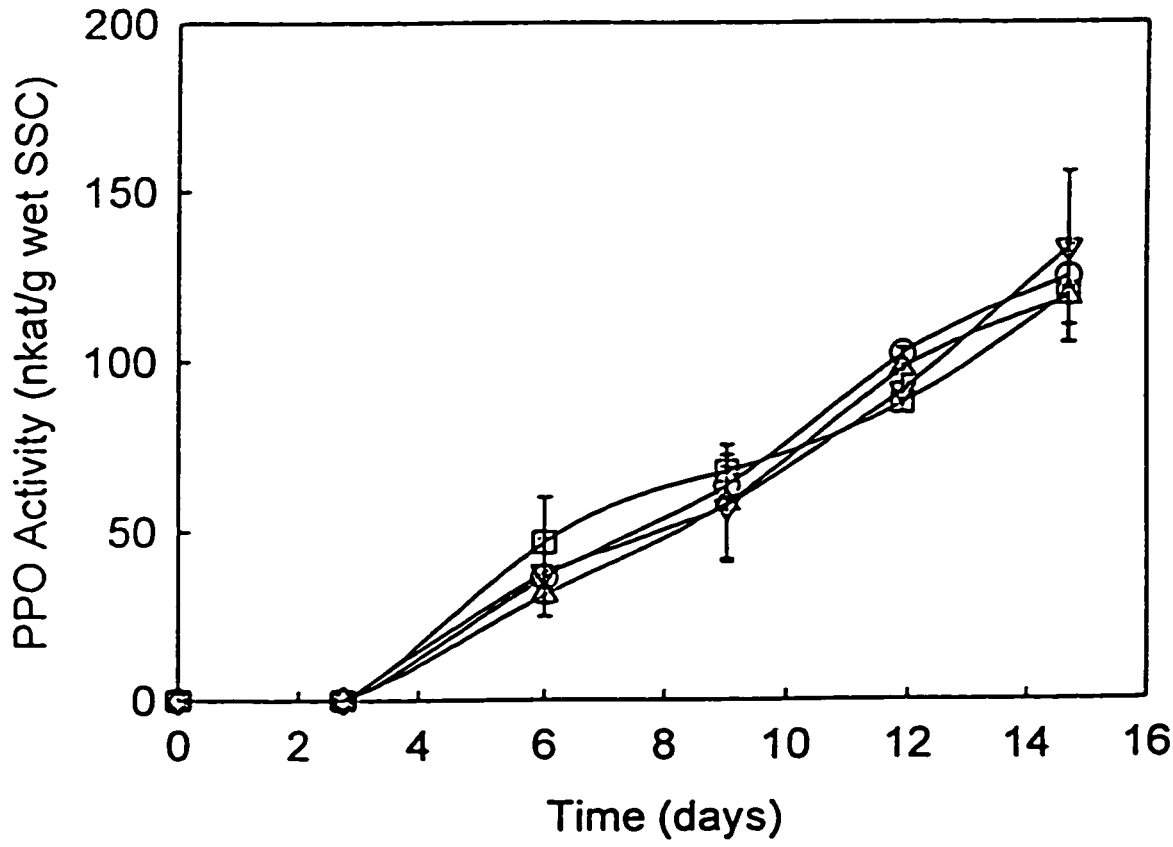

Figure 6-16: Effect of time of homogenization on PPO production during SSF on CM; Time of homogenization (s): (○)-10; (□)-30; (△)-75; (▽)-150

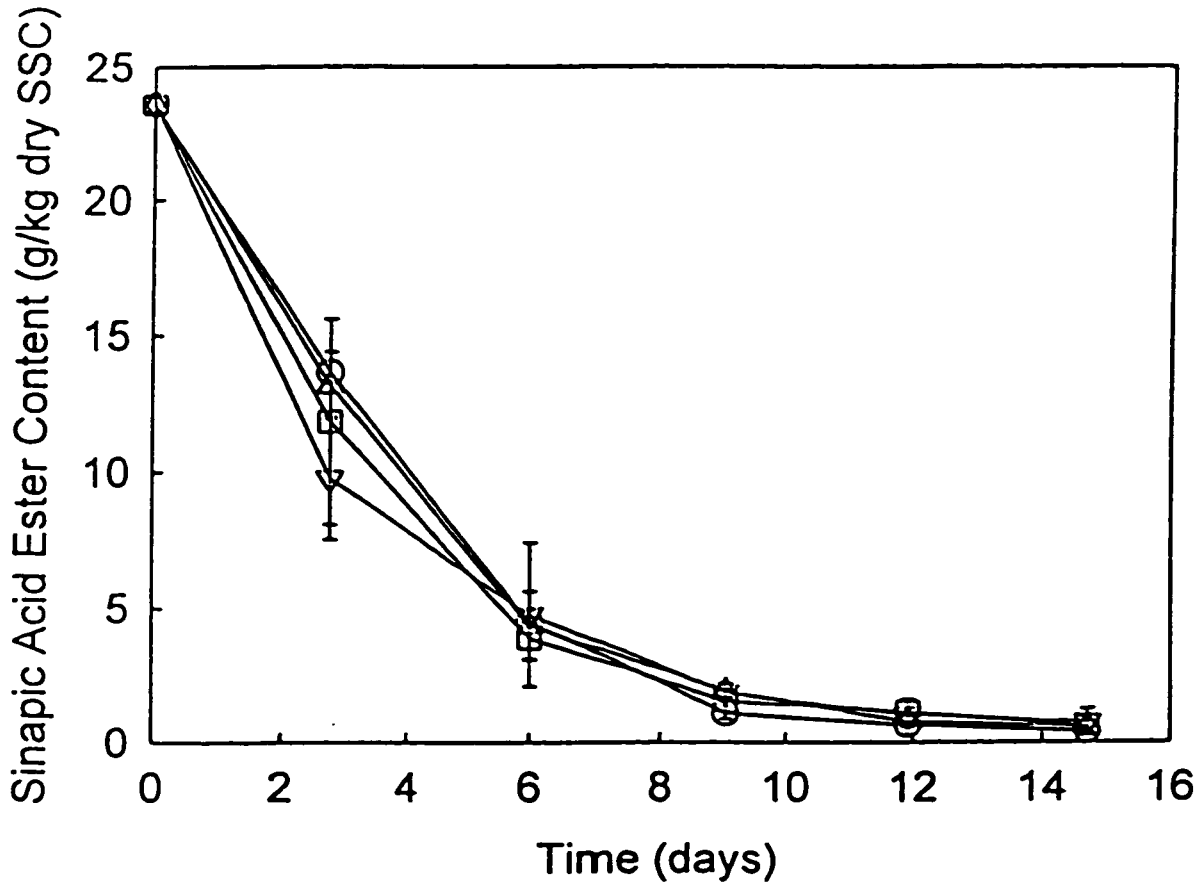

Figure 6-17: Effect of homogenization on SAE content decrease during SSF on CM; Time of homogenization (s): (○)-10; (□)-30; (△)-75; (▽)-150

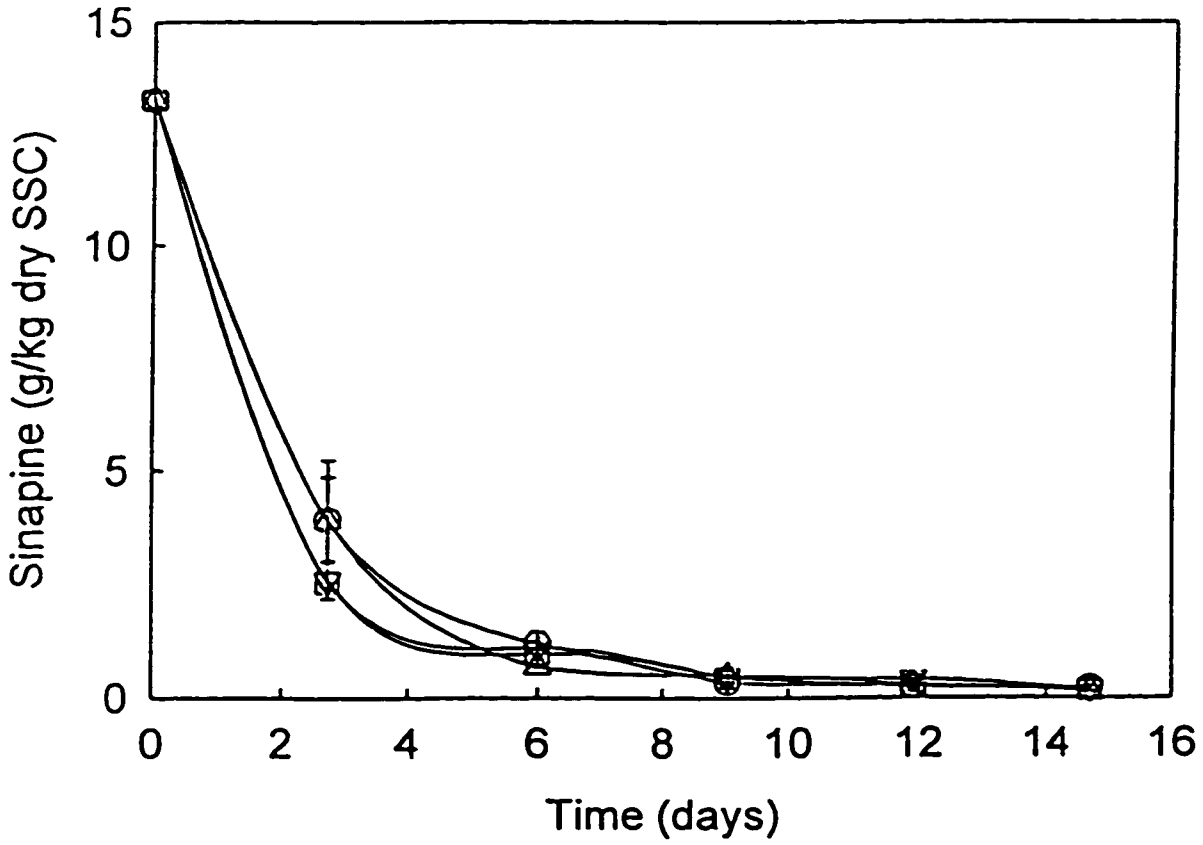

Figure 6-18: Effect of homogenization on sinapine content decrease during SSF on CM; Time of homogenization (s): (○)-10; (□)-30; (Δ)-75; ()-150

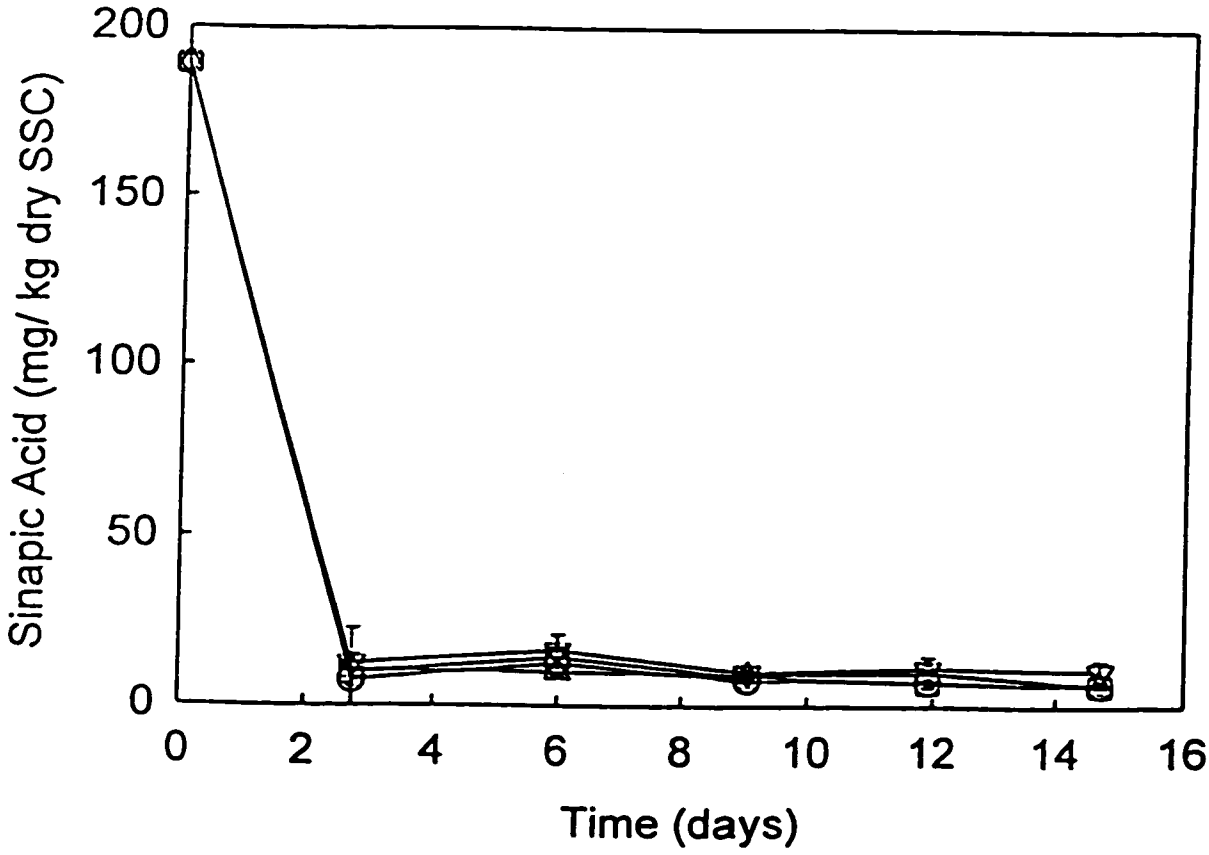

Figure 6-19: Effect of homogenization on sinapic acid content decrease during SSF on CM; Time of homogenization (s): (○)-10; (□)-30; (△)-75; (▽)-150

However, since these experiments were not followed kinetically, it is possible that the SBM culture reached a maximum activity earlier than the CM cultures and thus the fungus could have begun to consume the enzyme. Alternatively, it is also possible that the PPO production in the SBM cultures may not be parallel to biomass growth, as was the case of CM, which could mean that PPO production was just beginning at this sampling time. Finally, it is also possible that the solid SBM culture lacked an essential co-factor for PPO production which was supplied in the submerged media from the nutrient broth or yeast extract.

