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The Production of Phenolics-Degrading Enzymes in Submerged and Solid State Fermentation and the Decrease in Phenolic Content of Canola Meal using the White-Rot Fungus *Pleurotus ostreatus*

A Thesis Submitted to the School of Graduate Studies and Research in
Partial Fulfillment of the Requirements for the Degree of

Master of Applied Science
In the Department of Chemical Engineering
University of Ottawa

June 30, 1999

Abstract

Canola meal (CM) is a by-product of canola oil production. Because of the high nutritional composition CM has, it is often used as a protein source for livestock feeding. However, its usage is limited due to the presence of some undesirable compounds in CM, such as glucosinolates, phytic acid and phenolic compounds (mainly sinapic acid esters-SAE). Phenolic compounds reduce the digestibility of proteins in CM, and they are also responsible for the dark color and bitterness of the meal.

The objectives of this research project were: 1) to study the production of phenolics-degrading enzymes secreted by the white-rot fungus *Pleurotus ostreatus* in submerged and solid state fermentation processes; 2) to examine the degradation of phenolic compounds in CM during solid state process.

The effects of some operating parameters on enzyme production were investigated during the submerged process. It was found that the growth of biomass and enzyme production depended on the initial glucose concentration. An optimum of 4% was found for the enzyme production. An increase in inoculum concentration helped the initial biomass growth. However, the maximum enzyme activity was not found in the culture with the largest amount of inoculum. In the study of the effect of oxygen concentration, it was found that inhibition of enzyme production occurred at high concentrations of oxygen. Among the five chemical inducers tested, 2,5-xylidine was found to cause the highest increase in enzyme production, around 1.8 folds. When the effect of CM was studied, an optimum of 3% of CM was found for the enzyme production during the submerged process.

Enzyme prepared from the submerged process was characterized using three substrates, sinapic acid (SA), sinapine (SIN) and sinapaldehyde (SALD). The results showed that the enzyme was inhibited by high concentrations of all three substrates. The optimum pH values were 4.4, 5.0 and 4.7, using SA, SIN and SALD as the substrates, respectively. The optima temperatures for enzyme activity were in the range of 40°C to 60°C for all three substrates.

The production of enzyme and degradation of phenolics during solid state process were studied. The effect of moisture content was important for enzyme production and the optimum was around 60%. However, the highest rate of SAE degradation was found in the culture with the highest moisture content tested, at 75%. The highest enzyme activity was in the culture with the lowest inoculum concentration tested. The results indicated that rapid growth of biomass in later stage of the process helped the production of enzyme. An increase in homogenization time resulted in increases in biomass and enzyme production. However, the effect was not obvious on SAE degradation. Larger particle size of the CM favored the production of enzyme, biomass and the degradation of SAE.

Three surfactants, Triton X-100, Tween 80 and sodium oleate, were tested for their effects on the solid state process. Sodium oleate was found to cause the highest increase in enzyme production, at about 40%.

Modeling of biomass and enzyme production, as well as SAE degradation during solid state process have been attempted. The modeling equations were based on a logistic kinetic equation for biomass production. These equations were not able to fit all experimental data well. Better fitting was found when there was a short or no lag phase in biomass growth during the solid state process.

Résumé

Les tourteaux de canola (CM) sont des sous-produits du procédé de pressage des graines oléagineuses de canola. Étant donné leur contenu élevé en valeur nutritive, ils sont utilisés comme source de protéines dans l'alimentation animale. Cependant, leur usage est limité dû à la présence de composés indésirables tels des glucosinates, de l'acide phytique et des composés phénoliques (principalement les estères de l'acide sinapique, SAE). La présence de composés phénoliques occasionne la digestion difficile des protéines présente dans les tourteaux et sont également responsables de la coloration foncée et du goût amère des tourteaux.

Les objectifs de ce projet étaient: 1) d'étudier la production d'enzymes dégradantes des composés phénoliques par la moisissure *Pleurotus ostreatus* en milieu de culture submergée et en milieu de culture solide; 2) d'étudier la dégradation des composés phénoliques présents dans les tourteaux de canola en milieu de culture solide.

Les effets de certains paramètres d'opération sur la production d'enzyme ont été étudiés lors du procédé en milieu de culture submergée. Il a été démontré que la croissance de biomasse ainsi que la production d'enzyme dépendent de la concentration initiale de glucose dans le milieu de culture. La production d'enzyme est optimum à une concentration de glucose de 4%. Une augmentation de la concentration initiale de biomasse aide à la croissance initiale mais l'activité enzymatique maximum n'a pas été observée en milieu contenant la plus grande quantité de biomasse. Lors de l'étude de l'effet de la concentration d'oxygène, il a été démontré que la production d'enzyme est inhibée à des concentrations d'oxygène élevées. Parmi les 5 inducteurs chimiques testés, 2,5-xylidine provoque la plus grande augmentation de production d'enzyme, ce par un facteur d'environ 1.8. Lorsque l'effet des tourteaux de canola a été étudié, un optimum de 3% de CM a été obtenu pour la production d'enzyme en culture submergée.

La charactérisation d'enzyme produite à partir de cultures submergées était effectuée en utilisant trois substrats: l'acide sinapique (SA), le sinapine (SIN), et

l'aldéhyde de l'acide sinapique (SALD). Les résultats démontrent que l'enzyme est inhibée par des concentrations élevées de chacun de ces substrats. Les pH optimum sont respectivement de 4.4, 5.0 et 4.7 en utilisant SA, SIN, et SALD comme substrat. Les températures optimum pour l'activité enzymatique varient entre 40°C et 60°C pour les trois substrats.

La production d'enzyme et la dégradation des composés phénoliques lors du procédé en milieu de culture solide ont été étudiées. L'effet du contenu en eau était important pour la production d'enzyme et l'optimum a été déterminé comme étant un contenu d'eau de 60%. Par contre, le taux de dégradation de SAE le plus élevé a été observé dans les cultures contenant la plus grande quantité d'eau testée, soit 75%. L'activité enzymatique la plus élevée a été observée dans les cultures contenant la plus petite quantité de biomasse initiale. Les résultats indiquent que la croissance rapide de biomasse dans les stages avancés du procédé aide à la production d'enzyme. Une augmentation du temps d'homogénisation résulte en une augmentation de biomasse et de production d'enzyme mais ces effets ne sont pas évidents sur la dégradation de SAE. Une grosseur de particules plus élevée des tourteaux de canola favorise la production d'enzyme, la croissance de biomasse et la dégradation de SAE.

Trois surfactants, Triton X-100, Tween 80 et le sodium d'oléate ont été testés pour leurs effets sur le procédé en milieu de culture solide. Le sodium d'oléate cause la plus gande augmentation dans la production d'enzyme, ce à 40%.

La modélisation de la croissance de biomasse et de la production d'enzyme, ainsi que de la dégradation de SAE lors du procédé en milieu de culture solide ont été tenté. Les équations étaient basées sur l'équation logistique de la cinétique de la croissance de biomasse. Ces équations n'ont pas été efficaces à représenter toutes les données expérimentales. De meilleurs résultats ont été obtenus lorsque la période latente de croissance était courte ou absente.

Acknowledgements

I would like to express my gratitude to Dr. Duvnjak for his guidance and encouragement throughout this project. I would like to thank the Natural Science and Engineering Research Council (NSERC) for its scholarship. Many thanks are also extended to Mr. Jeff Woods, Mr. Hasan Atiyeh and Ms. My-Hanh Luu, for their help and encouragement.

I am forever thankful to my parents, my sister Jinghong and my friend Yidong for their love, support and encouragement.

Nomenclature

Abbreviations

ABTS - 2,2'-azinobis(3-ethylbenthiazoline-6-sulfonic acid)

CM - Canola Meal

DNS - 3,5-Dinitrosalicyclic acid

DMP - Dimethylpyrazine

i.d.w. - initial dry weight

LiP - Lignin Peroxidase

MnP - Mangnese Peroxidase

nKat - nanokatal (nmole/s)

PPO - polyphenol oxidase

RASD - Rapid Acceleration followed by Slow Deceleration

SA - Sinapic Acid

SALD - Sinapaldehyde

SIN - Sinapine

SSC - Solid State Culture

SSF - Solid State Fermentation

TMA - Trimethylamine

TTMP - Tetra-Methylpyrazine

Symbols

A_{ini} - initial absorbance

A_{fin} - final absorbance

Dil - Dilution factor for equation 4.5-1

k - exponential rate constant for RASD kinetics equation

K_1,K_2 - constants defined by equations 3.4-6,7

K',K'' - empirical constants from equation 3.4-3

K_m - Michaelis-Menten's constant

K_s - rate constant for SAE degradation (g dry SSC/g /day)

K_v - rate constant for enzyme production (nKat/g /day)

M_{CM} - mass of canola meal solid sample (g)

r - rate of increase in enzyme activity from equation 3.1-4

s - substrate concentration (nmol/mL)

SAE - Sinapic Acid Esters, concentration of SAE (g/g dry SSC)

SAE_0 - Initial SAE concentration (g/g dry SSC)

t - time (day)

t_1 - time when the maximum enzyme activity is attained

V_e - volume of enzyme (mL)

V_{ext} - volume of extract added to cuvette (mL)

V_{MeoH} - volume of methanol used for extraction (mL)

V_{sys} - volume of reaction system (mL)

X - biomass concentration (g/g dry SSC)

X_m - maximum biomass concentration (g/g dry SSC)

X_0 - initial biomass concentration (g/g dry SSC)

Y - yield coefficient for biomass

Greek Letters

β - parameter defined by equation 3.1-8

ε - extinction coefficient ($mol^{-1}cm^{-1}$)

ε' - slope of standard curve for sinapine determination

μ_m - maximum specific growth rate (day^{-1})

ν - enzyme activity (nKat/mL or nKat/g dry SSC)

ν_0 - initial enzyme activity (nKat/g dry SSC)

ν_{max} - maximum enzyme activity (nKat/mL)

ν_{opt} - enzyme activity at optimum pH (nKat/mL)

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Chapter 1

Introduction

The global production of rapeseeds, including canola varieties ranks third amongst oilseed crops (Shahidi, 1990). The history of canola goes back to the rapeseed plant, but canola and rapeseed are not the same. Canola is a genetic variation of rapeseed and the seeds it produces contain about 40% oil (Lacki, 1997). Canola seeds are crushed to obtain canola oil for human consumption, and the solid residue remaining after the de-oiling process, called canola meal (CM), is used as a high protein source for livestock feed. On average, CM contains 35-40% proteins (Lacki, 1997) and has a well-balanced amino acid composition.

A large amount of canola meal is produced in Canada each year, and the trend has been increasing (Lacki, 1997). Although CM is highly nutritional as livestock feed, it is only used in small portions. This is because of some anti-nutritional components contained in CM, such as glucosinolates, phytic acid and phenolic compounds (mainly sinapic acid esters or SAE).

Phenolic compounds (phenolics) in CM have strong binding affinities towards dietary proteins making them difficult to digest, and they are also responsible for the dark color and bitterness of the meal. The predominant phenolic compounds present in canola are phenolic acids. Phenolic acids in CM are in free, esterified and insoluble-bound forms and are derivatives of benzoic and cinnamic acids (Naczk et al., 1998).

The level of phenolics content in CM is around four to five times higher than that in soybean meal (Naczk et al., 1998). If the problems of CM can be solved or reduced,

CM can be a competitive protein source for livestock feeding and the market for CM can be greatly increased.

Phenolic compounds in CM can be removed or degraded by extraction, chemical reaction and enzymatic treatment. The methods of extraction or chemical reaction usually involve the use of large amount of organic solvent or chemicals. Although the removal efficiencies have been reported to be high, traces of organic solvent or chemicals left in the meal create another problem when CM is used to feed animals. On the other hand, enzymatic treatment avoids using organic solvent or chemicals. The reaction conditions involved are mild (Lacki, 1997) and the method reduces the risk of degradation of nutritional components in the meal.

Enzymes that are capable of degrading phenolic compounds can be produced by certain white-rot fungi during fermentation processes. The efficiency of phenolics degradation in CM can be high using enzymatic treatment as reported by Lacki and Duvnjak (1996). They used the fungus *Trametes versicolor* for the production of polyphenol oxidase, one type of phenolics-degrading enzymes, in the degradation of phenolic compounds in CM.

In this study, another type of white-rot fungus (*Pleurotus ostreatus)* was used. One of the enzymes this fungus can produce is called laccase. This enzyme is classified as a polyphenol oxidase. Although the characteristics of this laccase are expected to be different from those of the polyphenol oxidase produced by *T. versicolor*, their functions should be similar in degrading phenolic compounds. In fact, laccase has been reported to attack and degrade phenolics effectively (Martirani el al., 1995; Srinivasan, 1995), and the fungus *P. ostreatus* is one of the most active laccase producers. Therefore, effective

degradation of phenolic compounds in CM is expected using the enzymes produced by this fungal species during fermentation processes.

Fermentation processes can be in either submerged or solid state forms. Submerged process involves the growth of microorganisms in liquid medium with dissolved or suspended nutrients. Solid state process uses solid substrates or nutrients with the addition of certain amount of water. Although submerged process creates a more homogenous growing environment, solid state fermentation is closer to the natural habitat for fungi to grow. Studies show that solid state process could promote the production of enzymes, and the enzymes produced are more concentrated and more substrate specific (Smits et al., 1998).

The objectives of this research project are:

1. to study the effects of operating parameters on enzyme production during the submerged process using the fungus *P. ostreatus*;
2. to characterize the enzymes produced by this fungus using phenolic compounds found in CM as the substrates;
3. to examine the effects of operating parameters on enzyme production during solid state processes;
4. to study the degradation of phenolic compounds in CM during the solid state process;
5. finally, to test the suitability of some logistic model equations for describing the biomass production, enzyme production and degradation of phenolic compounds during solid state processes.

Chapter 2

Literature Review

This section reviews the literature on canola meal, phenolic compounds in canola meal and the production of phenolic-degrading enzymes during fermentation processes.

2.1 Rapeseed and Canola

Rapeseed was introduced as an alternative crop for Canadian producers during the World War II when the rapeseed oil was mainly used as a lubricant. Since then, efforts have been focused on rapeseed breeding towards improving both oil and meal quality (Wang, 1992). In the early 1970s, research conducted at the University of Manitoba produced the first genetically improved rapeseed variety with oil suitable for human consumption and meal for livestock feed (www.gov.mb.ca, 1999). A number of this type of cultivars have also been bred in Europe and other countries (Wang, 1992) and now, the global production of rapeseed ranks third amongst oilseed crops (Shahidi, 1990). These genetically improved rapeseeds are mostly referred to as "canola", a combination of two words "Canadian" and "oil". The name canola also became a certification mark that defines the quality parameters of less than 2% erucic acid in the oil and less than 30 μg/g glucosinolates in the meal.

2.1 .1 Canola

Canola is grown primarily in western Canada and the seeds contain approximately 40% oil. The oil content depends on the variety and the environment. High temperatures and low soil moisture during pod development can reduce the potential oil content (www.gov.mb.ca, 1999).

In Canada, rapeseed/canola has been ranked before soybean as the most important oilseed crops (Lacki, 1997). Canadians are the largest per capita consumers of canola oil in the world. For a total vegetable oil used in Canada for the production of margarine, shortenings and salad oil, canola has captured 40, 54 and 80% of the market respectively (www.astro.space.gc.ca, 1999).

Canola oil is also used in inedible products such as cosmetics, fungicides, herbicides, pesticides, plasticisers, suntan oil and anti-static for paper and plastic wrap. In Japan, it has been used as an organic fertilizer for tea and citrus fruit crops. Canola oil has high potentials of being used as industrial lubricant and fuel additive. The so called bio-diesel has been shown to reduce the level of engine-emission drastically (www.astro.space.gc.ca, 1999).

2.1.2 Canola Seed Processing

Solvent extraction process is commonly used to remove oil from canola seeds and leave canola meal as a byproduct. In this process, canola seeds are first heated to prevent shattering. Then, these seeds are flaked by roller mills to rupture the seed coat and many small oil cells contained in the seed. The flakes are then cooked/conditioned by steam to thermally rupture remaining oil cells. This processing step also serves to coagulate the protein and reduce oil viscosity. Solvent is used next to extract and separate oil from the remaining residue. The final stage of the process is desolventizing for the production of commercial canola meal. Hexane specially refined for use in the vegetable oil industry can be used for the extraction of canola oil from canola seeds (Al-Asheh, 1993). About 45% of canola produced in Canada is trucked to the nearest processor where it is crushed for oil and meal. Another 40% of Canada's canola seed is exported to Japan for

processing. The rest is exported to Mexico, the United States and Europe (www.astro.space.gc.ca, 1999).

2.2 Canola Meal (CM)

There were 1,739,500 tonnes of CM produced in Canada in the crop year 1997-98 and around 65% of the total is exported to other countries. The United States is the largest importer of canola oil and meal produced by the Canadian processing plants. The meal obtained after oil extraction contains about 40% protein (Naczk et al., 1998). Although the protein has a well-balanced amino acid composition for human use (Naczk et al., 1998), CM is mainly used as portions of livestock feed and poultry diets, and it is rarely used alone. This is primarily due to the presence of some undesirable components, such as glucosinolates, phytic acid and phenolic compounds (Al-Asheh, 1993, Naczk et al., 1998).

CM contains about 0.5% glucosinolates (Lacki, 1997). They are complex organic molecules containing nitrogen and sulphur. The hydrolytic products of glucosinolates, such as isothiocyanates, oxazolidinethiones and nitriles, can cause problems like off-flavor, anti-nutritive and toxic effects. Such hydrolysis occurs when the myrosinase enzyme (contained in the meal), in the presence of moisture, reacts with glucosinolates. In the industry of canola processing, the removal of glucosinolates is still not a standard practice in canola oil production. However, a number of new processes have been developed, including methods that involve chemical, microbial and physical treatment, for the removal of glucosinolates (Naczk et al., 1997).

Another type of anti-nutritional compound in CM is phytic acid, which represents 78 to 88% of total phosphorus in CM (Naczk et al., 1998). In foods and seeds, phytic

acid can bind mono and divalent metal ions to form complex phytates, thus reducing the bioavailability of these essential minerals (Erdman, 1979). Techniques for phytates removal include physical and/or chemical methods as well as solid state fermentation process (Al-Asheh, 1993).

In addition to glucosinolates and phytic acid, phenolic compounds are also seen as undesirable compounds in CM. The content of phenolic compounds is much higher compared to other oilseeds (Shahidi, 1992). It can be up to four or five times higher in CM than that found in soybean meal (canola meal, 15.4-18.4g/kg dry basis and soybean meal, 4.6g/kg dry basis) (Naczk et al., 1998). Phenolics are believed to cause off-flavor of the meal and decrease the protein digestion in CM. Hence, they are also a concern when considering CM as a source of human food-grade protein.

2.3 Phenolic Compounds:

The content of phenolic compounds in CM increases during the ripening of the seeds. The content of water-soluble phenolics reaches maximum between the final stage of the green seeds and the beginning of their browning stage, remaining at a stable level during the stage of ripeness (Naczk et al., 1998). The predominant phenolics present in rapeseed/canola are phenolic acids and condensed tannins.

2.3.1 Phenolic Acids:

Phenolic acids in rapeseed, including the canola varieties, account for about 2% of the oil-free meal mass (Xu and Diosady, 1997). There are three forms of these acids: the free, esterified and insoluble-bound forms, and they are derivatives of benzoic and cinnamic acids (Naczk et al., 1998).

Free phenolic acids:

Canola meal may contain over two grams of free phenolic acids per kg of meal. Sinapic acid, 3,5-dimethoxy-4-hydroxy cinnamic acid, constitutes 70-85% of the total free phenolic acids. There are also small quantities of p-hydroxy-benzoic, vanillic, protocatechuic, syringic, p-coumaric, ferulic, gallic, caffeic and chlorogenic acids in the free form (Naczk and Shahidi, 1989), as shown in Figure 2.1.

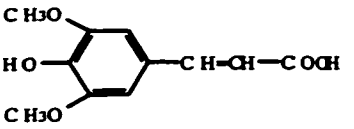
Esterified phenolic acids:

Esterified phenolic acids constitute up to 80% of the total phenolic acids, and sinapic acid esters (SAE) are predominant. Sinapine, choline ester of sinapic acid (3,5-dimethoxy-4-hydroxy cinnamoylcholine), is the most abundant phenolic ester (Figure 2.2) present in CM. This ester compound is the most important phenolic found in canola and rapeseed. It is thought that sinapine in germinating seedlings is hydrolyzed to choline and sinapic acid. Sinapic acid is required for the biosynthesis of lignin and flavonoids, while choline is an important metabolite in the methylation cycle. These compounds are known to exhibit antioxidant properties (Shihadi, 1992). There are also at least seven other phenolic esters (Fento et al., 1980).

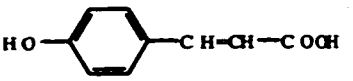
Insoluble-bound phenolic acids:

Canola meal contains about one gram of insoluble bound phenolic acids per kg of meal. Sinapic acid is found to be predominant as it constitutes 30-59% of the total insoluble fraction in rapeseed and mustard flours (Naczk et al., 1998).

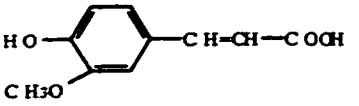
sinapic acid



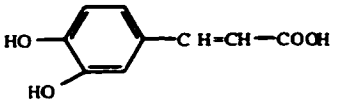
coumaric acid



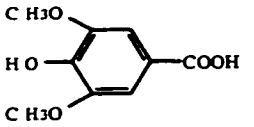
ferulic acid



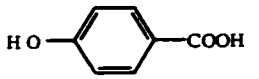
caffeic acid



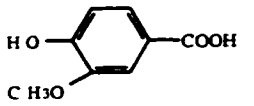
syringic acid



p-hydroxybenzoic acid



vanillic acid



gallic acid

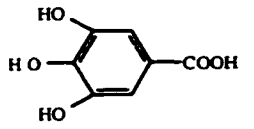


Figure 2.1. Chemical structures of phenolic acids in CM.

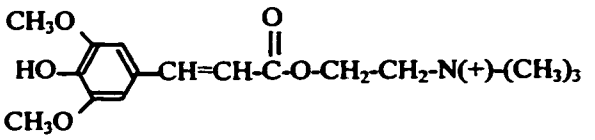


Figure 2.2 Chemical structure of sinapine

2.3.2 Condensed Tannins

Tannins are complex phenolic compounds that occur either in the hydrolyzable or condensed forms in plant materials. Canola hulls may contain up to 2% of condensed tannins, but the amount varies depending on growing conditions of canola (Naczk et al., 1998). The content of condensed tannins is lower in CM compared to canola hulls and is found to be around 0.68 to 0.77% (Shahidi and Naczk, 1989).

2.3.3 Determination of Phenolic Content in CM

A number of analytical techniques have been developed for the phenolic content analysis (Table 2.1). Each has its particular advantages and drawbacks. Hence, the choice of analytical method to use depends on individual preferences and the goals of a particular research project (Lacki, 1997). Extraction using organic solvents is usually required prior to these analyses.

Table 2.1 Methods involved in determination of phenolic content in CM

Methods	Comments	References:
1. Direct Spectrophotometric (DS)	Measures in terms of sinapic acid esters (SAE) plus free sinapic acids	Naczk et al., 1992; Pink et al., 1994
2. Chemical Spectrophotometric (ChS)	Measures only all SAE	Ismail and Eskin, 1979
	Also includes free sinapic acid	Lacki and Duvnjak, 1996c
3. Thin Layer Chromatography (TLC)	Based on separation techniques, gives detailed composition of analyzed phenolics;	Fenwick et al., 1986
	Not accurate enough	Xu and Diosady, 1997
4. Gas-Liquid Chromatography (GLC)	Based on separation techniques, gives detailed composition of analyzed phenolics	Krygier et al., 1982; Dabrowski and Sosulski, 1983
5. High Performance Liquid Chromatography (HPLC)	Based on separation techniques, gives detailed composition of analyzed phenolics;	Bouchereau et. al., 1991
	Methods 3, 4 and 5 are slower compared to spectrophotometric methods	Lacki, 1997
6. Gas Chromatography-Mass Spectrometry (GC-MS)	Requires extensive preparative work, instrumentation involved is expensive	Krygier et al., 1982, Xu and Diosady, 1997

7. Naczk et al.'s Wet Chemical method	Total phenolic acids are broken down into three fractions: free, esterified and insoluble-bound; involves many steps for the extraction and fractionation of the phenolic acids followed by colormetric measurement; it is tedious with many potential sources of experimental error	Naczk et al., 1992; Xu and Diosady, 1997
8. Ismail-Eskin's Wet Chemical method	Simpler compared to Naczk et al.'s wet chemical method; but it requires hazardous reagents including $TiCl_4$ in concentrated hydrochloric acid (HCl) for colormetric measurement	Ismail and Eskin, 1979; Xu and Diosady, 1997

2.3.4 Problems Caused by Phenolics in CM

A few major problems exist in CM production and when it is used as animal feed due to high phenolics content in the meal. First, the presence of phenolic compounds in CM may result in the dark color of the meal upon commercial processing. This is due to the fact that the oxidation of flavonoid tannins during heat-processing give rise to products which are dark colored, or reacted with other components of the meal to produce substances which have dark color (Durkee, 1971).