The SFM and SWM had about equal PPO levels and similar moisture contents, although only about 40% and 47% of the activity of CM, on a dry basis. Since the moisture content was shown to be very important for the production of PPO, it is not clear what effect this may have on the PPO production in SFM and SWM cultures. A separate experiment, using only one SFM culture, obtained a PPO level of 428 nkat/g dry SSC at a moisture content of 61%. Since this value is higher than that obtained at the moisture content of 58% used above, the effect of moisture content on PPO production with SFM and SWM should be studied further.

Table 6-1: Effect of Oilseed Meal Type on the Production of PPO During SSF

	nkat/g wet SSC	nkat/g dry SSC	Final Moisture Content
canola	241 ± 22	785 ± 94	69% ± 1%
soybean	24 ± 7	75 ± 26	67% ± 1%
sunflower	136 ± 60	314 ± 163	58% ± 6%
safflower	162 ± 18	368 ± 62	55% ± 6%

6.8 Flooding Experiments

A typical fermentation requires about 15 days to remove greater than 95% of the phenolic content. Furthermore, after about 5 or 6 days, the rate of decrease in phenolic content slows down considerably, due to the slow diffusion of SAE and enzymes within the SSF culture. It was expected that, once the culture had begun to produce PPO, the

addition of water would facilitate the diffusion of the SAE and hence the decrease in phenolic content. Experiments were performed in an attempt to speed the overall process for improving the meal quality.

A typical SSF containing 60% moisture and 10 mL/L Vogel's minerals was used for this experiment. The biomass, PPO activity and sugar concentrations are shown in Figure 6-20. The SAE, SIN and SA concentrations for this fermentation are shown in Figure 6-21. During the course of the process, samples were taken and water was added to them to obtain suspensions of 20% and 40% (culture in water). These are equivalent to adding 20 g or 40 g culture to 100 mL water and is called flooding.

Figures 6-23 and 6-24 show the concentrations of SAE, SA and SIN in the culture in relation to the length of time since the addition of the water. The data for these figures were calculated by analyzing three samples for each flooding data point separately and presenting the averages and standard deviations. The data set for the sterile culture for both flooding concentrations was obtained from a 20% suspension since it was originally intended to perform 10% and 20% flooding suspensions. It can be seen that at 5 and 7 days, the SAE concentration does not decrease further upon the addition of water. This would indicate that, in this particular batch, flooding was not useful after 7 days. On the other hand, for the 3 day culture, there were decreases in SAE content of approximately 45% and 60% after 16 hours for the 20% and 40% suspensions (Figures 6-22 and 6-23). Although there does not appear to be any PPO present (Figure 6-20) there are very low levels which cannot be measured due to the spectrophotometric interference, as discussed earlier. The 40% suspensions had a larger decrease in SAE since the enzyme and SAE concentrations were greater.

To further investigate the phenolic content decrease, the SAE, SIN and SA contents of each sample for the 3, 5 and 7 day culture was analyzed in addition to the SAE content presented above. Since only the 3 day cultures showed a further decrease in these phenolics, only these results are presented (Figures 6-24 and 6-25). After the 16

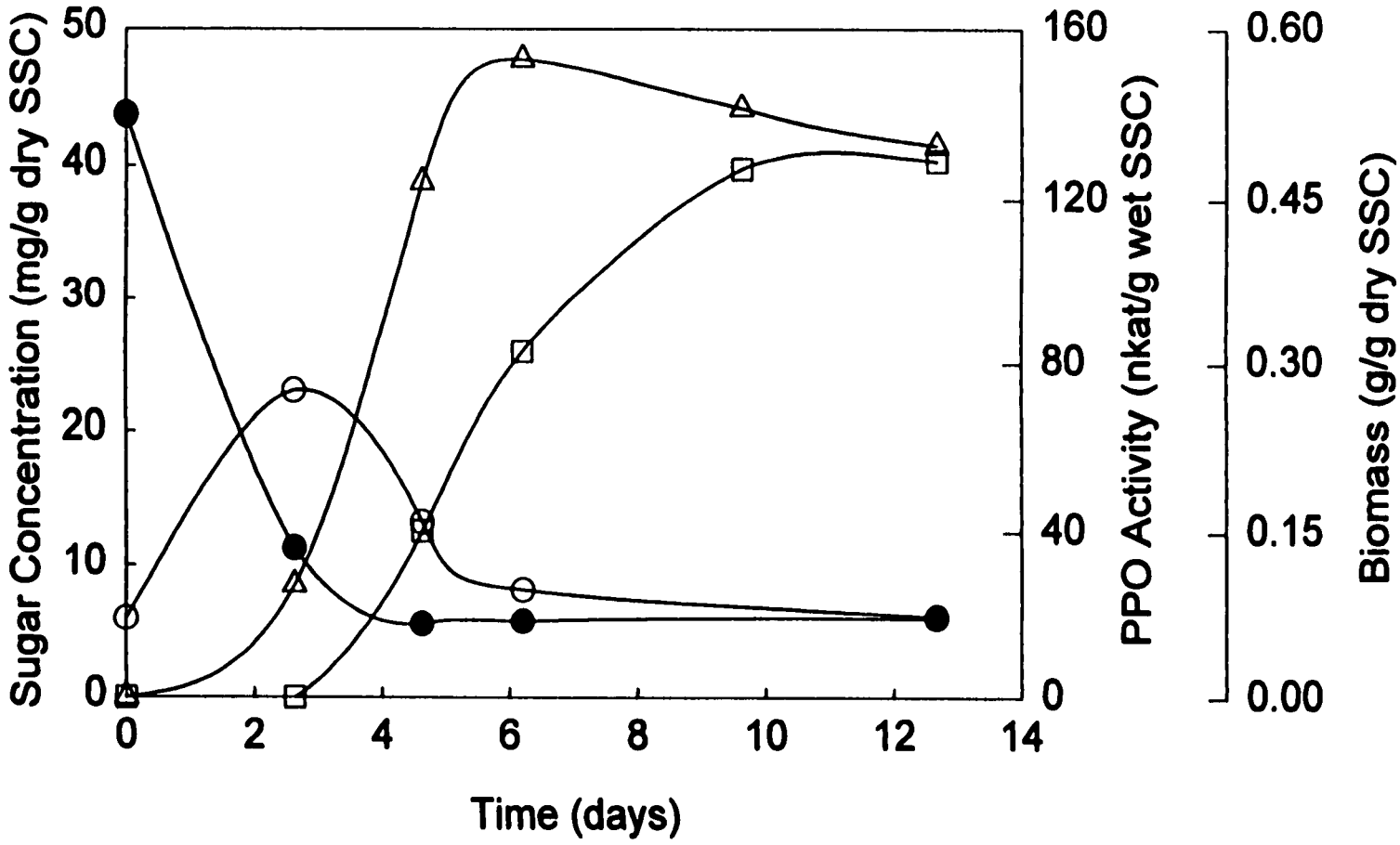

Figure 6-20: SSF fermentation for flooding experiment with 60% moisture and 10 mL/L Vogel's minerals at 29°C; (○)-glucose; (●)-non-reducing sugars; (Δ)-biomass; (□)-PPO

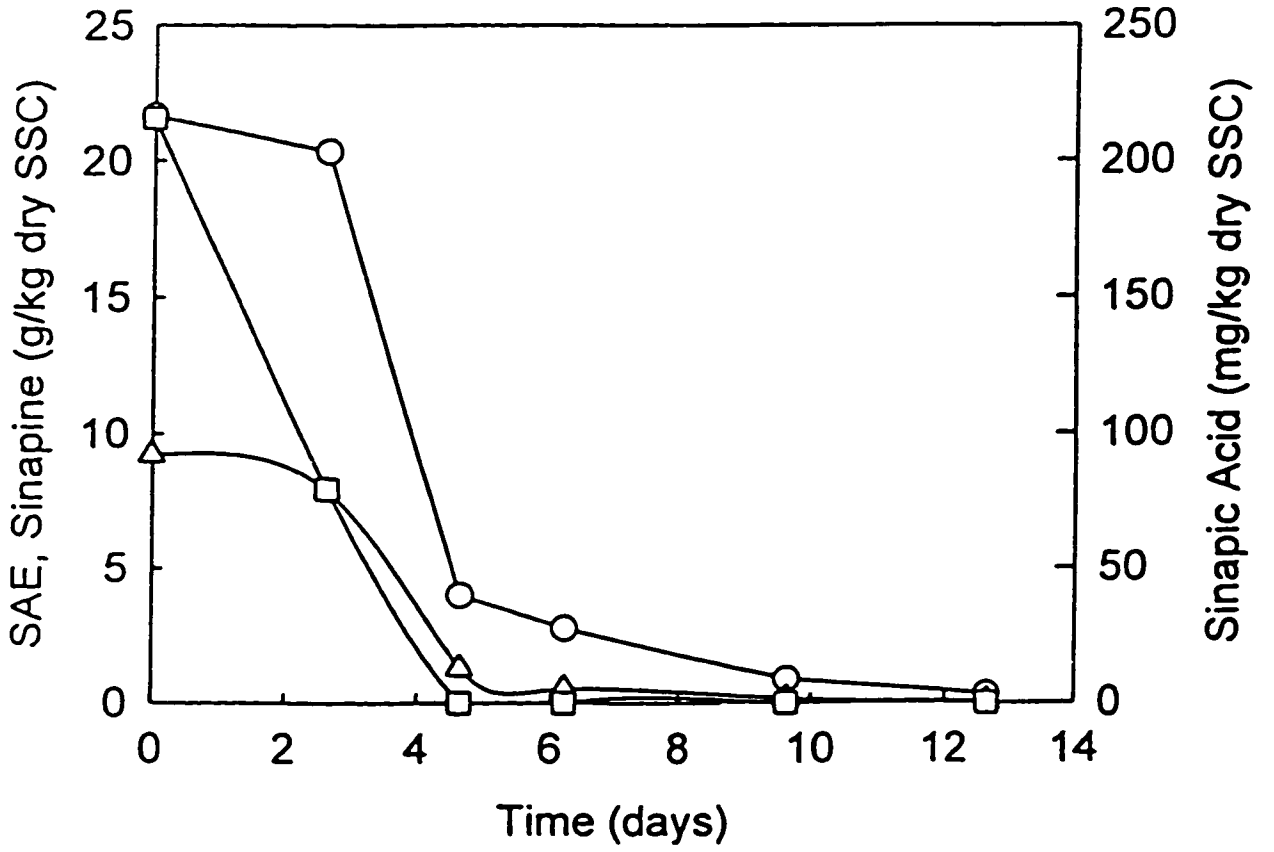

Figure 6-21: Decrease in phenolic content during SSF for flooding experiment with 60% Moisture and 10 mL/L Vogel's minerals at 29°C; (O)-SAE; (Δ)-sinapine; (□)-sinapic acid

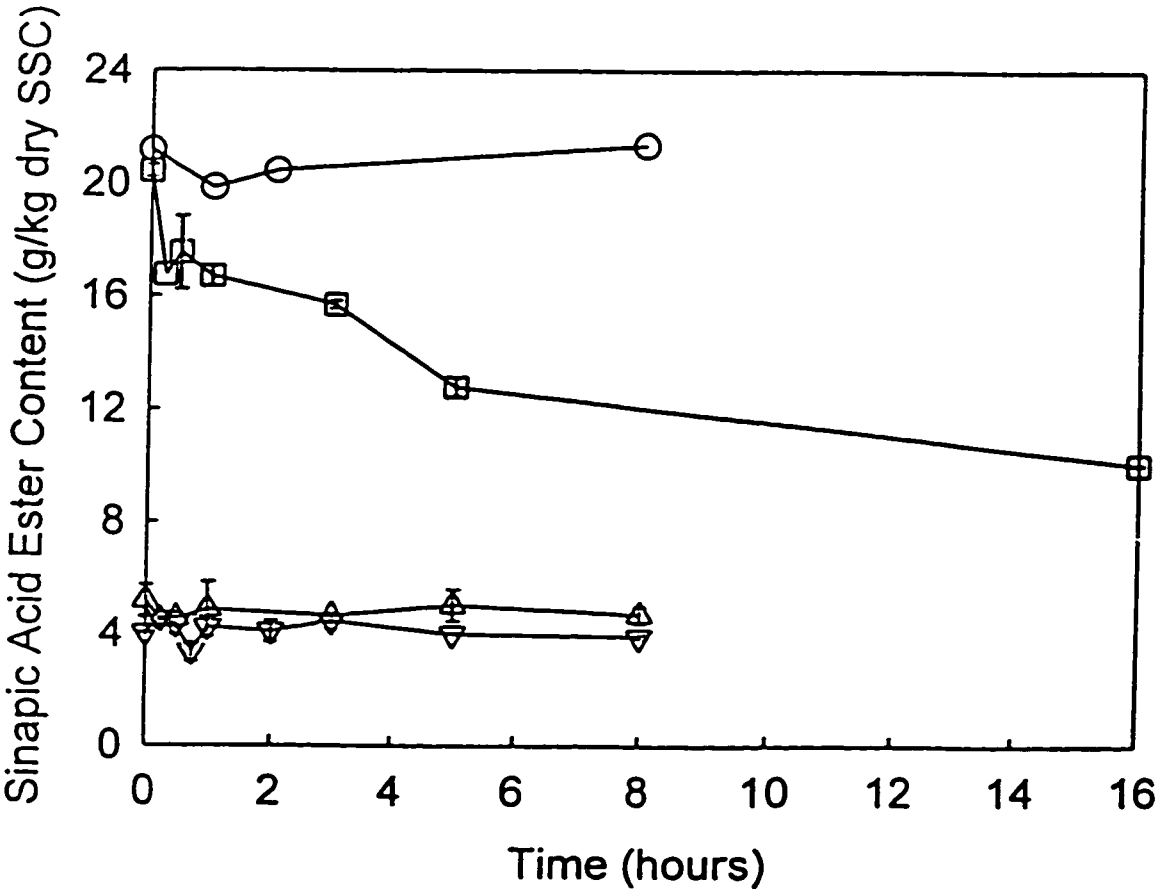

Figure 6-22: Effect of flooding in a 20% (w/v) suspension of culture in water on SAE content; (O)-sterile medium; (□)-3 day culture; (Δ)-5 day culture; (∇)-7 day culture

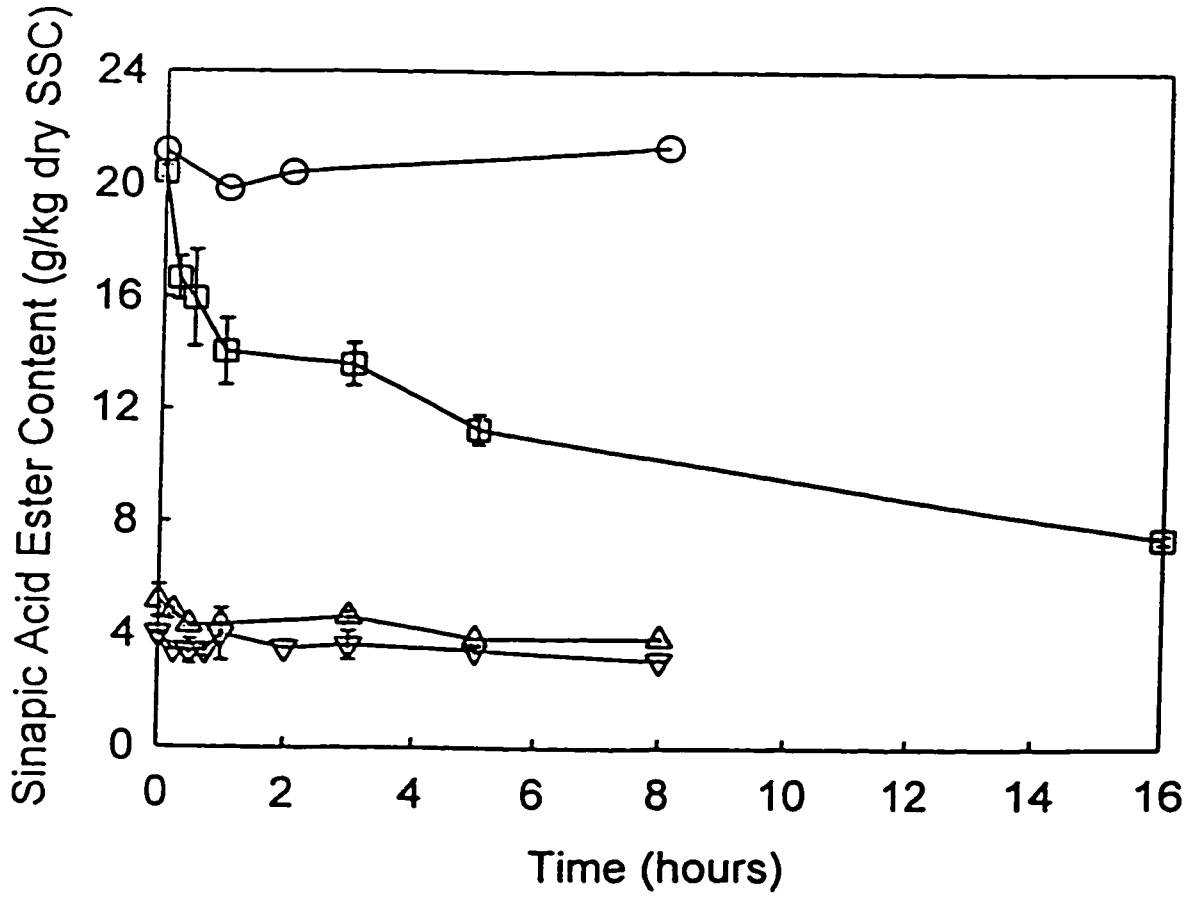

Figure 6-23: Effect of flooding in a 40% (w/v) suspension of culture in water on SAE content; (O)-sterile medium; (□)-3 day culture; (Δ)-5 day culture; (∇)-7 day culture

hour reaction time, the decreases in these components at 20% flooding are approximately: 45%, 50%, and 66%, respectively. For the 40% suspensions, the decreases are approximately 60%, 80%, and 83%. These results show that flooding is effective at speeding up the phenolic content decrease, however not to the final levels that can be obtained during a longer fermentation. It is still possible that a later time, for example 4 days, would have higher enzyme concentration and be capable of further decreasing the phenolic content.

6.9 Modeling of SSF System

The logistic growth model was used to describe the growth kinetics, PPO production and sinapine decrease during a solid state process containing 60% moisture and 10 mL/L Vogel's minerals solution. The sets of data from Figures 6-1, 6-20 and 6-21 were used for modeling. The parameters that provided the best fits to the models are given in Table 6-2. These values were determined using the Scientist software package (Micromath).

Table 6-2: Parameters for the logistic law during SSF of *T. versicolor* on CM

Parameter	Value
X_m	0.5165 (g biomass/ g dry SSC)
μ_m	1.366 (d^{-1})
k_v	33.006 (nkat/g dry SSC/ d)
k_s	7.74 (g dry SSC^2/g X^2/d)
k	.2976 (d^{-1})

The logistic law gives a sigmoidal curve for the growth of the fungus (Figure 6-26). Due to the errors discussed in the previous sections, there is some scatter among the data. As a result, it can be concluded that the model fits the data reasonably well. The value of X_m is consistent with the maximum biomass concentrations seen previously.

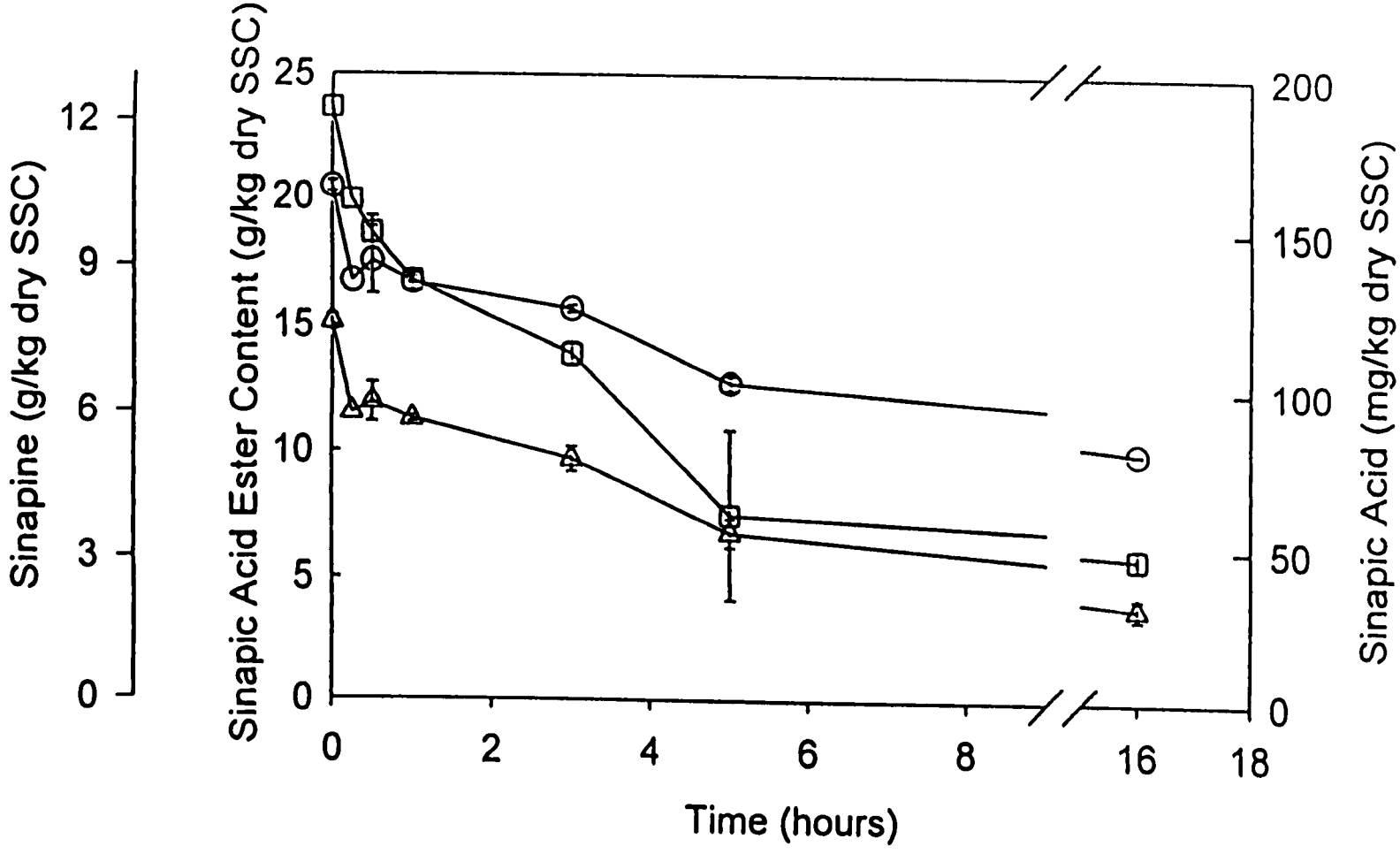

Figure 6-24: Decrease in phenolic content of a 3 day SSF culture due to addition of water to a concentration of 20% (w/v) culture in water; (O)-SAE; (Δ)-sinapine; (□)-sinapic acid

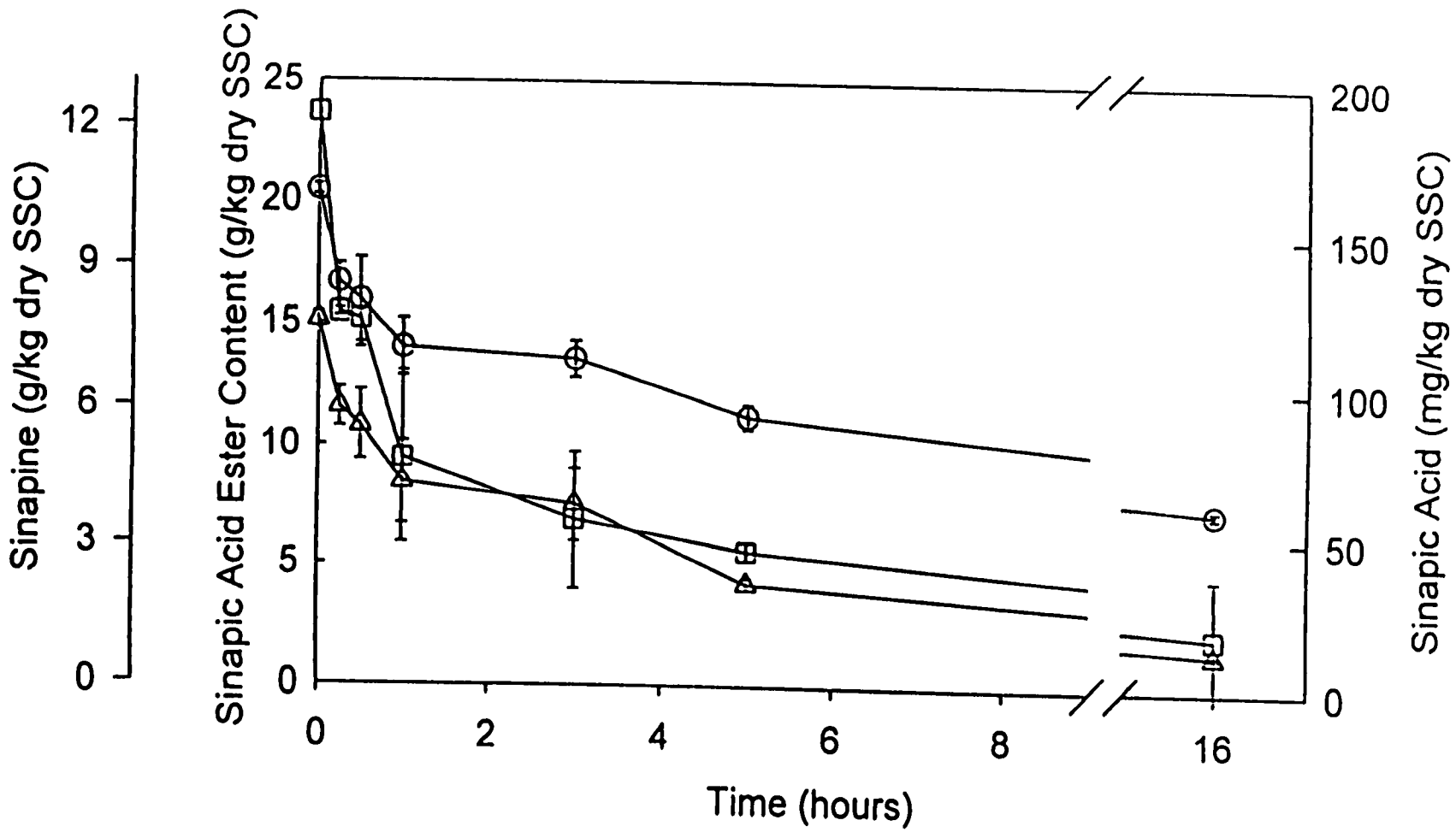

Figure 6-25: Decrease in phenolic content of a 3 day SSF culture due to addition of water to a concentration of 40% (w/v) culture in water; (O)-SAE; (Δ)-sinapine; (□)-sinapic acid