Second, sinapine, as the main component of phenolic content in CM, has been reported to be responsible for the bitter taste and astringency of CM and protein preparation (Ismail et al., 1981). It has been shown that the bitterness of sinapine is 20% higher than that of sinapic acid (Lacki, 1997). With high content of sinapine, the

production of "fishy tainted" brown shell eggs have been reported (Naczk et al., 1998). The fishy odor of these eggs is caused by the accumulation of trimethylamine (TMA) (Goh et al., 1979). TMA is normally oxidized to water soluble TMA oxide by the action of an oxidzing enzyme, TMA oxidase. However, sinapine and tannins tend to block the metabolism of TMA by inhibiting the activity of TMA oxidase.

Sinapine may also be involved in disagreeable taste of meat and milk when canola meal is fed to calves and dairy cows (Wang, 1992).

Finally, sinapic acid, sinapine and other phenolic compounds may interact with proteins (including enzymes) and other nutrients in the meal, render them unavailable for utilization or inhibit the activity of enzymes (Shahidi, 1992). The interaction of phenolics with proteins may be reversible or irreversible.

2.3.5 Removal of Phenolic Compounds from CM

A typical industrial CM process can reduce about 26% of total sinapine content in the meal without any treatment. Different methods have been developed to decrease the phenolic content in CM, making it a more valuable nutrition source. Lacki (1997) summarized the existing methods for this purpose. He stated that three groups of processes are available, which are methods based on 1) extraction, 2) extraction with chemical reaction, and 3) solely chemical reaction.

1). For the extraction methods, organic solvents such as ethanol, methanol, 1% HCl in methanol, or hexane-methanol mixture are used to extract phenolic compounds. These are usually analytical techniques used for the quantitative determination of phenolic content in the meal. Dabrowski and Siemieniak (1987) reported that a combination of various solvents and extraction conditions could remove most of the sinapine. A multi-stage countercurrent extraction with a mixed solvent of acetone-

methanol-water was reported to reduce sinapine in rapeseed meal by 97%. Although solvent extraction methods have achieved high efficiencies in removing phenolic compounds (up to 100%), the traces of organic solvent left in the meal create another problem (Lacki, 1997). To overcome this difficulty, methods using water as the extractant have been developed. For example, some of these methods involve soaking seeds at room temperature for 24 hours. Unfortunately, the efficiencies obtained with these methods are not as high as the methods using organic solvents.

2.) It is expected that the use of extraction with chemical reaction methods would decrease the amount of extractant needed due to a constant presence of the concentration gradient. This could be beneficial for meal quality because a smaller amount of the meal's valuable compounds would be extracted (Lacki, 1997). However, negative effects on the meal quality also exist from these treatments. For example, by using calcium hydroxide during the alkaline hydrolysis treatment, sinapine was reduced by 96% under certain process conditions. This decreases the egg tainting effect, but changes the protein-phenolics interaction from reversible to irreversible binding.

3.) Chemical reaction processes used for removal of phenolics from CM could consist of methods employing a temperature-governed hydrolysis or autolysis of meal. However, denaturation of protein is expected during these high temperature treatments. Another type of chemical reaction treatment is ammoniation, which has been reported to be effective in reducing phenolic compounds. McGregor et al. (1983) reported that gaseous ammoniation of one type of mustard meal removed up to 74% of its sinapine content. An even higher efficiency was achieved (90%) when canola seeds were treated (Fenwick et al., 1986; Shahidi, 1992).

Besides process treatments, plant breeding has also been studied and it is thought to be probably the most economic approach to reduce or eliminate sinapine from seed. However, this would only be possible if a plant with a low or sinapine-free genotype was found in or among the canola variety and its wild relatives. To date such a plant has not been identified. Studies in genetic engineering for producing cultivars that have defects in sinapine biosynthesis are being carried out (Wang, 1992).

Therefore, a procedure that can effectively remove phenolics from CM with little negative effect on the meal quality is still yet to be developed.

2.4 Enzymatic Processes for Degradation of Phenolics

In recent years, a lot of research has been done on enzymatic processes in the area of phenolic-compounds degradation. The enzymes can be produced from many different microorganisms (Walker, 1975; Lacki, 1997). These processes are usually less energy intensive and more environmental friendly compared to other industrial processes. One of the main sources of enzyme is from microorganisms that are usually involved in lignin degradation. Lignin is a random phenylpropanoid polymer (C_6-C_3) that includes polymerized phenolic material, a heterogeneous structure in plants. Among various species studied, white-rot fungi have been found to be the most efficient lignin degraders (Youn et al., 1995). White-rot basidiomycetes produce different extracellular enzymes involved in lignin degradation. The pathways involved in the degradation by different fungi also vary. Most of these fungi can produce lignin peroxidases (LiP), mangnese peroxidases (MnP), laccases and oxidases. These enzymes have been implicated in the ligninolytic system (Buswell et al., 1987). Studies show that, among these enzymes, laccases have broad substrate specificity toward aromatic compounds containing

hydroxyl and amine groups, especially phenolic compounds (Youn et al., 1995). While LiP have exhibited the characteristic of attacking non-phenolic lignin moiety (Munoz et al., 1997), MnP can degrade both phenolic and non-phenolic compounds under certain conditions. Therefore, the use of those laccase-rich fungi to produce extracellular enzymes for the degradation of phenolics has great potential in industrial applications.

2.4.1 Enzymatic Reaction Pathways

Laccase is classified as a polyphenol oxidase [benzenediol: oxygen oxidoreductase] (Youn et al., 1995) and is able to modify the aromatic structures in phenolic compounds. Although these modifications can not directly cause break down of aromatic compounds, they are essential for ring-cleavages to occur. After the modification, ring-cleavage reactions are catalyzed by another enzyme, dioxygenase (Khan and Overend, 1990). Laccase catalyses the abstraction of one electron from phenolic hydroxyl groups to give phenoxy radicals (Youn et al., 1995). When there is a methoxy group attached to the aromatic ring, a demethylation reaction would produce o-quinones (Ishihara, 1980). The o-quinones can then be reduced into catechol compounds which are known to be substrates of the dioxygenases responsible for ring cleavage. Side chain elimination is another important reaction catalyzed by laccase (Ishihara, 1980). These reactions produce p-quinones and can be reduced to hydroquinone-type compounds, which are also known to be substrates for oxygenase.

Figure 2.3 shows an example of this reaction pathway which was suggested by Ishihara and Ishihara (1976). Each phenolic compound can be oxidized to different quinone products. The structure of the original molecule shown in the figure is syringic acid. The reaction was initiated by abstraction of electron from the phenolic hydroxyl group, as mentioned before. The resulting phenoxy radical and its mesomeric forms

proceed to couple. Some of the structures are favored by the influence of the substituents (side chain and methoxyl), and therefore, the frequency of the coupled intermediates radicals varies according to the compounds involved (Ishihara, 1980). The product is usually a mixture of o- and p-quinones with various ratios for different substrates.

The reduction of quinones to catechol or hydroquinone compounds is catalyzed by cellobiose:quinone oxidoreductase (Westermark and Eriksson, 1974). A question naturally arises for how both oxidation and reduction, obviously competing reactions, can be physically compatible. According to Ishihara's (1976) speculation, one possibility is that the oxidizing enzyme and reducing enzyme appear at different growth stages.

These transformation mechanisms are applicable to the transformation of phenolic compounds in CM and are expected to reduce the problem with dietary protein bonding.

2.4.4 Production of Laccase

Laccases produced by various fungi have different characteristics. This section reviews the production of laccase by a few well-known laccase producers.

In 1975, Leonowicz and Trojanowski showed that laccase occurs in both inducible and constitutive forms. The constitutive forms of laccase are produced by fungi in nutrient medium during a fermentation process. The inducible forms of enzymes are produced by the promotion of certain substances, called inducers, in addition of the basic nutrients. Further study was done in 1978 (Leonowicz et al., 1978) to compare the activity towards various phenolic hydrogen donors, using enzymes from a few basidiomycetes species that were known to produce laccase. The fungi used in their study included *Trametes versicolor*, *Pleurotus ostreatus* and *Pholivta mutabilis*. Multiple forms of laccases were found, separable by DEAE – Sephadex chromatography.

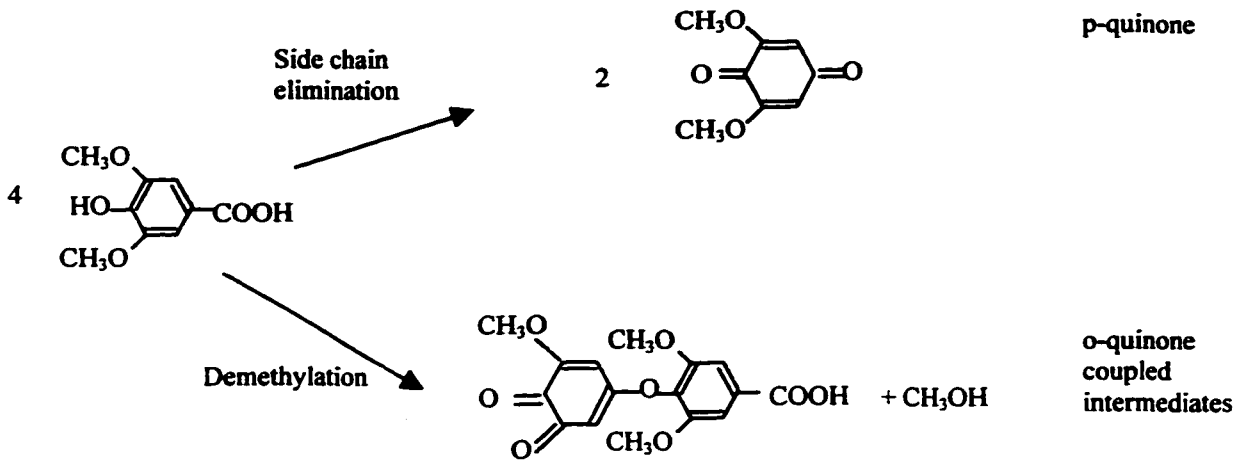


Figure 2.3 Example of a reaction pathway catalyzed by laccase

The number varied from two in *T. vesicolor* and *P. mutabilis* to five or possibly six in *P. ostreatus*. These fungal species oxidized o- and p-phenols (e.g., ferulic acid and hydroquinone) more actively than m-phenols (e.g., orcinol). The induced forms of laccases showed 2-5 folds higher activity than the constitutive forms. They were also more active towards phenolic compounds. It was also found that *P. ostreatus* had the highest specific activity compared to other fungi studied.

A more recent study by Bollag and Leonowicz (1984) shows that considerable amounts of laccase was released into the liquid growth medium by many fungal species, *Botrytis cinerea, Podospora anserina, Fomes annosus, Pholiota mutabilis, P. ostreatus, Rhizoctonia praticola, and T. versicolor*. However, in this study, *T. versicolor* produced the highest laccase activity in both inducible and constitutive forms. *P. ostreatus, F. annosus*, and *P. mutabilis* also produced laccase with higher activity in the induced cultures. The specific activity (U/mg of protein) of laccases from *T. versicolor* were

more than twice as high as the enzymes produced by *P. ostreatus*, in both induced or non-induced forms. The optima pH for enzyme production by most fungi were found to be in the acidic range. For example, the optimum pH for laccase from *P. ostreatus* and *T. versicolor* were 3.8 and 3.6, respectively, when sinapic acid was used as the substrate.

Another laccase producing fungus, *Pleurotus eryngii*, was studied (Munoz et al., 1997), and two protein bands with laccase activity were found in its liquid culture with and without inducers. The intensity of one band was strongly induced by vanillic and veratric acids (3 folds). It had an optimum pH value of 4.5 and was stable from pH 3 to 10 for 24 hours at room temperature. This enzyme had wide substrate specificity on hydroquinones, methoxy-substituted monophenols, and aromatic amines. The maximum activity was found three days after additions of the aromatic compounds.

Although 1mM xylidine has been described as the best elicitor of laccase for the fungus *Phlebia radiata*, for another type of fungus, *P. eryngii*, it was necessary to decrease the concentration to 0.02 mM for a positive effect in laccase production (Rogalski et al., 1991; Nunoz et al., 1997).

Fukushima and Kirk (1995) studied the characteristics of laccase produced by *Ceriporiopsis subvermispora*, one of the best species of fungi for biopulping. It was found that no significant increase of activity was measured after adding xylidine, and higher activity was developed in cultures grown on a wheat bran medium. Two iso-enzymes were purified to homogeneity, which had overlapping pH optima in the range of 3 to 5, depending on the substrates. A variety of usual laccase substrates, including lignin-related phenols and 2,2'-azinobis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) were oxidized.

Laccase produced *by Coriolus hirsutus* had typical biochemical characteristics of fungal laccases (Koroljova-Skorobogat'ko, 1998). The enzyme was stable over pH values of 3.5-6.5 and temperatures of 20-50°C. The K_m values for hydro-quinone, eugenol and sinapic acid were 61, 45 and 24 μM, respectively. These were much smaller compared to the general range (1-100 mM) of K_m values for different laccases. It was also found that syringaldazine was the most efficient inducer of extracellular laccase synthesis. With an addition of syringaldazine at concentration of 0.11μM, the enzyme activity increased by 1000%.

In conclusion, laccases from various fungal cultures differed markedly in their inducibility, number of enzyme forms and pH optimum. However, evidence showed that their main function, transformation of phenolic substances, is the same (Bollag and Leonowicz, 1984). Furthermore, the growth conditions are critical for the production of enzymes (Lacki, 1997).

Reasearch has been done by Lacki (1997) and Woods (1999) on the degradation of phenolic content in CM using fungus *T. versicolor*. They studied the characteristics of polyphenol oxidase (PPO) which is the main phenolic-degrading enzyme produced by this fungus. The enzyme was able to degrade phenolic compounds in CM.

P. ostreatus, is one of the most active producers of phenolics-degrading enzymes. It can be expected that this fungus also has the ability to effectively degrade the phenolic compounds in CM. *P. ostreatus* is an edible mushroom fungus (Sarikaya, 1996), its growth on CM could upgrade meal quality and at the same time produces edible mushrooms. This fungus was used in this research project.

2.4.5 Enzymes Produced by *P. ostreatus*:

P. ostreatus is well known for the production of lignin-degrading enzymes. It has been reported to produce lignin peroxidase (LiP), mangnese peroxidase (MnP), laccase and arylalcohol oxidase (Tuor et al., 1995). But initially, no LiP could be found from *P. ostreatus*. However, Kang et al. (1992), were able to purify an extracellular peroxidase from *P. ostreatus*. Results showed that this enzyme exhibited peroxidase activity for a wide variety of phenols and phenolic compounds and the activity was dependent on H_2O_2. This was confirmed by Akhmedova (1995) and Han et al. (1996) with different media and growth conditions.

Laccase from *P. ostreatus* can be induced by xylidine as studied by Bollag and Leonowicz (1984). They found that an excess of saccharose or glucose in the liquid medium repressed the biosynthesis of the inducible enzyme, but allowed the production of the constitutive laccase.

It is known that laccases produced by most fungi belong to the class of blue oxidases containing 4 copper atoms/molecule distributed in three different copper binding sites. However, Palmieri et al. (1997) has found a novel laccase which has 2 zinc, 1 iron and only 1 copper atom/molecule. This enzyme had similar substrate specificity as all laccases. In particular, it more efficiently oxidized o-dimethoxy-substituted phenols (such as sinapic acids) compared with o-monomethoxy-substitued phenols (such as ferulic acid).

It is evident that the enzymes produced by *P. ostreatus* have strong affinities towards phenolic compounds. This has been noticed by many researchers and *P. ostreatus* has been used by many applications with various purposes.

2.5 Applications and Researches of *P. ostreatus*:

P. ostreatus has been used in various fields for many purposes, such as in the degradation of organo-pollutants and agricultural waste, in biopulping and biobleaching processes.

Effective detoxification by this fungus has been found by Martirani et al. (1996), when they investigated reduction of phenol content and toxicity in olive oil mill waste water. It was concluded that treatment of the waste water with purified enzyme resulted in a significant reduction in phenolic content, but no decrease of toxicity was observed. On the other hand, the effect of processing the waste with the entire microorganisms resulted in a noticeable detoxification with reduction of the phenol content.

Decolorization of artificial dyes by this fungus has also been found effective. Shin et al. (1998) concluded that the decolorization was catalyzed by extracellular peroxidase produced by the fungus *P. ostreatus*. Like laccase, this enzyme also reacts with various phenolic compounds (Shin et al., 1998). Moreover, the enzyme was similar to Mn (II)-dependent peroxidase from *Phanerochaete chrysosporium* (a well studied lignin-degrading fungus) (Kang, 1993). It had high affinity towards phenolic compounds containing methoxyl and p-hydroxyl group, directly attached to the benzene ring (such as sinapic acid). However, it had no affinity for non-phenolic compounds.

P. ostreatus has also been used in pulp bleaching. It was reported that laccases are active in pulp bleaching in the absence of lignin-peroxidases (Addlemen and Archibald, 1993). Sermanni et al. (1994) also found that, some physical properties of the paper made from corn straw were improved, when corn straw was treated with the crude filtrate from submerged fermentation by *P. ostreatus*.

Biodegradation of the wheat straw by *P. ostreatus* and its use in cattle feeding have also been studied (Adamovic et al., 1998). It was found that a substantial amount of straw dry matter was degraded. The most noticeable effects were on cell-wall components, which allowed successful use of the straw as a substrate for mushroom growth. The spent *Pleurotus* compost was used as a part of the cattle diet, but, it was found that animals would not consume mixed ration with more than 17% compost.

Besides the enzymes, other compounds produced by *P. ostreatus* have also been studied. For example, volatile compounds secreted by *P. ostreatus* demonstrated strong antibacterial activity against some bacterial strains (Beltrangarcia et al., 1997).

The accumulation of heavy metal ions has been reported in several microorganisms (Sanglimsuwan et al, 1993). The uptake of copper, zinc and cadmium into mycelia of seven *Pleurotus* species were studied by Sanglimsuwan et al. (1993), it was found that *P. ostreatus* exhibited the highest resistance to all these heavy metals. In other words, the fungus collected the metals and its growth was not inhibited. It was also found that the increase in the uptake of the ions was proportional to the increase in the concentrations of these metals in the medium. The accumulation of potentially toxic elements such as cadmium and mercury in edible mushrooms must be given appropriate attention.

2.6 Submerged Fermentation Processes

In the study of enzyme production by microorganisms, submerged processes are often used. The growth medium is in liquid form, chemically defined or complex nutrients are added into the liquid. It usually consists of water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins and oxygen.

A submerged system offers an uniform growing environment for the fungus. The growing conditions have large effects on the growth of the fungus and the production of enzymes. For example, Schiesser et al. (1989) studied the effects of sunflower oil on the enzyme production during the growth of *P. ostreatus* on straw in submerged culture. It was found that the presence of straw and oil as carbon and energy sources stimulated the growth of mycelia and the production of phenol oxidase when compared to the use of a basic medium. Hence, nutrient source is important in such a fermentation process.

Besides nutrients, aeration is also important for the growth of biomass and production of enzymes. A study was done on the effect of air flow rate on phenol oxidase activities (Burla et al., 1992). Increasing the air supply from 0.5 to 2.0L/min enhanced the biomass yield. The phenol oxidase activities were also enhanced by higher air flow rates. They explained these effects by larger O_2 availability and more efficient gaseous exchange between mycelium and culture medium. It was concluded that the aeration rate is the determinant for phenol oxidase production.

Many other factors have influence on the behavior of such a submerged process and it is important to optimize the growth conditions for the purpose of enzyme production.

2.7 Solid State Fermentation Processes

Although *P. ostreatus* has been used in many industrial applications and researches that usually involve liquid cultures, solid state fermentation (SSF) has been found to be more effective in many cases.

2.7.1 Advantages of SSF

Al-Asheh (1993) studied some of the common SSF processes and summarized the characteristics of SSF process. It was stated that solid state fermentation involves cultivation of mycelia organisms on media comprising three phases. The first is the solid phase which is generally a vegetable substrate containing the microorganism's nutrients; the second is a liquid phase bound to the solid matrix where different mass transfer occurs; and the third is a gaseous phase.

Some of the advantages of SSF are:

1. The space required by the fermentation equipment is small relative to the yield of product because less water is used and substrate is concentrated (Al-Asheh, 1993);
2. Aeration is easily obtained since there are air spaces between each particle of the substrate (Sarikaya and Ladisch, 1997);
3. The medium is simple, usually the substrates contain all or most of the nutrients needed for growth and products formation, hence needs little or no additive (Al-Asheh, 1993);
4. The conditions under which fungus grows are more like the conditions of its natural habitat (Smits et al., 1998);

In particular, for the production of enzymes, researches have shown that:

5. SSF stimulates or induces the production of enzymes, which leads to a higher concentration of the product (Sermanni et al., 1994; Smits et al., 1998);
6. The enzymes produced are usually with high specificity toward the substrates (Smits et al., 1998);
7. Lower cost due to simpler plant, machinery and energy requirement.

2.7.2 Application of SSF

Most of the SSF processes that exist in industries are in the areas of food and enzyme productions. Food fermentations such as cheese making, vinegar and garlic acid production are examples of SSF that have existed for many years. New technologies and developments continue to use and improve solid state fermentation processes.

In the production of enzymes, solid state fermentation has the potential to produce inexpensive enzymes for use in high volume industrial applications. A good example is the production of pectinase from coffee bean fermentation. Pectinase is used in fruit juice extraction processes. When fruit tissue is crushed during processing, some of the pectin appears in either soluble or insoluble form in the juice and this can affect the quality or yield of the juice recovered. The addition of pectinase results in a rapid reduction of the viscosity of the juice and leads to particle coagulation and settling. Different microorganisms have been used for pectinase production in SSF processes, and a commonly used species is *Aspergillus niger*. However, it has been found that different strains showed different abilities in producing pectinase. Researches are being carried out in searching for better strains for higher production of enzymes (Antier et al., 1993).

Another example is the production of xylanase during solid state process on agricultural by-products. Xylanases cover a complex group of enzymes, which hydrolyze arabans, galactans, amnnans and xylans (Moo-Young, 1985). A study of the xylanase production using *Trichoderma longibrachiatus* on wheat bran was done by Ridder et al. (1998). The results showed that the level of xylanase produced from such a solid process depended on many process parameters. It was found that moisture content of substrate, depth of substrate and duration of fermentation all had significant effects on the production of enzymes.

Cellulase can also be produced using solid state fermentation on lignocellulosic material, such as wheat straw. Cellulases are currently used in cereal processing, brewing, alcohol production, fruit processing, wine manufacture and waste treatment. (Moo-Young, 1985) The main purpose of producing cellulases using lignocellulosic material is to reduce the cost of the enzyme production by using these cheap substrates, instead of costly materials such as pure cellulose or lactose. Studies show that the fermentation process is affected by the operating conditions. Muniswaran et al. (1994) examined the effects of nutrient level, substrate particle size and inoculum size for optimal production of cellulases by the fungal strain *A. niger*. It was found that at 20-50% inoculum and substrate particle size of 375μm, the enzyme production reached the highest level.

Bio-transformation of crop residues into animal feed by SSF has been studied intensively in recent years. Singh et al. (1996) found that SSF is inexpensive and capable of enriching the feed by building up biomass protein product and can degrade lignin so as to increase digestibility. Some special analysis has been done on cassava meal for protein enrichment with *A. niger*. A maximum of 9.5% protein increase was achieved under laboratory conditions (Lopez-Ulibarri, 1989).

Composting solid organic waste by SSF is another area that researchers have been paying more attention to lately, due to the rapid increase in solid waste production. Fujio (1997) studied the treatment of a dehydrated sewage-sludge cake using SSF. A large amount of heat generated by the composting was involved in the fermentation. Enzymes that can hydrolyze starchy materials were produced effectively on molding solid media. SSF is a great potential alternative for waste treatment.

In general, SSF can promote the production of enzymes. In a recent study of the ligninolytic enzyme production, Rodriguez et al. (1999) found that *P. ostreatus*, as one of the species studied, produced higher laccase activity when grew in solid state culture on whole oats compared with growth in a complex liquid medium.

2.6.3 Disadvantages of SSF

Some of the disadvantages of SSF are:

1. The types of organisms are limited to those which can grow at reduced moisture levels, namely fungi, some yeasts, and some bacteria including streptomycetes (Al-Asheh, 1993);
2. Growth is initially limited to the surface of the solid particles (Sarikaya and Ladisch, 1996); Large amount of inoculum is necessary to reduce lag phase of biomass growth;
3. Chances of contamination are high if water is to be added during the course of fermentation (Al-Asheh, 1993);
4. Using monitoring devices to determine moisture, pH, etc, becomes difficult (Al-Asheh, 1993); It is therefore more difficult to model a SSF process compared to a submerged fermentation process;
5. Power requirement may be high for continuous agitation of solid material;
6. Structurally and nutritionally heterogeneous substrates cause non-homogenous biomass growth (Sarikaya and Ladisch, 1996) which leads to non-uniform production of enzyme;
7. Extraction and separation of enzyme from solid cultures might be more difficult compared to liquid cultures;

8. Heat generated as a result of microbial activity is dissipated by evaporation of water and conduction through the solid medium. When the dissipation is insufficient, a temperature gradient is created (Smits el al., 1998).