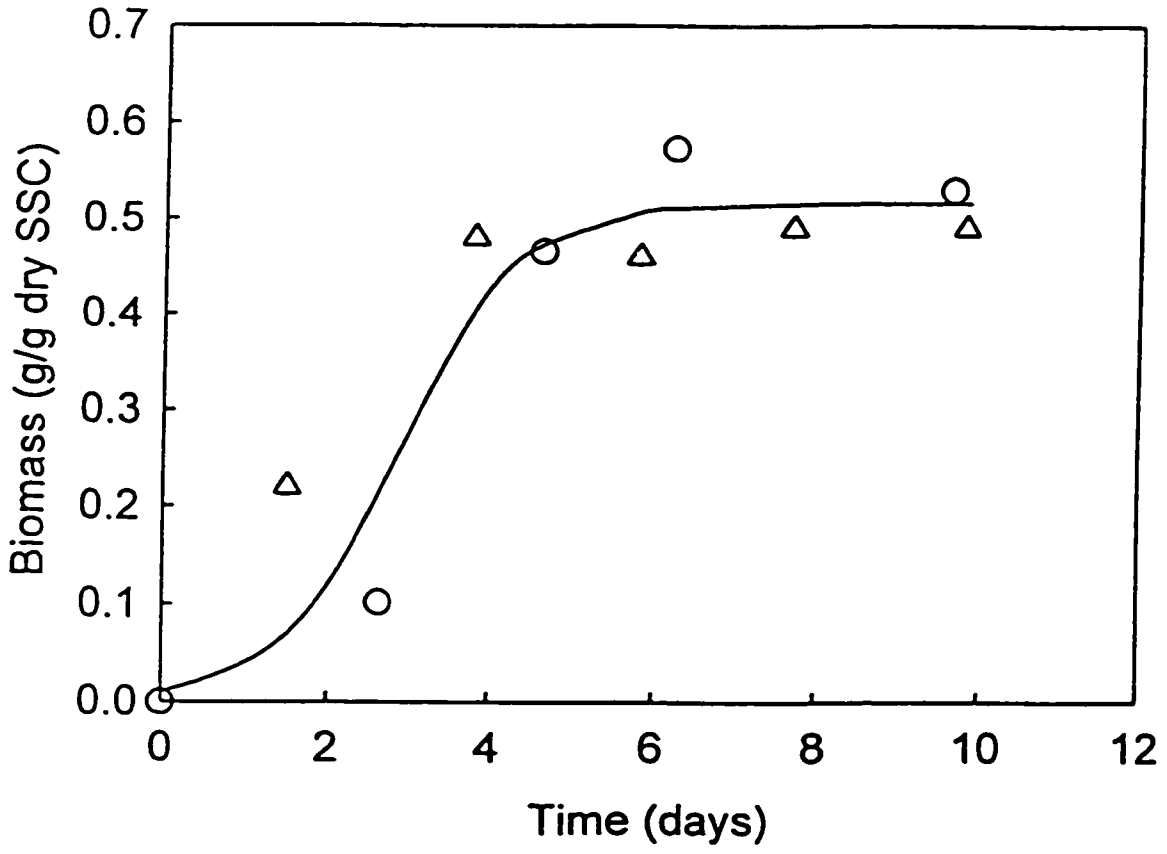

Figure 6-26: Comparison between experimental data and logistic model during exponential biomass production during a solid state process; (Δ)-Figure 6-1; (O)- Figure 6-20; (-)- logistic model

The μ_m is consistent with the value obtained by Al-Asheh (1993) for the growth of *Aspergillus carbonarius* on CM, which ranged from 1.344-1.464 d^{-1} when the meal was supplemented with glucose concentrations ranging from 0-12g per 50g dry CM.

The logistic model for enzyme production was used to model the PPO production during the SSF processes (Figure 6-27). Only the exponential portion of the PPO production was modeled since the model applies only to the exponential portion of the data and does not include the stationary phase. The values of X_m and μ_m found during the growth phase were used in this model. It is not possible to compare the value of K_v with literature data since none could be found for this enzyme in a similar system.

The model is less accurate when applied to the decrease in sinapine content (Figure 6-28). This is a result of the process of inoculation. Since there is a very low PPO concentration initially in the inoculum, although undetectable by the current procedure, the sinapine concentration decreases rapidly initially. With time, the fungus begins to produce PPO, which continues to decrease the SIN content. Up until this point, the process is rate-limited, due to the abundance of substrate and low concentration of enzyme. However, after about 5 days, there are only very low levels of SIN and the process becomes diffusion-limited since the SIN must diffuse from the interior of the CM particle before it can react with the abundant quantity of PPO in the culture. The logistic model assumes that the sinapine decrease is related solely to the growth of the fungus and resultant production of PPO as well as the SIN concentration. The slow diffusion of the substrate from within the particle is not taken into consideration. This means that the model predicts that the SIN concentration should reach zero around 7 days whereas it actually takes a few days longer. It can thus be concluded that the logistic model for substrate concentration is not applicable in this case, or should possibly incorporate diffusion effects. As an alternative, the first order differential equation was applied and was found to fit the data much more accurately. This model assumes that the rate of

decrease is dependent upon the substrate concentration, and not enzyme concentration, and should thus be considered empirical.

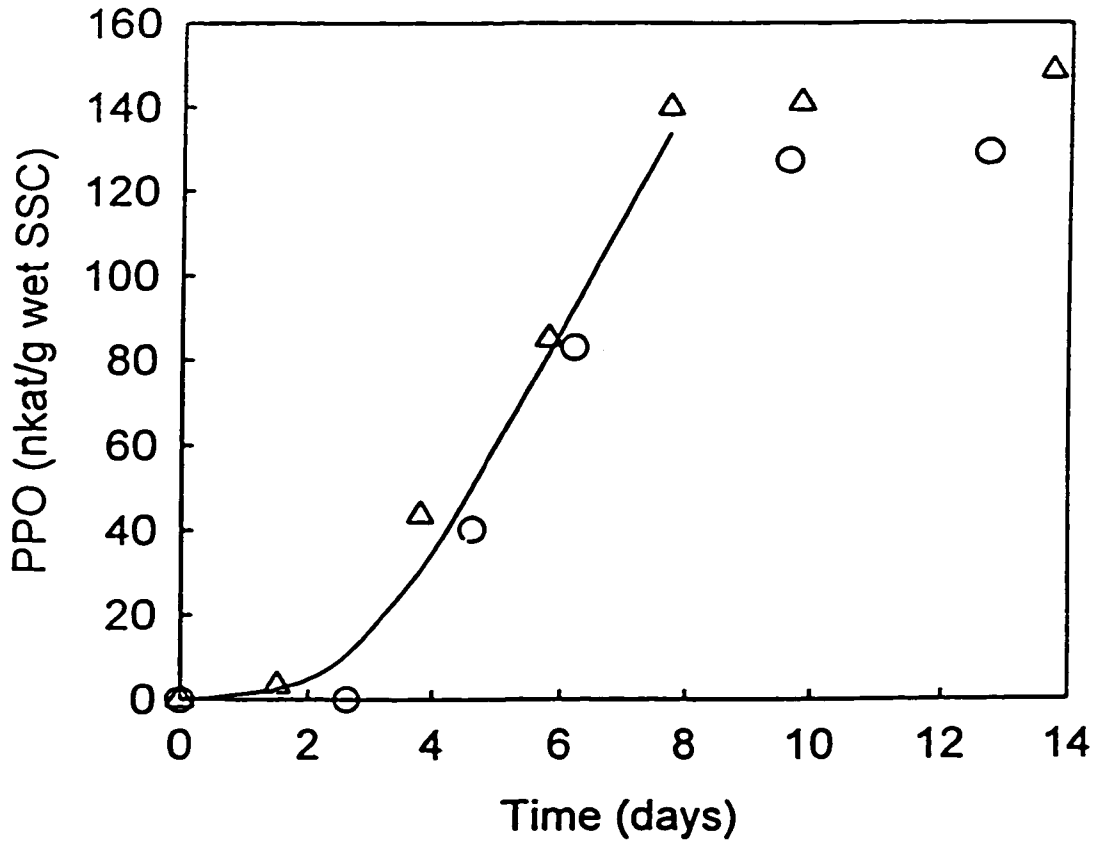

Figure 6-27: Comparison between experimental data and logistic model during exponential phase of PPO production for a solid state process; (Δ)-Figure 6-1; (O)- Figure 6-20; (-)- logistic model

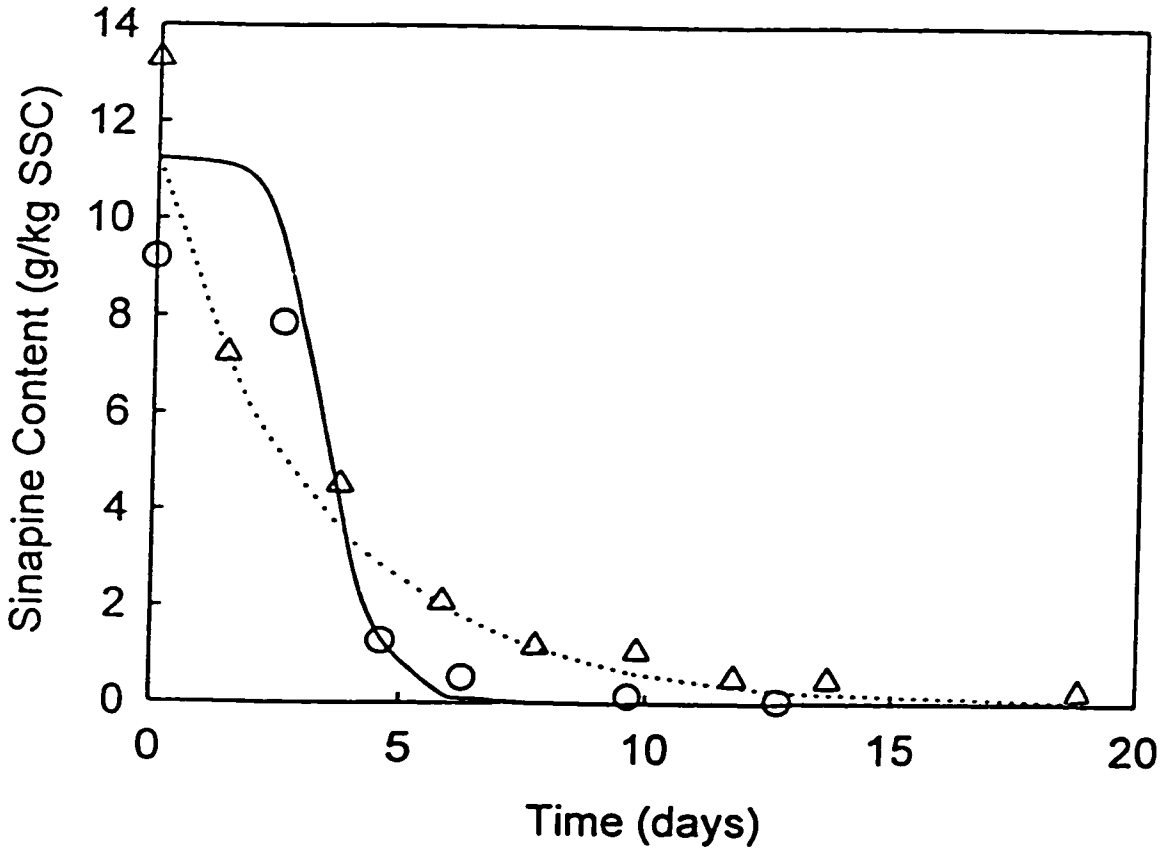

Figure 6-28: Comparison between experimental data and logistic model for the decrease in sinapine content during a solid state process; (Δ)-Figure 6-1; (O)- Figure 6-20; (–)- logistic model; (--) 1st order

Chapter 7: Conclusions

The following are some important findings from this research.

- The addition of canola, soybean or sunflower meal to a submerged fermentation culture stimulated polyphenoloxidase production to a greater extent than in the control medium without meal. The maximum levels of PPO in the three meal cultures were 140, 118 and 102 nkat/mL, respectively, compared to the 60 nkat/mL obtained in the control culture.

- Several phenolics were tested for the induction of PPO. 2,5-xylidene was found to be the best inducer, whereas sinapic acid, ferulic acid and syringaldazine had only small increases in PPO production. In all cases, the amount of PPO produced was less than in the 5% CM medium.

- Increasing the amount of aeration or protein content to a submerged culture stimulated PPO production. Vogel's minerals were found to be inhibitory towards biomass growth and PPO production.

- Growing the fungus in a solid state process on CM was found to be capable of decreasing the sinapine content of the meal by 98.5% after 6 days and by 99.8% after 18 days.

- The moisture content of the solid state process had a very profound influence on PPO production. The PPO activity increased with moisture content from negligible amounts at 50% moisture to 550 nkat/ g wet SSC at 80%.

- Temperatures ranging from 24-32°C did not have a profound influence on PPO production. However above 36°C, the activity decreased rapidly due to very little growth. Homogenization of the inoculum and initial biomass concentration did not have an influence on PPO production or the residual sinapine and sinapic acid ester content of the canola meal.

- The order, among solid state cultures with meals, was CM>SWM>SFM>SBM. Despite having similar PPO levels in submerged cultures, SBM had about 10% of the activity of CM in the solid state process. However, since the final moisture contents of the cultures were different, it is unsure what influence this would have had on the activity measured.

- Flooding experiments were carried out in an attempt to speed up the SAE decrease in CM. The SAE content of a 3 day culture was decreased by 45% and 60% using a 20% and 40% suspension of culture in water, respectively after 16 hours. Unfortunately, the decrease was less than that which could be obtained by the fermentation process.