In conclusion, study of the effects of operating parameters on SSF process is necessary for the success of enzyme production and phenolics degradation for the purposes of this project.

Chapter 3

Mathematical Modeling of Solid State Process and Enzyme Characterization

Understanding the kinetics of the microbial growth, enzyme production and substrate utilization is fundamental to a successful design, scale-up, operation and optimization of SSF bioreactors. Compared to modeling of submerged liquid fermentation systems, modeling of SSF is not well developed. Al-Asheh (1993) summarized some of the difficulties associated with modeling SSF processes:

1. The systems are geometrically complex due to non-homogenous spatial distribution of the components;
2. Growth is largely limited to the surface of the particle. This leads to formation of concentration gradients;
3. Solid substrates are usually relatively unprocessed agricultural by-products and may be structurally or nutritionally heterogeneous. In addition, significant variations can occur between batches;
4. Important parameters may be difficult or impossible to measure. A critical problem is the penetration of fungal hyphae into the solid substrates, which prevents direct recovery and determination of biomass;
5. Mixing of the system in order to obtain good representative samples for analysis highly damages the mycelia;

3.1 Modeling of Biomass Growth Kinetics

Different ways of measuring biomass have also been developed and the method that involves measurement of glucosamine has been used in this study for biomass analysis.

There are several phases that living cells will go through in a typical batch process. The lag phase, logarithmic phase, stationary and death phases are among them.

In the lag phase, no increase in cell number or growth in size is evident. The length of this phase usually depends on medium composition, physiological condition of the inoculum and its size.

After the lag phase, a period of rapid growth occurs, during which the cell numbers increase exponentially with time, usually referred to as exponential growth.

Naturally in a closed vessel, the cells cannot grow indefinitely, and a stationary phase follows the exponential growth. At this point, the population achieves its maximum size. Eventually, a decline in cell numbers occurs during the death phase. Many factors affect the growth kinetics, such as the inoculum. Bailey and Ollis (1986)[1] proposed that the inoculum should be as active as possible and going through the exponential growth phase.

As for the models describing biomass growth kinetics, Sangsurask et al. (1996) summarized four most commonly known correlations. Some of these attempt to describe biomass growth kinetics empirically, while others attempt to describe the kinetics simultaneously with other parameters in the system, the values of which affect growth. These model equations are described next.

3.1.1 Approximately Linear Growth Kinetics

Such kinetics take the form:

$$\frac{dX}{dt} = C \qquad (3.1-1)$$

where

X-biomass concentration;

t-time

C-constant.

3.1.2 Exponential Growth Kinetics

Another type of kinetic equation is:

$$\frac{dX}{dt} = \mu X \qquad (3.1-2)$$

where μ is the specific growth rate.

This model is most suitable for exponential growth phase. It has been noted (Bailey and Ollis, 1986[2]) that Malthus' Law gives one of the simpler form of this model, $F(X) = \mu X$. Malthus' prediction of death phase resulting from unbridled population growth has not (yet) been realized, and the transition to a stationary population is generally observed for microbial population. The use of exponential growth kinetics for modeling biomass is applicable in many SSF processes. For example, Sugama and Okazaki (Sangsurasak, 1980) have modeled the growth of Koji mold on solid substrate et al. based on the assumption of exponential kinetics.

3.1.3 Logistic Growth Kinetics

The logistic equation has been used often by researchers for modeling SSF. The equation is known to cover the exponential and stationary phases of biomass growth (Okazaki et al, 1980):

$$\frac{dX}{dt} = \mu_m X(1-\frac{X}{X_m}) \qquad (3.1-3)$$

where,

X_m-the maximum biomass concentration

μ_m-the maximum specific growth rate.

The logistic model is also incapable of describing the death phase. However, this equation has been used to model the underlying growth kinetics in many more complex mathematical models of SSF. Al-Asheh (1993) has successfully used this equation for the modeling of biomass concentration in SSF.

3.1.4 Rapid Acceleration Followed by a Slow Deceleration (RASD)

In SSF, it is not uncommon to have a period of rapid acceleration of growth early, followed by an extended period during which the growth rate decelerates slowly. An example given by Sangsurasak et al. (1996) described the modeling of glucoamylase activity and assumed the growth was directly proportional to the rate of hydrolysis of starch in the substrate. This hydrolysis depended on glucoamylase activity in the substrate which was modeled empirically. The growth equations used were:

1. During the early period of increase in glucosamylase activity: $(0 < t < t_l)$

$$X = X_o + \frac{Y.r.t^2}{2} \qquad (3.1-4)$$

where,

X-the biomass concentration

X_o-the initial biomass concentration

Y-the yield coefficient for biomass

r-the rate of increase in glucoamylase activity until the maximum activity is attained at time t_l.

2. During later period when glucoamylase activity decreases: $(t \geq t_l)$

$$X = (X_o + \frac{Y.r.t_l^2}{2}) + (\frac{Y.r.t}{K})(1 - \exp(-kt - t_l)) \qquad (3.1-5)$$

where k is the exponential rate constant for decay of activity after time t_1.

In this study, the growth of biomass is expected to be the bases for the production of extracellular enzymes. Although enzyme activity should also have effects on the biomass growth, similar rates of acceleration and deceleration of growth have been found in some cases in the experimental results. Therefore, logistic equations will be used for modeling the kinetics of solid state fermentation processes.

3.1.5 Using Logistic Equation

The logistic equation can be integrated providing the initial condition, when t = 0, X = X_o. X_o is the initial biomass concentration after inoculation. Then the integrated equation is:

$$X = \frac{X_m}{1 + \beta \exp(-\mu_m t)} \qquad (3.1-6)$$

$$\beta = \frac{X_m}{X_o} - 1 \qquad (3.1-7)$$

Experimental data can be fitted to the logistic equation with the fixed initial condition using the SCIENTIST software, so that each set of data will give different values of X_m and μ_m. The resulted equation with these calculated parameters can be used as a model equation to predict the biomass concentration in this system at any time of the fermentation.

3.2 Modeling of Enzyme Production

Based on the logistic equation for biomass production, and with the assumption that the enzyme production is proportional to biomass concentration, the following equation can be written:

(3.2 – 1)

$$\frac{dv}{dt} = K_v X$$

where,

ν - the enzyme activity (nKat/g dry SSC)

K_v - a rate constant (nKat/g /day)

Substituting the logistic equation for X into equation 3.2-1 would give:

$$\frac{dv}{dt} = \frac{K_v X_m}{1+\beta\exp(-\mu_m t)} \qquad (3.2-2)$$

with the initial condition, when t = 0, $\nu = \nu_o$ fixed (ν_o is the initial enzyme activity right after the inoculation), the equation can be integrated to:

$$v = v_o + (\frac{K_v X_m}{\mu_m}) \ln(\frac{\beta+\exp(\mu_m t)}{\beta+1}) \qquad (3.2-3)$$

Experimental data can be fitted to find the constant K_v, knowing the values of X_m and μ_m from biomass modeling.

3.3 Modeling of Sinapic Acid Esters (SAE) Degradation

The SAE content in CM was measured during the SSF processes in this study. It is reasonable to expect that the decrease of SAE content should be related to enzyme concentration and to the amount of SAE in the solid culture. Moreover, the enzyme concentration is assumed to be proportional to biomass concentration. Therefore, the following equation is proposed:

$$\frac{dSAE}{dt} = -K_s SAE \cdot X \qquad (3.3-1)$$

where,

K_s - the rate constant (g dry SSC/g /day)

SAE - remaining sinapic acid esters concentration in culture (g/g dry SSC)

Substitute the logistic equation of X into equation 3.3-1:

$$\frac{dSAE}{dt} = -K_s SAE \frac{X_m}{1+\beta \exp(-\mu_m t)} \qquad (3.3-2)$$

Rearranging, and integrating the equation above:

$$\int \frac{dSAE}{SAE_0} = \int \frac{-K_s X_m}{1+\beta \exp(-\mu_m t)} dt \qquad (3.3-3)$$

with the initial condition, when t = 0, SAE =SAE_o, the final model equation is:

$$\ln \frac{SAE}{SAE_o} = \frac{K_s X_m}{\mu_m} \ln\left(\frac{\exp(-\mu_m t)(1+\beta)}{1+\beta \exp(-\mu_m t)}\right) \qquad (3.3-4)$$

K_s is the new parameter to be found by fitting the experimental data. μ_m and X_m are parameters from the logistic equation for biomass modeling. The result is presented as fraction of SAE remaining after time t.

3.4 Enzyme characterization

The enzyme produced by the fungus *P. ostreatus* was partially characterized using three substrates: sinapic acid, sinapine and sinapaldahyde. Reaction kinetic parameters for this enzyme using the three substrates were calculated and compared.

3.4.1 Effect of Substrate Concentration on Enzyme Activity

The Michaelis-Menten (Bailey and Ollis, 1986[3]) equation was used to study the effect of substrate concentration on enzyme activity:

$$v = \frac{v_{max} S}{K_m + S} \qquad (3.4-1)$$

where,

v is enzyme activity (nKat/mL)

v_{max} is the maximum enzyme activity (nKat/mL)

K_m is the Michaelis-Menten constant (nmol/mL), and

s is the substrate concentration (nmol/mL).

A smaller K_m value would result in a larger activity when the other terms are fixed. The activity is proportional to the other parameter, v_{max}.

This equation was proposed based on a few features of the reaction:

1. The rate of reaction is first order in concentration of substrate at relatively low values of concentration.
2. As the substrate concentration is continually increased, the reaction order in substrate diminishes continuously from one to zero.
3. The rate of reaction is proportional to the total amount of enzyme present.

If these features were assumed to be valid or at least at low concentrations of substrate, by rearranging equation 3.4-1, the following equation (known as Lineweaver-Burk plot) is obtained and can be used to plot data and evaluating the parameters:

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_m}{v_{max}} \frac{1}{S} \qquad (3.4-2)$$

A plot of $1/v$ vs. $1/s$ will give the intercept at $1/v_{max}$, then v_{max} can be calculated accurately. However, K_m was to be calculated using a v vs. s plot (Bailey and Ollis, 1986[4]). Equation 3.4-2 is more valid when $1/s$ is small. When it increases, error also increases and the slope calculated in this range would be less accurate. The proposed way to determine K_m is to find the substrate concentration for ½ v_{mas} which corresponds to K_m. This method was used to calculate K_m and v_{max} values in this study.

While the Michaelis-Menten equation successfully describes the kinetics of many enzyme-catalyzed reactions, it is not universally valid. In this study, it is clear to see that not all the features were met. To model the substrate concentration effect on the enzyme activity, a semi-empirical equation was developed:

$$v = \frac{v_{max}}{1 + \frac{K'}{s} + \frac{s}{K''}} \qquad (3.4-3)$$

where K' and K'' are the empirical constants.

3.4.2 Effect of pH on Enzyme Activity

Proteins in enzymes contain basic, neutral, or acidic groups. Such ionizable groups are often part of the active sites of enzymes for acid- or base- type of catalytic actions. For an appropriate catalysis to be possible, the ionizable groups can only exist in one particular ionization state. Thus, the catalytically active enzyme may be a large or small fraction of the total enzyme present, depending on the pH (Bailey and Ollis, 1986[5]). Effect of pH on enzyme activity can be modeled based on the following model of active site ionization state:

$$E \rightleftharpoons E^- \rightleftharpoons E^{2-}$$

E^- denotes the active enzyme form while E and E^{2-} are inactive forms obtained by protonation and deprotonation of the active site of E^-, respectively. Therefore, if e_0 represents the total enzyme concentration,

$$e_0 = e + e^- + e^{2-} \qquad (3.4\text{-}4)$$

the active fraction y^- is e^-/e_0 and is given by:

$$y^- = \frac{1}{1 + h^+ / K_1 + K_2 / h^+} \qquad (3.4-5)$$

and

$$K_1 = \frac{h^+ e^-}{e}; \qquad (3.4-6)$$

$$K_2 = \frac{h^+ e^{2-}}{e^-} \qquad (3.4-7)$$

where $h^+ = 10^{-pH}$.

Equation 3.4-5 is one of the Michaelis' pH functions. Bailey and Ollis (1986[6]) stated an equation relating the maximum reaction velocity v_{max} to pH values. Further work was done by Al-Asheh (1993) to represent enzyme activity as a function of pH values:

$$v = v_{opt} \frac{1 + 2\sqrt{K_2 / K_1}}{1 + h^+ / K_1 + K_2 / h^+} \qquad (3.4-8)$$

where v_{opt} is the activity at optimum pH. K_1 and K_2 can be determined by fitting the experimental data, v vs. pH values.