- The logistic model of Okazaki *et al.* (1980) was used to model biomass concentration, PPO production and sinapine decrease in the solid state process. The model fit the exponential growth and enzyme production phases, however it was not effective at describing the decrease in sinapine content. A first order model was found to provide a better fit for the sinapine concentration.

Chapter 8: Recommendations

There are many concerns that should be addressed in future studies. These include the following.

Submerged fermentation.

(1) Several different compounds were examined individually for PPO production, such as sugars and proteins, however, interactions among the compounds were not examined. This would be interesting to study as well as comparing the control media with a synthetic 5% CM media.

(2) The oxygen content of the media was found to be important for PPO production. Furthermore, since the oil content of the media can affect oxygen transfer, the effect of meal residual oil content on PPO production should be examined.

Solid state fermentation.

(1) Since the PPO activity in SSF was not quantifiable early in the process, yet it was detectable (for example, due to further decrease in phenolic content during flooding experiments), the binding of PPO onto CM should be examined. This could be done by adding a known amount of enzyme to a fixed mass of CM and then extracting the mixture to determine if the same amount of enzyme could be obtained. Furthermore, another non-spectrophotometric assay technique could be used to measure the PPO activity early in the process, such as using HPLC to measure the loss in absorbance in ferulic acid.

(2) Aeration was found to be an important parameter for the submerged process. A preliminary experiment showed this was equally important in SSF. As a

result, the effect of this parameter on PPO production and SAE decrease should be examined.

(3) The flooding experiments, applied to a 3 day culture, were effective in decreasing the phenolic content in CM, however not to the extent possible through fermentation. It was suggested that a later sampling day should be tested, for example 4 days, when there would be slightly higher levels of PPO in the culture.

(4) Since one of the primary goals of this project was to upgrade CM for potential use in livestock diets, the quality of the meal after the SSF process should be examined. In particular, the protein content should be assessed as well as identifying the final phenolic products. This information could then be used in studies with livestock to examine the suitability of the fermentation process to upgrade CM.

(5) The direct addition of PPO to CM has been found to be successful in decreasing the SAE content of CM (Lacki and Duvnjak, 1996c). The products of the enzymatic transformation should be compared with those obtained during the SSF process to see which decreases the final phenolic content to the greatest extent.

(6) Due to the large amount of PPO produced during SSF, it may be possible to use this enzyme in a complete process to upgrade CM. This would involve producing the enzyme preparation during SSF, addition to untreated CM, then combining the meals from fermentation and enzymatic treatments. This resultant meal would have a lower phenolic content and would also add additional nutrients due to fermentation process.

(7) Other oilseed meals with high phenolic contents (e.g. SFM) should be followed kinetically in a SSF process to determine if there is potential for upgrading these meals.

(8) Finally, since the nutritional value of CM is also limited by the phytic acid content, the residual phytic acid content should be measured following the SSF process. A two microbe process, using *T. versicolor* and a fungus that degrades phytic acid should be examined since this would further improve the nutritional qualities of the meal.

Chapter 9: References

Aidoo, K.E., Hendry, R. and Wood, B.J.B. "Estimation of Fungal Growth in a Solid State Fermentation System." *European J. Appl. Microbiol. Biotechnol.*, **12**, 6-9 (1981).

Al-Asheh, S. "Production of Phytase and Reduction of Phytic Acid Content in Canola Meal by Solid-State Fermentation Using *Apergillis Carbonarius*" MASc. in Chemical Engineering Thesis, University of Ottawa, Ottawa, Ontario (1993).

Asiegbu, F.O., Paterson, A. and Smith, J.E. "Inhibition of Cellulose Saccharification and Glycolignin-Attacking Enzymes of Five Lignocellulose-Degrading Fungi by Ferulic Acid." *World J. Microbiol. Biotechnol.*, **12**, 16-21 (1996)

Austin, F.L. and Wolff, I.A. "Sinapine and Related Esters in Seed Meal of *Crambe Abyssinica.*" *J. Agr. Food Chem.*, **16** (1), 132-135 (1968).

Bailey, J.E. and Ollis, D.F. "Biochemical Engineering Fundamentals." 2nd edition, McGraw-Hill Book Company, p. 317 (1986)

Bell, J.M. "Factors Affecting the Nutritional Value of Canola Meal: A Review." *Can. J. Anim. Sci.*,**73** (4), 679-697 (1993).

Blix, G. "The Determination of Hexosamines According to Elson and Morgan." *Acta Chem. Scand.*, *2*, 467-473 (1948).

Bollag, J.M. and Leonowicz, A. "Comparative Studies of Extracellular Fungal Laccases." *Appl. Environ. Microbiol.*, **48** (4), 849-854 (1984).

Bouchereau, A., Hamelin, J., Lamour, I., Renard, M. and Larher, F. "Distibution of Sinapine and Related Compounds in Seeds of *Brassica* and Allied Genera." *Phytochemistry*, **30** (6), 1873-1881 (1991).

Brumm, T.J. and Hurburgh, Jr., C.R. "Estimating the Processed Value of Soybeans." *JOACS*, **67** (5), 302-307 (1990).

Buswell, J.A. and Eriksson, K.E.L. "Effect of Lignin-Related Phenols and Their Methylated Derivatives on the Growth of Eight White-Rot Fungi." *World J. Microbiol. Biotechnol.*, **10**, 169-174 (1994).

Butt, V.S. "Direct Oxidases and Related Enzymes." In The Biochemistry of Plants, ed. Davies, D.D., **2** (3), 81-123 (1980).

Chen, M. and Rohani, S. "Recovery of Canola Meal Proteins." *Biotechnol. Bioeng.*, **40**, 63-68 (1992).

Clandinin, D.R. "Effect of Sinapine, the Bitter Substance in Rapeseed Oil Meal, on the Growth of Chickens." *Poultry Sci.*, **40**, 484-487 (1961).

Collins, P.J. and Dobson, A.D.W. "Extracellular Lignin and Manganese Peroxidase Production by the White-Rot Fungus *Coriolus versicolor* 290." *Biotechnol. Lett.*, **17** (9), 989-992 (1995).

Dabrowski, K.J. and Sosulski, F. "Composition of Free and Hydrolyzable Phenolic Acids in Defatted Flours of Ten Oilseeds." *J. Agric. Food Chem.*, **32**, 128-130 (1984).

Dehorter, B. and Blondeau, R. "Extracellular Enzyme Activities During Humic Acid Degradation by the White-Rot Fungi *Phanerochaete chrysosporium* and *Trametes versicolor*." *FEMS Microbiol. Lett.*, **94**, 209-216 (1992).

Dittmer, J.K., Patel, N.J., Dhawale, S.W. and Dhawale, S.S. "Production of Multiple Isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency." *FEMS Microbiol. Lett.*, **149** (1), 65-70 (1997).

Dosoretz, C., Chen, A.H.C. and Grethlein, H.E. "Effect of Oxygenation Conditions on Submerged Cultures of *Phanerochaete chrysosporium*." *Appl. Microbiol. Biotechnol.*, **34**, 131-137 (1990).

Eggert, C., Temp, U. and Eriksson, K.L. "The Lignolytic System of the White-Rot Fungus *Pycnoporus cinnabarulinus*: Purification and Characterization of the Laccase." *Appl. Environ. Microbiol.* **62** (4), 1151-1158 (1996).

Ekblad, A. and Nasholm, T. "Determination of Chitin in Fungi and Mycorrhizal Roots by an Improved HPLC Analysis of Glucosamine." *Plant and Soil*, **178**, 29-35 (1996).

Fahraeus, G. "Formation of Laccase by *Polyporus versicolor* in Different Culture Media." *Physiol. Plant.*, **5**, 285-291 (1952).

Fahraeus, G. and Reinhammar, B. "Large Scale Production and Purification of Laccase From Cultures of the Fungus *Polyporus versicolor* and some Properties of Laccase A." *Acta Chem. Scand.*, **21** (9), 2367-2378 (1967).

Fenton, T.W., Leung, J. and Clandinin, D.R. "Phenolic Components of Rapeseed Meal." *J. Sci. Food Agric.*, **30** (7), 661-663 (1979).

Gattinger, L.D., Duvnjak, Z. and Khan, A.W. "The Use of Canola Meal as a Substrate for Xylanase Production by *Trichoderma reesei*." *Appl. Microbiol. Biotechnol.*, **33**, 21-25 (1990).

Gillberg, L. and Tornell, B. "Preparation of Rapeseed Protein Isolates." *J. Food Sci.*, **41**,1063-1069 (1976).

Harkin, J.M. and Obst, J.R. "Syringaldazine, an effective Reagent for Detecting Laccase and Peroxidase in Fungi." *Experientia*, **29**, 381-387 (1973).

Hirabayashi, M., Matsui, T. and Yano, H. "Fermentation of Soybean Meal with *Aspergillu usamii* Improves Zinc Availability in Rats." *Biological Trace Element Research.*, **61**, 227-234 (1998).

Hobson-Frohock, A., Land, D.G., and Griffiths, N.M. and Curtis, R.F. "Eggs Taint; Association with Trimethylamine." *Nature*, **243**, 303-305 (1973).

Howard, B. "Oil and Oilseeds to 1996: New Patterns of Supply and Demand." Special Report M703, The Economic Intelligence Unit Limited: New York (1993). Cited in: Klockeman, D.M., Toldeo, R. and Sims, K.A. "Isolation and Characterization of Defatted Canola Meal Protein." *J. Agric. Food. Chem.*, **45** (10) 3867-3870 (1997).

Ibrahim, H.M., Mohamed, H.A., Shams, E. and Atia, A.M. "Comparative Studies on Safflower and Sunflower Meals." *Egypt. J. Food. Sci.*, **20** (2), 273-284 (1992).

Jones, H.L. and Worrall, J.J. "Fungal Biomass in Decayed Wood." *Mycologia*, **87** (4), 459-466 (1995).

Katagiri, N., Tsutsumi, Y. and Nishida, T. "Correlation of Brightening with Cumulative Enzyme Activity Related to Lignin Biodegradation of Kraft Pulp by White-Rot Fungi in the Solid-State Fermentation System." *Appl. Environ. Microbiol.*, **61** (2), 617-622 (1995).

Khan, W.A. and Overand, R.P. "An Oxygenase Enzyme System from *Trametes versicolor*." *FEMS Microbiology Letters*, **66**, 215-220 (1990).

Kirk, T.K., Connors, W.J. and Zeikus, G. "Requirement for a Growth Substrate During Lignin Decomposition by Two Wood-Rotting Fungi." *Appl. Environ. Microbiol.*, **32** (1), 192-194 (1976).

Kirkpatrick, N., Reid, I.D., Ziomek, E., Ho, C. and Paice, M.G. "Relationship Between Fungal Biomass Production and the Brightening of Hardwood Kraft Pulp by *Coriolus versicolor*." *Appl. Environ. Microbiol.*, **55** (5) 1147-1152 May (1989).

Klockeman, D.M., Toldeo, R. and Sims, K.A. "Isolation and Characterization of Defatted Canola Meal Protein." *J. Agric. Food. Chem.*, **45** (10) 3867-3870 (1997).

Kozlowska, H., Rotkiewica, D.A. Zadernowski, R. and Sosulski, F.W., "Phenolic Acids in Rapeseed and Mustard." *JAOCS*, **60** (6), 1119-1123 (1983).

Krygier, K., Sosulski, F. and Hogge, L. "Free, Esterified and Insoluble-bound Phenolic Acids. 1. Extraction and Purification Procedure." *J. Agric. Food Chem.*, **30**, 330-334 (1982a).

Krygier, K., Sosulski, F. and Hogge, L. "Free, Esterified and Insoluble-bound Phenolic Acids. 2.Composition of Phenolic Acids in Rapeseed Flour and Hulls." *J. Agric. Food Chem.*, **30**, 334-336 (1982b).

Lacki, K. and Duvnjak, Z. "Transformation of 3,5-dimethoxy, 4-hydroxy Cinnamic Acid and its Derivatives Using Enzyme From White-Rot Fungus *Trametes versicolor*: Enzyme Characteristics and Its Application." *J. Chem. Tech. Biotechnol.*, **65**, 211-220 (1996a).

Lacki, K. and Duvnjak, Z. "Modeling of the Enzymatic Transformation of 3,5-dimethoxy, 4-hydroxy Cinnamic Acid by an Enzyme of White-Rot Fungus *Trametes versicolor*." *Biotechnol. Bioeng.* **51**, 249-259 (1996b).

Lacki, K. and Duvnjak, Z. "Comparison of Three Methods for the Determination of Sinapic Acid Esters Content in Enzymatically Treated Canola Meal." *J. Appl. Microbiol. Biotechnol.*, **45**, 530-537 (1996c).

Lacki, K. "A Novel Method for the Decrease of Phenolic Content in Commercial Canola Meal Using an Enzyme Preparation Secreted by the White-Rot Fungus *Trametes versicolor*." Ph.D. in Chemical Engineering Thesis, University of Ottawa, Ottawa, Ontario, (1997).

Leitgeb, R., Luger, K. and Ulberth, F. "Einsatz von Soja- und Sonnenblumen-extraktionsschrot und Sonnenblumensamen als Eiweibfuttermittel fur Milchkuhe." *Fett/Lipid*, **98** (11), 360-362 (1996).

Leonowicz, A. and Grzywanowicz, K. "Quantitative Estimation of Laccase Forms in Some White-rot Fungi Using Syringaldazine as a Substrate."*Enzyme microbiol. Technol.*, **3**, 55-58 (1981).

Leonowicz, A. and Trojanowski, J. "Induction of Laccase by Ferulic Acid in *Basidiomycetes*." *Acta Biochemica Polonica*, **22** (4), 291-295 (1975).

Leung, J., Fenton, T.W. and Clandinin, D.R. "Phenolic Components in Sunflower Meal." *J. Food Sci.*, **46**, 1386-1393 (1981).

Maga, J.A. and Lorenz, K. "Gas -Liquid Chromatography Separation of the Free Phenolic Acid Fractions in Various Oilseed Protein Sources." *J. Sci. Food Agric.*, **25**, 797-801 (1974).

Martin, C. and Manzanares, P. "A Study of the Decolourization of Straw Soda-Pulping Effluents by *Trametes versicolor*." *Biores. Technol.*, **47**, 209-214 (1994).

Menton, J.F.M., Pesti, G.M. and Bakalli, R.I. "A New Method for Determining the Availability of Choline in Soybean Meal." *Poultry Sci.*, **76** 1292-1297 (1997).

Mikolajczak, K.L., Smith Jr., C.R. and Wolff, I.A. "Phenolic and Sugar Components of Armavirec Variety Sunflower (*Helianthus annuus*) Seed Meal." *J. Agr. Food Chem.*, **18** (1) 27-32 (1970).

Miller, G.L. "Use of Dinitrosalicyclic Acid Reagent for the Determination of Reducing Sugars." *Anal. Chem.*, **31**, 426-428 (1959).

Mohme, H., Toska, M. and Gunther, K.D. "Teilentfettete Sonnenblumenkerne als Protein-Ressource in der Mast von Broilern." *Fett/Lipid*, **99** (3), 78-80 (1997).

Morin, L. "Mass-Production of Fungi for Bioherbicides." *Plant Protection Quarterly*, **74** (4), 143-148 (1992).

Morohoshi, N., Wariishi, H., Muraiso, C., Nagai, T. and Haraguchi, T. "Degradation of Lignin by the Extracellular Enzymes of *Coriolus versicolor*, IV." *Mokuzai Gakkaishi*, **33** (3), 218-225 (1987).

Mudgett, R.E. "Solid State Fermentations" in Manual of Industrial Microbiology and Biotechnology. Eds. Demain, A.L. and Solomon, N.A. American Society for Microbiology. Washington, DC. 59-65 (1986).

Mueller, M.M., Ryl, E.B., Fenton, B. and Clandinin, D.R. "Cultivar and Growing Location Differences on the Sinapine Content of Rapeseed." *Can. J. Anim. Sci.*, **58**, 579-583 (1978).

Mustafa, A.F., Christensen, D.A., and McKinnon, J.J. "Chemical Characterization and Nutrient Availability of High and Low Fiber Canola Meal." *Can. Journ. Anim. Sci.*,**76** , 579-586 (1996).

Naczk, M., Wanasundara, J.P.D. and Shahidi, F. "Facile Spectrophotometric Quantification Method of Sinapic Acid in Hexane-Extracted and Methanol-Ammonia-Water Treated Mustard and Rapeseed Meals." *J. Agric. Food. Chem.*, **40** (3), 444-448 (1992).

Nair, V. "Reduction of Phytic Acid Content in Canola Meal by Solid State Fermentation." MASc. in Chemical Engineering Thesis, University of Ottawa, Ottawa, Ontario (1990)

Nampoothiri, K.M. and Pandey, A. "Solid State Fermentation for L-Glutamic Acid Production Using *Brevibacterium* sp." *Biotechnol. Lett.*, **18** (2), 199-204 (1996).

Niazi, A.H.K., Kasur, T., Shah, F.H. and Ishaque, W. "Effect of Reducing Crude Fibre Content on the Nutritive Value of Sunflower Meal." *Pak. J. Sci. Ind. Res.*, **34** (9), 321-323 (1991).

Nielsen, H. "The Decrease of Phenolics in Soybean Meal Using a Polyphenoloxidase Enzyme Preparation from the White-Rot Fungus *Trametes versicolor*." B.A.Sc. in Chemical Engineering Thesis. University of Ottawa, Ottawa, Ontario (1998)

Okazaki, N., Sugama, S. and Tanka, T., "Mathematical Model for Surface Culture of Koji Mold." *J. Ferment. Technol.*, **58**, 471-476 (1980).

O'Quinn, P.R., Knabe, D.A., Gregg, E.J. and Lusas, E.W. "Nutritional Value for Swine of Soybean Meal Produced by Isopropyl Alcohol Extraction" *J. Anim. Sci.*, **75**, 714-719 (1997).

Pal, M., Clavo, A.M., Terron, M.C., Gonzalez, A.E. "Solid-State Fermentation of Sugarcane Bagasse with *Flammulina velutipes* and *Trametes versicolor*." *World J. Microbiol. Biotechnol.*, **11**, 541-545 (1995).

Penaloza, W., Davey, C.L., Hedger, J.N. and Kell, D.B. "Stimulation by Potassium Ions of the Growth of *Rhizopus oligosporous* During Liquid- and Solid-Substrate Fermentations." *World J. Microbiol. Biotechnol.*, **7**, 260-268 (1991).

Prasad, T. "Proteins of the Phenolic Extracted Sunflower Meal: 1. Simple Method for Removal of Polyphenolic Components and Characteristics of Salt Soluble Proteins." *Lebensm. -Wiss. u. Technol.*, **23**, 229-235 (1990).

Ramakrishna, M.B.V, Mital, B.K., Gupta, K.C. and Sand, N.K. "Determination of Phenolic Acids in Different Soybean Varieties by Reversed Phase High Performance Liquid Chromatography." *J. Food Sci. Technol.*, **26**, 154-155 (1989).

Reid, I.A. and Seifer, K.A. "Effect of an Atmosphere of Oxygen on Growth, Respiration and Lignin Degradation by White-Rot Fungi." *Can. J. Bot.*, **60**, 252-260 (1982).

Reid, I.D. "The Influence of Nutrient Balance on Ligin Degradation by the White-Rot Fungus *Phanerochaete chrysosporium*." *Can. J. Bot.*, **57**, 2050-2058 (1979).

Rizk, I.R.S. "Characteristics of Phytase in Sunflower Meal and Broad Beans." *Egypt. J. Food Sci.*, **19** (1-2) 17-30 (1991).

Rogulski, J., Dawidowica, A.L. and Leonowicz, A. "Purification and Immobilization of the Inducible form of Extracellular Laccase of the Fungus *Trametes versicolor*." *Acta Biotechnol.*, **10** (3), 261-269 (1990).

Rogulski, J., Wojtas-Wasilewska, M., Apalovic, R. and Leonowicz, A. "Affinity Chromatography as a Rapid and Convenient Method for Purification of Fungal Laccases." *Biotechnol. Bioengin.*, **37**, 770-777 (1991).

Rozan, P., Lamghari, R., Linder, M., Villaume, C., Fanni, J., Parmentier, M. and Mejean, L. "*In Vivo* and *In Vitro* Digestibility of Soybean, Lupine and Rapeseed Meal Proteins After Various Technological Processes." *J. Agric. Food Chem.* **45**, 1762-1769 (1997).

Sabir, M.A., Sosulski, F.W. and Kernan, J.A. "Phenolic Constituents in Sunflower Flour." *J. Agr. Food Chem.*, **22** (4), 572-574 (1974a).

Sabir, M.A., Sosulski, F.W. and Finlayson, A.J. "Chlorogenic Acid- Protein Interactions in Sunflower" *J. Agr. Food Chem.*, **22** (4), 575-578 (1974b).

Sakurai, Y., Lee, T.H. and Shiota, H. "On the Convenient Method for Glucosamine Estimation in Koji." *Agric. Biol. Chem.*, **41** (4), 619-624 (1977).

Salas, C., Lobos, S., Larrain, J., Salas, L., Cullen, D. and Vicuna, R. "Properties of Laccase Isoenzymes Produced by the Basidomycete *Ceriporiopsis subvermispora*." *Biotechnol. Appl. Biochem.*, **21** (3), 323-333 (1995).

Shahidi, F. "Phenolic Compounds of Brassica Oilseeds." Ch. 10 in Phenolic Compounds in Food and Their Effects on Health, **I**, 2-7 (1992).

Shahidi, F. and Naczk, M. "An Overview of the Phenolics of Canola and Rapeseed: Chemical, Sensory and nutritional Significance." *JAOCS*, **69** (9), 917-9 24 (1992).

Sharma, P.D., Fisher, P.J. and Webster, J. "Critique of the Chitin Assay Technique for Estimation of Fungal Biomass." *Trans. Br. Mycol. Soc.*, **69** (3), 479-483 (1977).

Siebrits, F.K. "Review Article: The Use of Sunflower Oilcake Meal in South African Pig Diets." *Pig News and Information* **17** (3), 83N-85N (1996).

Siljander-Rasi, H., Valaja, J., Alaviuhkola, T., Rantamaki, P. and Tuasela, T. "Replacing Soya Bean Meal with Heat-Treated Low-Glucosinolate Rapeseed Meal Does Not Affect the Performance of Growing-Finishing Pigs." *Anim. Feed Sci. Technol.*, **60**, 1-12 (1996).

Skorobogat'Ko, O.V., Stepanova, E.V., Gavrilova, V.P. and Yaropolov, A.I. "Effects of Inducers on the Synthesis of Extracellular Laccase by *Coriolus hirsutus*, a Basidial Fungus." *Prikladnaya Biokhimiya i Mikrobiologiya,* **32** (5), 524-528 (1996).

Smits, J.P., Rinzema, A., Tramper, J., van Sonsbeek, H.M., Hage, J.C., Kaynak, A. and Know, W. "The Influence of Temperature on Kineticsa in Solid State Fermentation." *Enzyme and Microb. Technol.*, **22**, 50-57 (1998).

Sotillo, E. and Hettiarachchy, N.S. "Corn Meal- Sunflower Extrudates and Their Physiochemical Properties." *J. Food Sci.* **59** (2), 432-435 (1994).

Srinivasan, C., D'Souza, T.M., Boominathan, K. and Reddy, C.A. "Demonstration of Laccase in the White-Rot Basidiomycete *Phanerochaete chrysosporium* BKM-F1767." *Appl. Environ. Microbiol.*, **61** (12), 4274-4277 Dec. (1995).

Stanford, K., McAllister, T.A., Xu, Z., Pickard, M. and Cheng, K.J. "Comparison of Lignosulfonate-Treated Canola Meal and Soybean Meal as Rumen Undegradable Protein Supplements for Lambs." *Can. Journ. Anim. Sci.*,**75**, 371-377 (1995).

Stanford, K., McAllister, T.A., Lees, B.M., Xu, Z.J. and Cheng, K.J. "Comparison of Sweet White Lupin Seed, Canola Meal and Soybean Meal as Protein Supplements for Lambs." *Can. Journ. Anim. Sci.*,**76**, 215-219 (1996).

Statistics Canada. *Catalo. 22-007*, **16** (1), (1996).

Statistics Canada. *Catalo. 22-007*, **18** (4), (1998).

Swift, M.J. "The Estimation of Mycelial Biomass by Determination of the Hexosamine Content of Wood Tissue Decayed by Fungi." *Soil Biol. Biochem.*, **5**, 321-332 (1973).

Szklarz, D.G., Antibus, R.K., Sinsabaugh, R.L. and Kinkins, A.E. "Production of Phenol Oxidase and Peroxidases by Wood-Rotting Fungi." *Mycologia*, **81** (2), 234-240 (1989).

Taylor, A.G., Paine, D.H. and Paine, C.A., "Sinapine Leakage from *Brassica* Seeds." *J. Amer. Soc. Hort. Sci.*, **118** (4), 546-550 (1993).

Tsujyama, S., Azuma, J. and Okamura, K. "Influence of Plant Hormones on a Wood-Rotting Fungus, *Coriolus versicolor*." *Trans. Mycol. Soc. Japan*, **34**, 369-376 (1993).

Valmaseda, M., Martinez, M.J. and Martinez, A.T. "Kinetics of Wheat Straw Solid-State Fermentation with *Trametes versicolor* and *Pleurotus ostreatus*- Lignin and Polysaccharide Alteration and Production of Related Enzymatic Activities." *Appl. Microbiol. Biotechnol.*, **35**, 817-823 (1991).

Westermark, U. and Eriksson, K.E. "Cellobiose:Quinone Oxidoreductase, a New Wood-Degrading Enzyme from White-Rot Fungi." *Acta Chem. Scand. B*, **26**, 209-214 (1974).

World Oilseed Situation and Outlook. United States Department of Agriculture, Washington, DC. June (1992). Cited in Zhang, Y. and Parsons, C.M. "Effects of Overprocessing on the Nutritional Quality of Sunflower Meal." *Poultry Sci.*, **73**, 436-442 (1994).

Yadav, J.S. and Tripathi, J.P. "Optimization of Cultivation and Nutrition Conditions and Substrate Pretreatment for Solid-Substrate Fermentation of Wheat Straw by *Coriolus versicolor*." *Folia Microbio.*, **36** (3), 294-301 (1991).

Zhang, Y. and Parsons, C.M., "Effects of Overprocessing on the Nutritional Quality of Sunflower Meal." *Poultry Sci.*, **73**, 436-442 (1994).

Ziomek, E., Kirkpatrick, N. and Reid, I.D. "Effect of Polydimethylsiloxane Oxygen Carriers on the Biological Bleaching of Hardwood Kraft Pulp by *Trametes versicolor*." *Appl. Microbiol. Biotechnol.*, **35**, 669-673 (1991).