Chapter 4

MATERIALS AND METHODS

This chapter describes the experimental procedures used in this study and the materials used in all procedures.

4.1 Microorganism and Its Maintenance

The white-rot fungus *Pleurotus ostreatus* (DAOM 197961) was used in the study and was obtained from Agriculture Canada, Ottawa, Ontario.

It has been maintained on agar slants medium at 277K (4°C), and transferred every 2-3 months to fresh slants. Agar slant contained (%, w/v): malt agar (4.5), glucose (0.5), yeast extract (0.5) and distilled-deionized water (94.5). To prepare this medium, the mixture was first stirred and heated until agar was completely dissolved. Then, five mL of the medium was transferred into a 10mL test tube and covered with a metal or plastic cap. The medium was sterilized at 388K (115°C) for 20 minutes in an autoclave and then cooled on angle. When the medium solidified, it was inoculated and incubated at 303K (30°C).

White mycelia started growing after day two and covered the surface of the solid medium completely after day five.

4.2 Growth and Preparation of Inoculum

Liquid medium was used for the growth of the fungus for the inoculum preparation. This medium composed of (%, w/v): glucose (2.0), yeast extract (0.5), pepton (0.8) and distilled water (96.7). 0.5 mL/L of Vogel's minerals was also added. This mineral solution contained 5.0 g citric acid-H_2O, 5.0 g $ZnSO_4$-$7H_2O$, 1.0 g $Fe(NH_4)_2(SO_4)_2$-

$6H_2O$, 0.25 g $CuSO_4$-$5H_2O$, 0.05 g $MnSO_4$-H_2O, 0.05 g H_3BO_3 and 0.05 g $NaMoO_4$-$2H_2O$. It was prepared by first adding these components to 95 mL of distilled water and stirred well. Then the solution was adjusted to 100 mL. It has been stored at 277K (4°C).

The liquid medium was prepared by first mixing the components in distilled water until dissolved and adjusted it to pH value 5.5 using 1 N sulphuric acid. 100 mL of the medium was transferred to an Erlenmeyer flask (500mL) and covered by a foam plug. It was then sterilized for 20 minutes at 388K (115°C) in an autoclave and then cooled to room temperature. The medium was inoculated with a piece of mycelium from an agar slant. The flask was finally placed on a shaker operating at 150 rpm and 303K (30°C) for 7 to 8 days when small round pellets of fungus appeared.

The fresh inoculum was prepared using the 7 to 8 day old fungus in the liquid medium. The content of the inoculum flask was washed with distilled water by adding 50 mL of water and decanting similar amount of liquid for a couple of times. The washed inoculum was transferred into a blender (Sunbean-Osterizer 8) and homogenized at "liquefy" for 30 seconds. This was used as the inoculum for fermentation.

4.3 Growth Media and Fermentation Processes

Media were prepared for submerged and solid state fermentations.

4.3.1 Submerged Fermentation

The liquid medium used in submerged fermentation composed of (%, w/v): yeast extract (0.5), pepton (0.8), 0.5 mL/L of Vogel's minerals, and various amount of additives such as glucose, inducers and CM as their effects were studied during the fermentation processes. Distilled water was added to make up the rest of the medium.

Study for the effects of additives was done one at a time. Most fermentation processes were carried out in 250mL Erlenmeyer flasks. Different sizes of flasks were used when oxygen concentration effect was studied. Around 75 mL of liquid medium was transferred into each flask. The flask was sterilized at 388K (115°C) for 20 minutes, cooled to room temperature, then inoculated. All flasks were placed in a shaker operating at 150 rpm and 303K (30°C).

The parameters studied in this project for submerged fermentation process included:

1) Initial glucose concentration: 1%, 2%, 4% and 6%;
2) Inoculum concentration: 1.5mg/mL, 3mg/mL and 6mg/mL (dry biomass/medium);
3) Oxygen Concentration: different volumes of the medium were added into 500mL Erlenmeyer flasks with the same size and shape, so that oxygen dissolved in liquid media would be different due to different air space above liquid. The volumes used were: 80 mL, 160 mL and 240 mL;
4) Inducers: 2,5-xylidine (0.25mM), caffeic acid (0.2mM), sinapic acid (0.2mM), sinapine (0.2mM) and syringaldazine (0.11×10^{-3}mM);
5) CM concentration: 0.5%, 1%, 2%, 3% and 4%.

4.3.2 Solid State Fermentation

The medium for solid state fermentation was prepared by mixing 10 g of CM with water (<75%) in a 250mL flask and covered with foam plug. The flask was sterilized for 45 minutes at 388K (115°C) and cooled to room temperature. Fresh inoculum was added and well mixed. Solid culture was placed in a stationary incubator at 302K (29°C) during the fermentation.

Process parameters, such as the composition of the solid culture and process conditions, were varied to study their effects on solid state fermentation. Parameters studied included:

1. Moisture content: 50%, 60%, 70% and 75%;
2. Inoculum concentration: 1.1mg/g, 2.2mg/g, 3.3mg/g, 4.4mg/g and 5.5mg/g (dry biomass/CM);
3. Homogenization time of inoculum: 15, 30, 60, 120 and 200 seconds;
4. Particle size of canola meal: S1<0.18mm, 0.18mm<S2<0.425mm, 0.41mm<S3<0.81mm, 0.81mm<S4<1.0mm, 1.0mm<S5<1.4mm and S6>1.4mm.

Fermentation process was carried out so that one parameter was changed at a time.

4.4 Enzyme Preparation

In order to characterize the enzymes produced by the fungus *P. ostreatus*, submerged fermentation was used. The enzyme activity in a submerged culture was followed and when the enzyme concentration reached its maximum, several flasks were combined and the culture was centrifuged (Beckman L7 ultracentrifuge) at 5000 rpm for 15 minutes. The supernatant was then filtered through a filter paper (0.45μm) and frozen in the freezer until use. This filtered supernatant was called the enzyme preparation. The activity was tested at the time of preparation and later on to ensure no significant loss in enzyme activity.

4.5 Analysis of Samples

Samples taken from the submerged cultures were analyzed for glucose and soluble carbohydrates contents in the medium, as well as biomass and enzyme

production. For solid state fermentation processes, enzyme production, biomass concentration and sinapic acid esters (SAE) content in the solid culture were measured.

4.5.1 Submerged Fermentation

1. Biomass

A simple procedure was used for biomass measurement. The content of the flask was filtered through filter paper (Whatman, No. 1) and the filtrate was collected in a flask. The biomass left on the filter paper was washed with distilled water for a few times. The filter paper and biomass were dried in an oven at 363K (90°C) overnight. The dry weight of biomass was then calculated knowing the weight of the filter paper prior to use.

2. Glucose (reducing sugars)

The filtrate was centrifuged at 5000 rpm for 15 minutes and the supernatant was used for analysis of glucose content by the dinitrosalicylic acid (DNS) method (Weiner, 1978; Chaplin and Kennedy, 1994). The solution used for DNS method was prepared by first making solutions A and B as follows:

A. 5 g of dinitrosalicylic acid was added in to 300 mL distilled water, stirred continuously until dissolved;

B. 1 g phenol, 0.25 g sodium bisulfite, 5 g sodium hydroxide and 100 g sodium potassium tartarate were dissolved in 200 mL distilled water.

Solution B was added to A while stirring. The final solution (DNS) was kept in the dark. The first step of the analysis method was to mix one mL of supernatant with three mL of DNS solution. The mixture was then boiled for five minutes and cooled for one minute in an ice bath. 20 mL of distilled water was added and mixed. The absorbance was measured at 600 nm against a reference sample. The reference sample was prepared

using one mL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curves (Appendix A) were prepared in the range of 0 to 2.5 g of glucose per liter of liquid, and the samples were diluted when necessary to fit in the range of the calibration curve.

3. Soluble Carbohydrates (non-reducing sugars)

The supernatant was hydrolyzed first and then analyzed using the DNS method. First, 0.5 mL of supernatant was mixed with one mL of 1N hydrochloric acid (HCl). The mixture was then boiled for 15 minutes and cooled in an ice bath. One mL of sodium hydroxide (NaOH) was added to neutralize the mixture. The final solution was used in the DNS method, and the measurements were taken the same way as for glucose determination.

4. pH measurement

The pH of the supernatant was measured using an ORION Research digital ionalyzer (Model-1501).

4. Enzyme activity

Laccase activity was measured using a method described by Leonowicz and Grzywnowicz (1981). The reaction mixture contained 2.7 mL citric-phosphate buffer at pH 5 (0.93 g of citric acid and 1.46 g of sodium phosphate-dibasic dissolved in 200 mL of distilled water), 0.2 mL of 0.5 mM syringaldazine in ethanol and 0.1 mL sample or its dilution. The dilution was made such that the increase in absorbance at 525nm was linear for the first five minutes of the test.

A total volume of three mL was well mixed in a cuvette and the reaction was followed by measuring the absorption at 525 nm using a spectrophotometer (Beckman-

Du640). The reaction mixture showed pink color and increased in intensity at the initial stage of the reaction, known as the oxidation of the substrate to its quinone. The enzyme activity was calculated using the following equation (Lacki, 1997):

$$v = \frac{(A_{ini} - A_{fin})}{\Delta t}\left(\frac{1}{\varepsilon}\right)\left(\frac{V_{sys}}{V_e}\right)Dil \times 10^6 \qquad (4.5-1)$$

where v is the enzyme activity (nKat/mL), A_{ini} and A_{fin} are the initial and final absorbencies, respectively, Δt is the reaction time (s), V_{sys} is the volume of the system (mL), V_e is the volume of the enzyme added (mL), Dil is the dilution factor, ε is the extinction coefficient, in this case, 65,000 mol^{-1} cm^{-1} for syringaldazine. The term (A_{ini}-A_{fin}) /Δt was evaluated from the linear part of the progress curve displayed on the spectrophotometer.

4.5.2 Solid State Fermentation

1. Biomass in Solid Culture

Determining biomass in a solid state process is much more difficult than in a submerged process. A method was adopted from Sakurai et al. (1976), which allowed biomass determination indirectly through measuring glucosamine content in the solid samples. Al-Asheh (1993) found this method suitable for biomass analysis in CM. The procedure was as follows:

a. 2 mL of 85% sulphuric acid was added to 2 g of wet solid sample in a 25 mL test tube. This was left overnight at room temperature, or even longer to hydrolyze completely;

b. Distilled water was added to the test tube and the content was transferred into a 100mL flask. Concentration of the sulphuric acid in this 100mL flask should be around 1 N. Based on the calculation, this required an addition of about 60 mL of distilled water;

c. The flask was covered with tin paper and autoclaved at 388K (115°C) for one hour;

d. The hydrolyzed and autoclaved mixture was then neutralized with 4 N sodium hydroxide to pH 7.0 and was diluted to 100 mL;

e. Two mL of acetylacetone solution was mixed with two mL of the hydrolyzed and diluted sample in a 25 mL test tube; acetylacetone solution was prepared by adding 1.5 mL acetylacetone into 50 mL of 1.25 N sodium carbonate;

f. This mixture was boiled in a water bath for one hour with the test tubes closed, and then cooled to room temperature;

g. 20 mL of 95% ethanol was added into the tube and well mixed;

h. Two mL of Ehrlich's reagent was added. The Ehrlich's reagent was made by adding 1.6 g of p-dimethylamino benzaldehyde and 30 mL of concentrated hydrochloric acid into 30 mL of 95% ethanol.

i. The resulting system was allowed to stand for one hour;

j. Absorbance was read at 530 nm.

Similarly treated distilled water was used as a reference. Calibration curves were made using pure biomass (fungus *P. ostreatus*) and biomass with CM. The influence of CM in the absorbance was taken into consideration and the calculation for each sample was adjusted for more accurate results (Appendix A).

2. *Sinapic Acid Esters (SAE) content:*

Soluble SAE content was determined by using a modified Muller's method (Lacki, 1997). Free phenolic acids and soluble SAE were first extracted from CM using 2% HCl in methanol (MeOH) mixture.

a. Samples of CM were transferred into 100 mL boiling flasks (2 to 5 grams in each) and MeOH/HCl mixture was added with solvents/sample ratio of 50 for the first extraction.
b. These flasks were connected with condensing tubes with flowing cold water and placed in a water bath at 353K (80°C).
c. The extraction continued for 30 minutes and repeated two more times with MeOH/HCl mixture to meal ratio of 50 and 25.
d. After each extraction, the liquid was taken out and combined at the end.