Appendix A
Experimental Data

Figure 5-1: Production of PPO by *T.versicolor* in a submerged process

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.03		23.42		0.11	
1.67	0.89	0.06	19.59	0.45	0.13	0.03
3.54	3.52	0.65	12.95	0.17	5.73	0.26
5.81	4.25	0.45	0.89	0.07	29.66	1.53
7.73	5.52	1.45	0.71	0.17	51.13	5.03
9.94	6.07	0.49	0.76	0.01	50.62	3.93

Figure 5-2: Effect of 2,5-xylidene on glucose consumption and biomass and PPO production

Control

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.07	0.00	23.75		0.03	
1.79	1.18	0.03	23.01	1.36	0.35	0.04
2.74	3.14	0.05	21.06	0.16	3.31	0.39
3.74	4.26	0.20	12.89	0.70	11.79	0.08
6.03	4.69	0.11	1.07	0.04	44.04	0.47
7.67	5.09	0.06	1.12	0.04	50.18	4.88
9.69	6.61	0.28	0.80	0.09	52.58	3.16

2,5-xylidene induced

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.07	0.00	23.75		0.03	
1.79	1.18	0.03	23.01	1.36	0.35	0.04
2.74	3.14	0.05	21.06	0.16	3.31	0.39
3.74	4.17	0.16	11.42		41.76	
6.03	4.14	0.31	1.28	0.02	71.02	1.16
7.67	4.83	0.29	1.08	0.03	84.14	4.28
9.69	6.57	0.09	0.89	0.12	80.72	1.48

Figure 5-3: Effect of addition of 5% CM on glucose consumption and PPO production

Control

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.03		23.42		0.11	
1.67	0.89	0.06	19.59	0.45	0.13	0.03
3.54	3.52	0.65	12.95	0.17	5.73	0.26
5.81	4.25	0.45	0.89	0.07	29.66	1.53
7.73	5.52	1.45	0.71	0.17	51.13	5.03
9.94	6.07	0.49	0.76	0.01	50.62	3.93

5% CM

time (d)	Glucose	bio error	PPO	ppo error
	(g/L)		(nkat/mL)	
0.00	24.63	1.10	0.11	
0.77	24.12			
1.67	24.17	0.14	0.14	
3.54	2.60	0.75	6.28	0.35
5.81	1.73	0.18	27.91	0.56
7.73	2.07	0.25	95.05	16.09
9.94	1.23	0.66	108.38	7.90

Figure 5-4: Effect of SA on biomass growth, PPO production and glucose consumption

control

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.02		23.75	1.89	0.01	
1.88	0.65	0.06	22.49	0.67	0.06	0.03
3.44	2.01	0.04	17.31	0.36	1.16	0.46
5.88	3.53	0.10	3.22	0.31	20.96	3.07
7.88	5.94	0.18	0.58	0.00		
8.96	5.05	0.67	0.63	0.02	24.16	2.26
12.88	4.63	0.01	1.12	0.40	25.17	5.92

SA medium

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.02		23.75	1.89	0.01	
1.88	0.75	0.01	21.32	0.47	0.04	0.00
3.44	2.14	0.03	16.74	0.05	1.58	0.23
5.88	3.94	0.27	1.51	0.53	22.30	2.52
7.88	5.80	0.11	0.55	0.00	24.08	6.47
8.96	5.99	0.05	0.56	0.01	26.49	1.08
12.86	3.93	0.18	0.86	0.14	30.41	1.52

Figure 5-5: Effect of SA on biomass growth, PPO production and glucose consumption

control

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.06		23.84	0.01	0.16	0.04
2.94	0.95	0.12	22.60	0.05	0.27	0.04
4.71	3.14	0.17	14.82	1.01	5.07	0.09
6.69	4.28	0.10	1.88	0.18	27.32	4.45
8.00	4.67	0.04	1.16	0.04	30.02	1.71
9.61	6.11	0.28	0.85	0.01	38.56	4.20
10.92	4.73	0.38	1.25	0.02	41.06	0.23
11.69	5.90	0.01	0.85	0.00	37.07	1.62

SA induced

time (d)	Biomass	bio error	Glucose	glu error	PPO	ppo error
	(g/L)		(g/L)		(nkat/mL)	
0.00	0.06		23.84	0.01	0.16	0.04
2.94	0.95	0.12	22.6	0.05	0.27	0.04
4.71	2.85	0.16	13.89	0.09	8.06	0.6
6.69	3.92	0.04	1.52	0.23	27.6	3.3
8.00	4.55	0.23	1.14	0.02	35.97	1.7
9.61	6.19	0.17	0.82	0	40.82	1.5
10.92	4.83	0.05	1.27	0.1	46.17	2.63
11.69	6.11		0.81	0.01	36.9	

Figure 5-6: Effect of phenolics on the production of PPO in a submerged process

time (d)	control	xylidene	SA	ferulic acid	syringaldazine
0	0	0	0	0	0
3	5.34	5.34	5.34	5.34	5.34
4	21.05	77.42	36.56	24.07	32.15
6	45.07	127.36	53.93	53.38	62.78
8	55.66	150.75	68.06	55.44	57.31
10.3	36.86	105.52	44.52	53.35	
12.2	29.91	77.53	35.91	40.31	43.86

Figure 5-7: Effect of phenolics on biomass production in a submerged process

time (d)	control	xylidene	SA	ferulic acid	syringaldazine
0.00	0.05	0.05	0.05	0.05	0.05
3.00	3.94	3.94	3.94	3.94	3.94
4.00	4.94	5.06	4.81	5.19	4.19
6.00		5.81	5.56		5.81
8.00	5.87	3.69	6.31	6.81	5.69
10.30	5.31	2.69	2.94	5.31	3.69
12.20	4.19	2.06	2.56	4.19	4.56

Figure 5-8: Effect of phenolics on biomass production in a submerged process

time (d)	control	xylidene	SA	ferulic acid	syringaldazine
0.00	23.52	23.52	23.52	23.52	23.52
4.00	12.11	11.48	0.77	9.93	0.78
6.00	0.45	0.62	0.59	0.44	0.61
8.00	0.54	0.61	0.59	0.62	0.58
10.30	0.56	0.44	0.42	0.57	0.40

Figure 5-9: Effect of Vogel's minerals concentration on PPO production in a submerged process

Time (d)	0 mL/L	0.5 mL/L	1 mL/L	5 mL/L	10 mL/L
0.0	0	0	0	0	0
2.4	2.88	3.05	2.55	1.05	1.2
3.9	24.71	19.58	19.98	19.03	17.46
6.0	59.07	53.6	48.31	45.35	
7.9	67.26	64.11	50.14	43.82	31.19
9.9	65.07	64.42	47.26	47.17	39.1

Figure 5-10: Effect of Vogel's minerals concentration on glucose consumption in a submerged process

Time (d)	0 mL/L	0.5 mL/L	1 mL/L	5 mL/L	10 mL/L
0.00	25.00	25.00	25.00	25.00	25.00
2.40	20.00	20.57	21.24	23.86	25.78
3.90	8.12	9.57	12.77	18.91	23.68
5.98	0.96	1.05	1.14	6.35	
7.89	1.20	1.17	1.26	1.21	22.53
9.94	0.64	0.62	0.65	1.33	21.39

Figure 5-11: Effect of Vogel's minerals concentration on biomass production in a submerged process

Time (d)	0 mL/L	0.5 mL/L	1 mL/L	5 mL/L	10 mL/L
0.00	0.00	0.00	0.00	0.00	0.00
2.40	2.31	2.44	1.81	1.19	0.06
3.90	3.69	3.19	2.81	2.19	0.81
5.98	3.44	3.56	3.69	4.44	1.69
7.89	4.44	4.19	3.69	5.31	1.06
9.94	5.31	5.69	5.94	4.94	1.56

Figure 5-12: Effect of aeration on PPO production in a submerged process

Time (d)	Control (no CM)	50 mL	80 mL	110 mL	140 mL
0.00	1.02	0.61	0.74	2.40	1.62
2.17	1.72	0.91	1.75	1.70	1.33
3.79	18.52	47.23	51.31	18.40	7.04
6.04	41.88	89.59	75.00	105.91	34.30
8.10	47.27	67.16	167.18	145.93	25.64
10.78	43.62	36.80	152.95	47.17	34.91

Figure 5-13: Effect of aeration on glucose consumption in a submerged process

Time (d)	Control (no CM)	50 mL	80 mL	110 mL	140 mL
0.00	23.76	23.00	24.31	24.76	21.79
2.17	18.14	20.14	17.04	12.61	15.62
3.79	7.51	3.30	2.63	2.95	3.01
6.04	0.79	2.58	2.16	2.40	2.11
8.10	1.17	2.27	2.10		2.17
10.78	0.90	1.78	1.72	1.78	2.04

Figure 5-14: Effect of aeration on SAE decrease in a submerged process

Time (d)	50 mL	80 mL	110 mL	140 mL
0.00	7.17	6.91	5.96	6.09
0.13	4.81	4.70	4.10	3.44
2.17	1.68	1.39	1.27	1.36
3.79	1.04	1.03	0.90	0.92
6.04	0.80	0.64	0.92	0.94

Figure 5-15: Effect of CM concentration on PPO production in a submerged process

Time (d)	control	no CM+5%	1% CM	3% CM	5% CM	5% reduced (6 g/kg)
0.00	1.29		1.17	2.73	0.93	
1.71	1.10		5.81	3.33	2.72	
3.60	14.92		95.95	68.28	32.70	
4.65	39.52	39.52	112.51	127.11	92.85	108.30
6.94	46.64	75.81	106.02	150.69	165.27	158.38
8.65	43.10	68.48	113.50	104.88	118.22	138.93

Figure 5-16: Effect of CM concentration on glucose consumption in a submerged process

Time (d)	control	1% CM	3% CM	5% CM
0.00	20.51	22.89	23.36	24.41
1.71	19.97	16.56	20.73	25.40
3.60	8.62	1.46	2.05	2.82
4.65	1.22	2.00	2.09	2.11
6.94	1.02	1.27	1.66	1.90
8.65	1.16	0.98	1.51	1.73

Figure 5-17: Effect of SBM concentration on PPO production in a submerged process

Time (d)	control	1% SBM	3% SBM	5% SBM	10% SBM
0.00	0.00	0.00	0.00	0.00	0.00
2.08	1.29	1.58	1.41	1.63	1.82
3.88	13.33	38.65	49.28	19.08	2.42
5.79	44.01	71.97	81.62	61.66	11.52
7.08	48.93	52.01		90.23	39.30
8.54	48.02	47.55	68.74	62.44	32.88

Figure 5-18: Effect of SBM concentration on glucose consumption in a submerged process

Time (d)	control	1% SBM	3% SBM	5% SBM	10% SBM
0.00	20.05	21.37	21.86	22.49	23.13
2.08	19.90	16.76	17.79	19.35	32.79
3.88	8.79	1.12	1.89	2.56	19.07
5.79	0.62	0.93	1.37	1.59	1.52
7.08	0.73	0.89	1.38	1.58	1.20
8.54	0.63	0.92	1.17	1.30	1.04

Figure 5-19: Effect of SFM concentration on PPO production in a submerged process

Time (d)	control	1% SFM	3% SFM	5% SFM	10% SFM
0.00	0.00	0.00	0.00	0.00	0.00
1.83	0.79	1.34	2.10	0.92	0.77
3.75	18.23	28.41	37.76	7.00	3.74
5.69	17.62	44.35	77.27	48.20	9.74
7.67	42.26	12.88	86.30	60.10	26.73
10.00	40.91	7.42		42.44	15.12

Figure 5-20: Effect of SFM concentration on glucose consumption in a submerged process

Time (d)	control	1% SFM	3% SFM	5% SFM	10% SFM
0.00	21.52	21.73	22.10	22.48	23.00
1.83	17.22	10.84	10.23	17.58	24.07
3.75	5.72	0.76	0.91	1.42	7.38
5.69	0.74	0.72	1.17	1.56	2.77
7.67	0.57	0.64	0.99	1.39	2.82
10.00	0.60	0.68		1.37	2.20

Figure 5-21: Comparison of three oilseed meals for PPO production in a submerged process

Time (d)	Control	5% SBM	3% SFM	5% CM
0.00	0.00	0.00	0.00	0.00
2.17	0.29	0.12	1.91	0.28
4.10	24.74	17.00	46.09	66.65
6.00	58.30	103.26	102.66	113.33
7.88	46.97	111.32	46.75	133.74
9.90	38.23	74.51	26.03	141.35
11.92	31.06		61.78	63.55

Figure 5-22: Comparison of three oilseed meals for glucose consumption in a submerged proces

Time (d)	Control	5% SBM	3% SFM	5% CM
0.00	21.52	21.34	22.10	21.39
2.17	18.23	18.35	8.45	16.40
4.10	5.55	1.98	1.19	1.93
6.00	0.70	1.23	1.15	1.83
7.88	1.50	0.58	1.71	1.16
9.90	0.65	1.39	1.11	1.43

Figure 6-1: Typical SSF of T. versicolor on CM Medium

Time (d)	biomass	glucose	non-reducing	PPO	SIN
	(g/g)	(g/g)	sugars	(nkat/g)	(g/kg)
0.00	0.01	5.96	43.71	0.00	13.26
1.50	0.22	22.50	23.73	3.26	6.77
3.80	0.48	11.58	5.63	43.57	4.11
5.80	0.46	9.19	5.85	84.98	1.67
7.70	0.49	8.38	5.87	139.90	1.16
9.80	0.49	8.18	5.22	141.07	0.77
11.80		7.81	6.83		0.44
13.70	0.42	8.41	5.82	148.50	0.49
18.80	0.24	9.04	7.65	138.06	0.20

Figure 6-2: Effect of temperature on PPO in SSF with 60% moisture

Temp	PPO	PPO error	PPO	PPO error
(°C)	(nkat/g wet)		(nkat/g dry)	
24.00	385.82	35.94	142.62	14.05
28.00	372.34	62.54	135.68	22.63
32.00	354.90	69.24	135.97	23.84
36.00	10.98	6.75	4.36	2.68
40.00	0.00	0.00	0.00	0.00

Figure 6-3: Effect of temperature on residual level of CM phenolics in SSF with 60% moisture

Temp	SAE	SAE error	SIN	SIN error	SA	SA error
(°C)	(g/kg)		(g/kg)		(mg/kg)	
24	4.43	0.51	0.50	0.06	10.46	0.90
28	2.89	0.30	0.29	0.03	7.38	0.74
32	2.58	0.34	0.22	0.05	8.33	1.29
36	10.44	1.71	4.04	2.09	75.01	16.02
40	13.32	0.77	3.89	1.12	73.78	22.95

Figure 6-4: Effect of temperature on residual glucose concentration in SSF with 60% moistur