The liquid extract or supernatant was analyzed for SAE content as follows:

e. Two mL of MeOH/HCl mixture was transferred into a glass cuvette and used as a blank at 400 nm for the spectrophotometer;
f. 50 to 100 μL of combined extract from the solid sample was added and the absorbance was recorded (A_{ini});
g. One mL of 1 N sodium hydroxide was then added and the final absorbance (A_{fin}) was recorded within five seconds after addition of base;

The concentration of SAE was evaluated based on the difference in absorbencies, $\Delta A = A_{fin} - 2/3\ (A_{ini})$. A standard curve prepared using sinapine bisulphate was used which was described by Lacki (1997) in sinapine determination. SAE calculated using

this procedure are expressed in terms of sinapine equivalent. The following formula was used to calculate SAE content in solid samples in terms of g/kg:

$$SAE = \left(\frac{\Delta A}{\varepsilon'}\right)\left(\frac{2.0}{V_{ext}} + 1\right)\left(\frac{V_{MeoH}}{M_{CM}}\right)(1 + X) \qquad (45\text{–}2)$$

where ΔA is the difference in absorbance, V_{MeoH} is the volume of MeOH/HCl mixture used for extraction (mL), M_{CM} is the mass of solid sample used for extraction (mg), V_{ext} is the volume of the extract added to the cuvette (mL), ε' is the slope of the standard curve for sinapine bisulphate determination (Lacki, 1997) and is equal to 0.03481 (mL/μg) and X is the moisture content of the meal (w/w) (Lacki, 1997)

3. *Moisture Content*

   The moisture content was determined by drying the solid sample in an oven at 378K (105°C) overnight.

4. *Glucose, Carbohydrate, pH values and enzyme activity*

Two to three grams of sample were taken from each flask and mixed with 20-30 mL of distilled water. The mixture was placed in a shaker to mix for one hour. It was then centrifuged at 5000 rpm for 15 minutes. The supernatant was used for soluble sugar (glucose and carbohydrates), pH and enzyme activity analyses. Procedures used for these analyses were the same as for samples from the submerged processes. For expressing enzyme activity, ν calculated by equation 4.5-1 was converted to activity per gram of dry culture. This was done as: ν(nKat/mL)*(amount of water/weight of dry culture). The weight of dry culture was determined by measuring the sample moisture content.

4.6 Chemicals

This section summarizes the chemicals used in this study.

Table 4.1 List of Chemicals used in this study

Acetylacetone (Sigma): $C_5H_8O_2$, FW=100.1
Boric acid (Sigma): H_3BO_3, FW=61.83
Caffeic acid (Sigma): anhydrous, FW=180.0
Citric acid (Sigma): monohydrate, $C_6H_8O_7$, FW=192.1
Cupric sulfate (BDH): pentahydrate, $CuSO_4$-$5H_2O$, FW=249.5
Dinitrosalicylic acid (Sigma): $C_7H_4N_2O_7$, FW=228.1
p-Dimethylamino benzaldehyde (Sigma): $C_9H_{11}NO$, FW=149.2
Ferrous ammonium sulfate (Sigma): hexahydrate, $Fe(NH_4)_2(SO_4)_2$-$6H_2O$, FW=390
Ethanol (UO): 95%, C_2H_5OH
D-Glucose (BDH): anhydrous, $C_6H_{12}O_6$, FW=180.16
Hydrochloric acid (BDH): HCl, FW=36.46
Malt agar (DIFCO): dehydrated
Manganous sulfate (BDH): monohydrate, $MnSO_4$-H_2O, FW=169.0
Methanol (EM SCIENCE): Omnisolv distilled, 99.90%, CH_3OH
Peptone (DIFCO): Bacto Peptone
Phenol (BDH): C_6H_5OH, FW=94.0
Sinapic acid (Aldrich)
Sinapine (Woods)
Sinapaldehyde (Aldrich)

Sodium bisulfite (Fisher): a mixture of $NaHSO_4$ and $Na_2S_2O_5$

Sodium carbonate (Sigma): anhydrous, Na_2CO_3, FW=106.0

Sodium hydroxide (BDH): NaOH

Sodium molybdate (BDH): dihydrate, Na_2MoO_4-H_2O, FW=241.95

Sodium phosphate-dibasic (Sigma): anhydrous, Na_2HPO_4, FW=142.0

Sodium potassium tartrate (Sigma): tetrahydrate, $C_4H_4KNaO_6$-$4H_2O$

Sulphuric acid (BDH): H_2SO_4, FW=98.08

Syringaldazine (Aldrich): $C_{18}H_{20}N_2O_6$, FW=360.4

2,5-Xylidine (Aldrich): FW=480.4

Yeast Extract (DIFCO)

Zinc sulfate (Sigma): heptahydrate, $ZnSO_4$-$7H_2O$

Aldrich – Aldrich Chemical Co., Milwanke, WI., USA
BDH – BDH Inc., Toronto, ON., Canada
DIFCO – DIFCO Laboratories, Detroit, MI., USA
EM SCIENCE – EM SCIENCE, Gibbstown, NJ., USA
Fisher – Fisher Scientific, Nepean, ON., Canada
Sigma – SIGMA Chemical Company, St. Louis, MO., USA
UO - University of Ottawa Chemistry Store, ON., Canada
Woods – prepared by Mr. Woods during his research at the University of Ottawa

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Submerged Fermentation Process

Although many factors have to be considered to design a suitable medium for a specific fermentation process, certain basic requirements must be met. These requirements include the elemental constituents for cell biomass and metabolite production as well as an adequate supply of energy for biosynthesis and cell maintenance. In this study, *Pleurotus ostreatus* was grown in a chemically complex medium, which was described by Lacki (1997). The medium was classified as a complex medium because not only pure chemicals were used, such as glucose, and Vogel's minerals solution, but other nutrients like pepton and yeast extract with uncertain compositions have also been added. This medium was determined to be optimum for polyphenol oxidase production using the fungus *T. versicolor* (Lacki, 1997). Although a different species is involved in this study, this was used as the start point of optimization for the submerged fermentation process. Improvement was made through this study for higher production of phenolics-degrading enzymes. The effects of initial glucose concentration, oxygen concentration, addition of inducers and CM have been examined.

5.1.1 Typical Submerged Fermentation with *P. ostreatus*

A typical submerged process for laccase production is shown in Figure 5.1. The process started with slow growth of biomass. But this lag phase was not long, approximately 2-3 days. Then rapid growth was during day three to day seven. This was the exponential phase of biomass growth. The maximum biomass production was

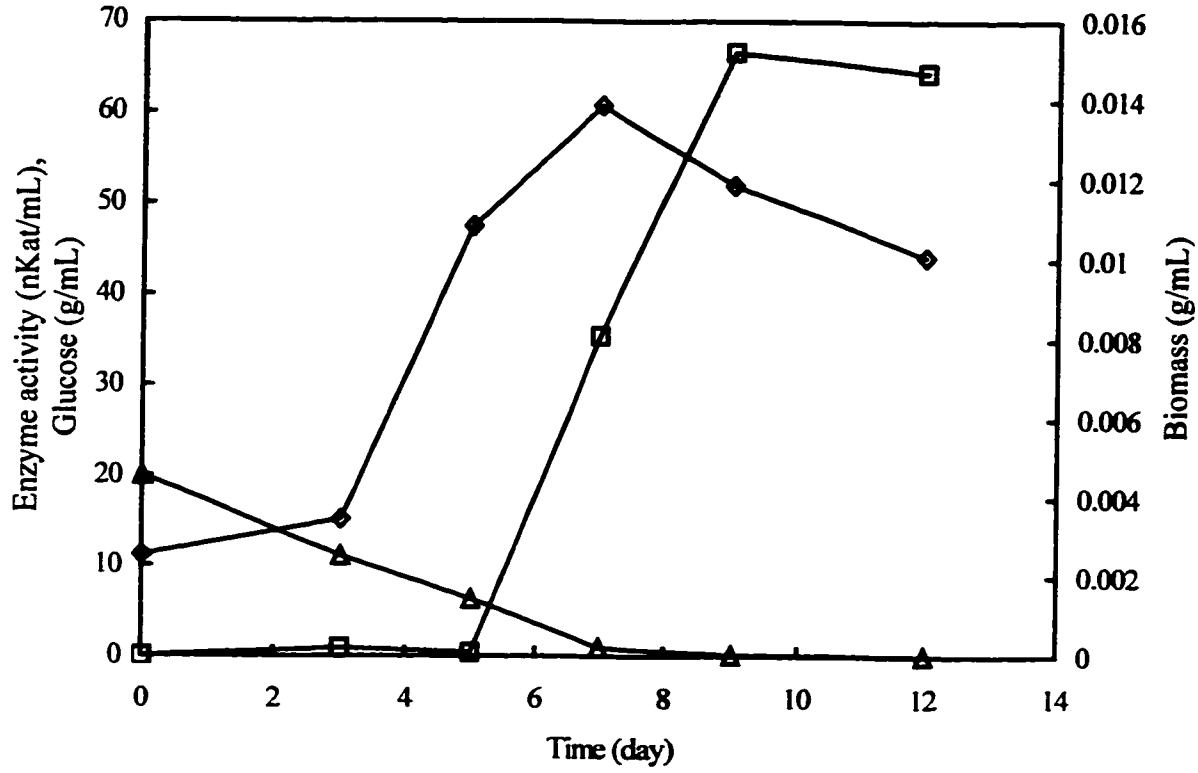


Figure 5.1 A typical submerged process

reached on day seven, which was about 0.014 g/mL. The lag phase of the growth of *P. ostreatus* was longer compared to the growth of *T. versicolor* (Lacki, 1997). In the same medium used, *T. versicolor* started growing rapidly within two days. However, the maximum biomass production obtained with *P. ostreatus* was much higher (0.014 g/mL), than that produced by *T. versicolor* (0.006 g/mL).

The glucose concentration decreased quickly and almost linearly, even at the beginning of the process when the growth of biomass was low. Glucose, as a carbon and energy source, is not only used for the growth of microorganisms, but also utilized to maintain the lives of the cells. Once the cells adapted to the environment, more glucose is used in the biosynthesis, which is the basis for the growth of biomass.

The production of laccases appeared around day five, which was during the exponential phase of biomass growth. Similar results were found with polyphenol oxidase production by *T. versicolor* (Woods, 1999).

The maximum enzyme activity was reached on day nine. Then, a slight decrease occurred thereafter. The decrease in enzyme activity after reaching a maximum was also found by Burla et al. (1992) when they tested *P. ostreatus* (strain Florida) for the production of phenol oxidase. An even faster decrease was found in their study. This decrease could be due to utilization of the enzymes by the fungus, or denaturation caused by other enzymes secreted at the end of the process.

5.1.2 Effect of Initial Glucose Concentration

A small but significant amount of a cell in microorganisms is composed of polysaccharides (Madigan et al., 1997). Polysaccharides are key constituents of the cell walls of many organisms. The sugars in polysaccharides are primarily six-carbon sugars called hexoses. The most common hexose in polysaccharides is glucose. Six–carbon

sugars needed for biosynthesis either can be obtained from the environment or can be synthesized within the cell from nonsugar materials. However, when a microorganism has a hexose or glucose as an energy and carbon source, this hexose becomes the precursor. The growth medium used in this study for submerged process contains glucose. Knowing the importance of glucose in the biosynthesis of microorganisms, the concentration of such sugar is expected to have large effects on biomass and enzyme production.

Tests with four initial concentrations were carried out. Figure 5.2 shows that biomass had slow increase for the first 3 days (the lag phase) and then started growing rapidly (exponential phase). The stationary phase was not long for any of the processes, especially those ones with low initial glucose concentration. There was a maximum point reached for each process on day 5, 7, 7 and 9 for 1%, 2%, 4% and 6% initial glucose concentration, respectively. As the initial glucose concentration increased, biomass production also increased. The maximum biomass production was approximately 0.018 g biomass/mL produced in the flask with the highest initial glucose concentration, 6% (w/v). This was about 1.6 times as the biomass in the culture with 1% (w/v) initial glucose concentration. These results indicated that glucose was critical for biomass production.

The kinetics of decrease of glucose concentration was also followed in these tests. Comparing these results (Figure 5.3) with those of biomass (Figure 5.2), it is evident that there was a strong relationship between the growth of the fungus and the concentration of glucose. Lacki (1997) also found that initial glucose concentration had positive effect on biomass production.