Temp (°C)	reducing (mg/g)	red. error	non-reducing	non-red. error
24.00	29.64	5.11	10.95	1.82
28.00	25.05	1.09	9.13	0.42
32.00	22.87	1.18	8.79	0.54
36.00	73.66	4.87	36.12	2.47
40.00	70.33	10.78	34.91	0.28

Figure 6-5: Effect of moisture content on biomass production in SSF after 10 days

Moisture (%)	dry bio (g/g)	dry error	wet bio (g/g)	wet error
30	0.11	0.01	0.15	0.01
40	0.26	0.02	0.44	0.01
50	0.23	0.01	0.49	0.03
55	0.20	0.02	0.50	0.05
60	0.16	0.01	0.48	0.02
65	0.13	0.00	0.46	0.02
70	0.10	0.01	0.41	0.07
75	0.08	0.01	0.40	0.03
80	0.06	0.01	0.36	0.04

Figure 6-6: Effect of moisture content on residual sugar concentration in SSF after 10 days

Moisture (%)	reducing (mg/g)	red. error	non-reducing (mg/g)	non-red. error	total sugar (mg/g)	total error
30	64.23	4.78	35.19	13.05	99.42	12.28
40	28.54	1.01	20.57	1.91	49.11	0.90
50	25.02	1.34	20.54	1.66	45.56	0.61
55	25.36	0.47	16.55	0.79	41.91	0.38
60	22.69	1.49	17.51	0.60	40.20	1.96
65	20.14	0.54	20.28	1.38	40.42	1.02
70	17.16	1.09	23.76	0.91	40.92	0.92
75	17.58	0.94	26.15	0.09	44.09	0.89
80	15.61	0.46	27.38	1.91	43.00	2.10

Figure 6-7: Effect of moisture content on final PPO activity in SSF after 10 days

Moisture	PPO	PPO err	PPO	PPO error
(%)	(nkat/ g dry)		(nkat/ g wet)	
30	0.00	0.00	0.00	0.00
40	0.00	0.00	0.00	0.00
50	31.97	12.47	14.86	5.87
55	210.42	9.68	83.91	4.25
60	442.72	38.09	150.73	10.34
65	806.28	81.24	227.80	23.65
70	1612.89	100.13	398.09	35.09
75	2011.07	389.29	420.10	64.40
80	3319.37	206.98	556.06	28.55

Figure 6-8: Effect of moisture content on residual level of CM phenolics in SSF after 10 days

Moisture	% SAE	% SIN	% SA
(%)			
30	92.20	72.99	70.26
40	39.61	22.69	26.97
50	27.68	14.47	16.82
55	13.83	7.31	3.33
60	5.37	2.22	0.00
65	0.00	1.08	0.00
70	0.50	1.41	0.00
75	0.39	1.44	0.00
80	1.50	2.67	0.00

Figure 6-9: Effect of Vogel's minerals on PPO production

Time	no Vogels	no Vogels	1 mL/L	1 ml/l	10 mL/L	10 ml/L	50 mL/L	50 ml/L
(d)		error		error		error		error
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.8	4.19	0.01	2.70	0.05	3.29	0.32	7.07	3.71
5.8	29.59	1.46	51.98	1.87	43.57	3.60	60.11	3.85
7.7	60.24	0.91	91.73	16.28	84.98	23.49	116.93	9.89
9.8	115.77	1.24	144.81	1.51	139.90	0.34	132.60	4.89
13.7	130.63	3.57	159.62	5.70	141.07	17.79	178.94	22.46
18.8	133.78	29.74	138.06	3.64	148.50	12.15	153.35	

Figure 6-10: Effect of Vogel's minerals on SIN content decrease

Time	no Vogels	no error	1 mL/L	1 ml/l	10 mL/L	10 ml/L	50 mL/L	50 ml/L
(d)		error		error		error		error
0	13.26		13.26		13.26		13.26	
1.5	6.85	0.02	7.23	0.05	6.77	0.42	6.88	0.03
3.8	3.51	0.29	4.52	1.32	4.11	1.46	3.39	0.97
5.8	2.35	0.31	2.08	0.25	1.67	0.08	1.41	0.22
7.7	1.28	0.01	1.17	0.10	1.16	0.04	1.03	0.02
9.8	0.76	0.04	1.07	0.18	0.77	0.07	0.55	0.14
11.8	0.64	0.19	0.52	0.08	0.44	0.08	0.42	0.18
13.7	0.57	0.03	0.47	0.07	0.49	0.13	0.56	0.33
18.8	0.22	0.02	0.24	0.01	0.20	0.01	0.11	

Figure 6-11: Effect of Vogel's minerals on SA content decrease

Time	no Vogels	no Vogels	1 mL/L	1 ml/l	10 mL/L	10 ml/L	50 mL/L	50 ml/L
(d)		error		error		error		error
0	189.13		189.13		189.13		189.13	
3.8	89.61	13.62	70.82	33.23	79.71	37.62	61.67	6.34
5.8	42.62	7.64	33.07	10.27	30.55	0.90	18.75	3.77
7.7	17.57	0.79	16.65	0.92	13.80	2.27	14.42	1.09
9.8	6.96	0.88	10.46	0.33	6.23	0.81	6.47	0.63
11.8	5.83	0.43	4.57	0.63	4.39	1.58	4.31	0.89
13.7	5.48	0.12	5.47	0.69	4.29	0.51	4.37	1.05
18.8	4.68	1.36	3.50	0.13	3.53	0.13	2.48	

Figure 6-12: Effect of inoculum size on PPO production during SSF on CM

Time (d)	2.5mL	2.5 mL	5 mL	5 mL	10 mL	10 mL
		error		error		error
0.00	0.00		0.00		0.00	
2.75	0.00		0.00		0.00	
6.00	43.97	2.60	46.76	13.19	31.83	9.24
9.04	82.55	2.06	67.51	7.81	67.55	0.48
11.92	115.72	6.39	87.55		81.09	29.80
14.71	241.45	33.43	119.59	14.29	170.98	35.35

Figure 6-13: Effect of inoculum size on SAE content decrease during SSF on CM

Time (d)	2.5mL	2.5 mL	5 mL	5 mL	10 mL	10 mL
		error		error		error
0.00	23.60		23.60		23.60	
2.75	12.64	1.99	11.83	3.76	8.31	0.16
6.00	3.35	0.09	3.88	0.75	3.87	0.74
9.04	1.66	0.10	1.53	0.68	1.15	0.08
11.92	0.48	0.31	1.14	0.49	0.62	0.50
14.71	0.59	0.19	0.59	0.14	0.63	0.45

Figure 6-14: Effect of inoculum size on SIN content decrease during SSF on CM

Time	2.5mL	2.5 mL	5 mL	5 mL	10 mL	10 mL
(d)		error		error		error
0.00	13.26		13.26		13.26	
2.75	4.82	1.02	2.54	0.11	2.17	0.01
6.00	0.98	0.19	0.98	0.12	0.61	0.18
9.04	0.54	0.09	0.44	0.09	0.34	0.02
11.92	0.30	0.06	0.26	0.11	0.13	0.04
14.71	0.23	0.02	0.16	0.06	0.13	0.01

Figure 6-15: Effect of inoculum size on SA content decrease during SSF on CM

Time	2.5mL	2.5 mL	5 mL	5 mL	10 mL	10 mL
(d)		error		error		error
0.00	189.13		189.13		189.13	
2.75	23.26	3.53	9.63	4.10	12.98	0.29
6.00	11.42	1.69	14.25	2.74	14.46	1.87
9.04	9.39	0.29	8.83	1.89	8.30	0.25
11.92	6.80	0.81	6.87	0.84	8.31	2.78
14.71	7.65	0.55	6.07	0.63	6.94	0.76

Figure 6-16: Effect of homogenization on PPO production during SSF on CM

Time	10 s	10 s	30 s	30 s	75 s	75 s	150 s	150s
(d)		error		error		error		error
0.00	0.00		0.00		0.00		0.00	
2.75	0.00		0.00		0.00		0.00	
6.00	36.25	11.50	46.76	13.19	31.10	2.58	37.88	0.00
9.04	63.32	5.49	67.51	7.81	58.03	17.24	57.01	15.25
11.92	101.90	1.75	87.55		97.46	5.97	91.00	6.04
14.71	124.58	3.05	119.59	14.29	118.29	13.48	132.97	22.73

Figure 6-17: Effect of homogenization on SAE content decrease during SSF on CM

Time	10 s	10 s	30 s	30 s	75 s	75 s	150 s	150s
(d)		error		error		error		error
0.00	23.60		23.60		23.60		23.60	
2.75	13.65	0.42	11.83	3.76	13.15	1.25	9.76	2.25
6.00	4.48	0.04	3.88	0.75	4.33	1.29	4.75	2.66
9.04	1.09	0.23	1.53	0.68	1.89	0.35	1.86	0.13
11.92	0.65	0.06	1.14	0.49	0.78	0.51	1.04	0.08
14.71	0.41	0.16	0.59	0.14	0.57	0.20	0.75	0.50

Figure 6-18: Effect of homogenization on sinapine content decrease during SSF on CM

Time	10 s	10 s	30 s	30 s	75 s	75 s	150 s	150s
(d)		error		error		error		error
0.00	13.26		13.26		13.26		13.26	
2.75	3.92	0.94	2.54	0.11	3.90	1.33	2.61	0.42
6.00	1.20	0.15	0.98	0.12	0.69	0.04	1.11	0.37
9.04	0.34	0.09	0.44	0.09	0.49	0.12	0.46	0.14
11.92	0.26	0.01	0.26	0.11	0.28	0.08	0.42	0.10
14.71	0.23	0.00	0.16	0.06	0.14	0.00	0.20	0.09

Figure 6-19: Effect of homogenization on SA content decrease during SSF on CM

Time	10 s	10 s	30 s	30 s	75 s	75 s	150 s	150s
(d)		error		error		error		error
0.00	189.13		189.13		189.13		189.13	
2.75	7.48	7.75	9.63	4.10	10.38	12.53	12.38	2.27
6.00	12.52	3.63	14.25	2.74	9.91	1.85	16.31	4.66
9.04	7.49	0.84	8.83	1.89	9.18	1.09	10.03	0.32
11.92	7.12	0.13	6.87	0.84	9.92	4.59	11.31	3.46
14.71	6.28	0.01	6.07	0.63	6.40	0.43	10.63	2.97

Figure 6-20: SSF fermentation for flooding experiment

Time (d)	Biomass	reducing	non-reduci	PPO
	(g/g)	(mg/g)	(mg/g)	(nkat/g)
0.00	0.01	5.96	43.71	0.00
2.63	0.10	22.96	11.28	0.00
4.63	0.47	13.24	5.55	40.21
6.21	0.57	8.17	5.75	82.96
9.63	0.53			127.38
12.67	0.50	6.10	5.99	129.22

Figure 6-21 Decrease in phenolic content during SSF for flooding experiment

Time (d)	SAE	SIN	SA
	(g/kg)	(g/kg)	(mg/kg)
0.00	21.66	9.21	215.69
2.63	20.33	7.87	79.19
4.63	3.98	1.27	0.00
6.21	2.76	0.52	0.00
9.63	0.88	0.15	0.00
12.67	0.32	0.04	0.00

Figure 6-22and 23: Effect of flooding in a 20% and 40% (w/v) suspension of culture in water

Time (h)	20% Sterile
0	21.19
0.25	
0.5	
1	19.83
2	20.44
8	21.34

3 day culture

Time (h)	20%	20% error	40%	40% error
0	20.42	0.22	20.42	0.22
0.25	16.75		16.66	0.77
0.5	17.53	1.29	15.94	1.72
1	16.68	0.34	14.04	1.19
3	15.69	0.14	13.59	0.76
5	12.77	0.31	11.27	0.52
16	10.03	0.02	7.41	0.16

5 day culture

Time (h)	20%	20% error	40%	40% error
0	5.16	0.57	5.16	0.57
0	4.45	0.25	4.80	0.13
1	4.53	0.23	4.30	0.08
1	4.85	0.98	4.35	0.05
3	4.64	0.06	4.62	0.20
5	5.01	0.57	3.85	0.04
8	4.68	0.31	3.88	0.06

7 day culture

Time (h)	20%	20% error	40%	40% error
0	4.02	0.04	4.02	0.04
0.25	4.54	0.25	3.43	0.01
0.5	4.33	0.38	3.39	0.43
0.75	3.36	0.36	3.42	0.22
1	4.22		3.99	0.92
2	4.08	0.38	3.52	0.00
3	4.44	0.32	3.62	0.51
5	4.00	0.06	3.43	0.11
8	3.90	0.08	3.11	0.03

Figure 6-24: Decrease in phenolic content of a 3 day culture due to addition of water to 20%

Time (h)	SAE (g/kg)	SAE err	SIN (g/kg)	SIN error	SA (mg/k	SA error
0	20.42	0.22	7.87	0.16	189.13	
0.25	16.75		5.99		159.15	
0.5	17.53	1.29	6.21	0.42	148.79	5.07
1	16.68	0.34	5.86	0.07	134.16	0.96
3	15.69	0.14	5.04	0.26	111.12	3.74
5	12.77	0.31	3.54	0.29	60.15	26.85
16	10.03	0.02	2.02	0.22	47.18	3.54

Figure 6-25: Decrease in phenolic content of a 3 day culture due to addition of water to 40%

Time (h)	SAE (g/kg)	SAE err	SIN (g/kg)	SIN error	SA (mg/k	SA error
0	20.42	0.22	7.87	0.16	189.13	
0.25	16.66	0.77	6.02	0.41	123.56	1.13
0.5	15.94	1.72	5.63	0.75	121.46	9.32
1	14.04	1.19	4.38	0.92	75.79	28.74
3	13.59	0.76	3.91	0.79	54.97	23.07
5	11.27	0.52	2.19	0.14	44.58	2.94
16	7.41	0.16	0.78	0.03	17.59	19.62