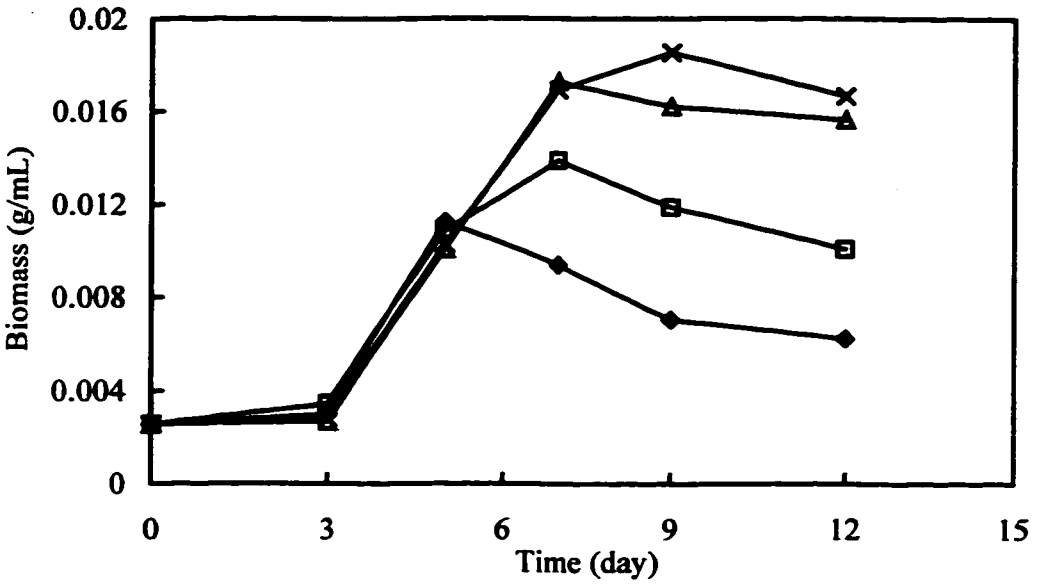
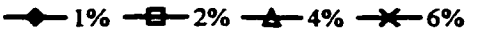


Figure 5.2 Effect of initial glucose concentration on biomass production during submerged process



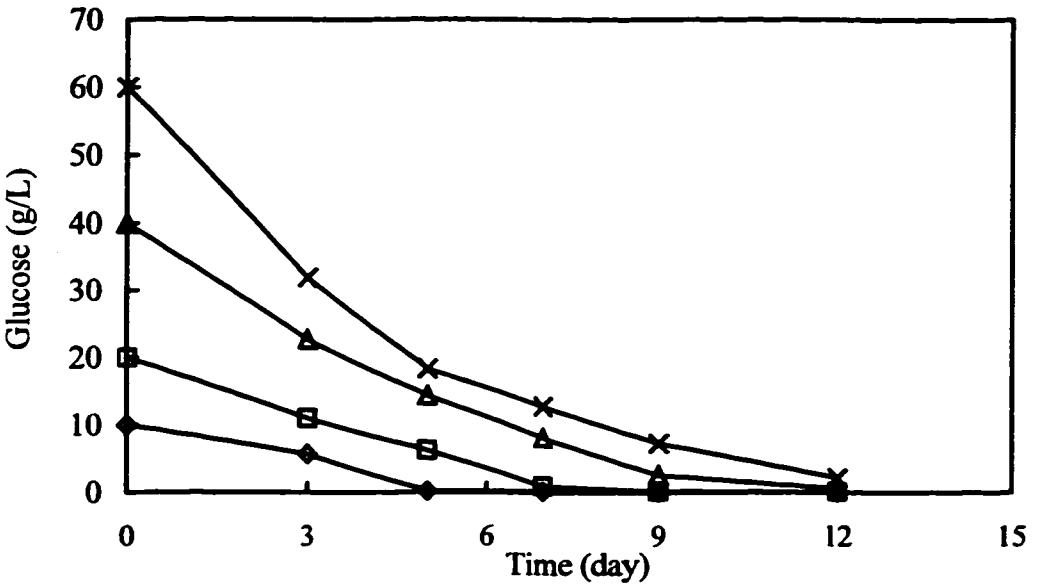
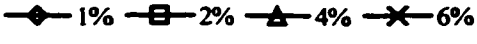


Figure 5.3 Effect of initial glucose concentration on glucose consumption during submerged process



The beginning of the enzyme production appeared in the later part of the exponential growth phase of biomass. The highest enzyme levels were reached on day nine for all processes regardless of the difference in glucose concentration (Figure 5.4). This was in the stationary phase of biomass growth for two processes with higher glucose concentrations and in the death phase for the other two. From this figure, it is clear that enzyme production did not follow the trend of the biomass production. The highest enzyme activity obtained was in the culture with initial glucose concentration of 4%. A higher level of initial glucose at 6% inhibited the enzyme formation, and therefore enzyme level was lower. Table 5.1 summarizes the maximum enzyme activities and maximum biomass concentrations. The specific activity was calculated for the results obtained on cultivation day nine, and it was found that 4% of initial glucose was the optimum concentration for enzyme production in this submerged process.

Table 5.1 Productivity of enzyme*, maximum biomass and maximum enzyme concentrations

Initial glucose concentrations (%)	Maximum enzyme concentration (nKat/mL)	Maximum biomass (g/mL)	Productivity of enzyme (nKat/g day)
1	46.5	0.011	459.9
2	66.5	0.014	532.4
4	88.6	0.017	569.8
6	57.5	0.019	344.3

* the productivity was calculated for cultures on day nine of the process.

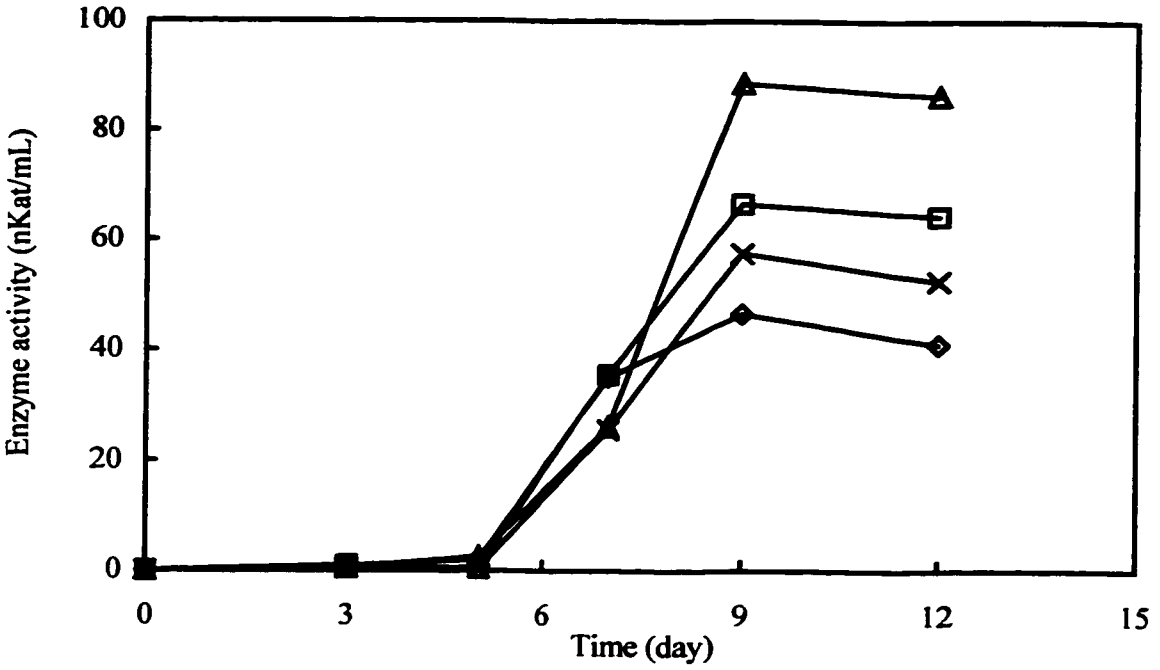


Figure 5.4 Effect of initial glucose concentration on enzyme production during submerged process

5.1.3 Effect of Inoculum Concentration

Inoculum is important in the success of a fermentation process. It should be in good physiological condition to allow biomass to grow quickly at the beginning of the process so that the lag phase is minimized. The amount of inoculum is also important. The quantity of inoculum normally used is between 3 and 10% of the medium volume (Stanbury et al., 1995). A relatively large inoculum volume is used to minimize the length of the lag phase and to generate the maximum biomass in the production fermenter in as short a time as possible, thus increasing vessel productivity. However, too much inoculum usually requires larger vessel for inoculum preparation or needs a number of stages to build up the biomass. This procedure could increase the risk of contamination and would become expensive when used in large scale. To study the effect of inoculum on enzyme production in this submerged process, tests were carried out with the inoculum concentration of 1.5 mg, 3.0 mg and 6.0 mg/mL (dry biomass/medium volume).

Figures 5.5 and 5.6 show the kinetics of the enzyme production and glucose consumption, respectively. It is clear that with the largest concentration of inoculum tested (6.0 mg/mL), the enzyme production was higher at the beginning of the fermentation, but its maximum enzyme activity was slightly lower than that in the culture with a lower inoculum concentration at 3.0mg/mL. This could indicate that a larger inoculum concentration could have a negative effect on enzyme formation. This was also found by Lacki (1997) using *T. versicolor* for the production of polyphenol oxidase. He concluded that the amount of enzyme produced was reversibly proportional to the initial biomass concentration. This conclusion was not obvious in this study, since an increase in inoculum concentration from 1.5mg/mL to 3.0mg/mL resulted in a large increase in

enzyme production. The maximum enzyme activity in the culture with 3.0mg/mL inoculum was about 1.6 times as high as that in the medium inoculated with 1.5mg/mL inoculum (Figure 5.5). The rates of decrease in glucose concentration (Figure 5.6) in the liquid cultures were in agreement with the enzyme production, with the slowest rate found in culture inoculated with the lowest inoculum, 1.5 mg/mL.

There is evidence that morphology could influence the productivity of a culture. When fungi are grown in submerged cultures, the types of growth varies from the pellet form, consisting of compact discrete masses of hyphae, to the filamentous form in which the hyphae form a homogeneous suspension dispersed through the medium. In industrial processes, fermentations are carried out with organisms growing in either form depending on their production. Some are optimum with filamentous growth, such as penicillin production by *P. chrysogenum*, and others are best in pellet form, such as citric acid production using *Aspergillus niger* (Bailey and Ollis, 1986[7]). Usually, a high spore inoculum will tend to produce a dispersed form of growth whilst a low one will favor pellet formation (Carlile and Watkinson[1], 1994). Thus, in the production of fungal products, it is critical to grow the organisms in the desired morphological form. The decrease of enzyme production at a higher inoculum concentration found in this experiment could be due to the undesirable morphological form of fungi growth.

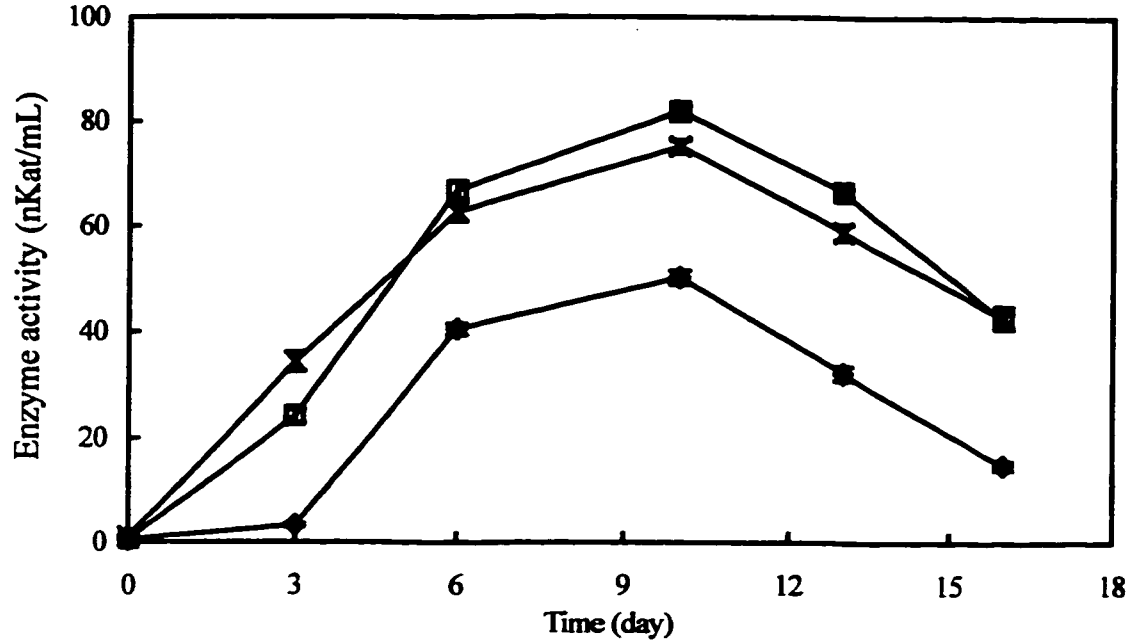


Figure 5.5 Effect of inoculum concentration on enzyme production during submerged process

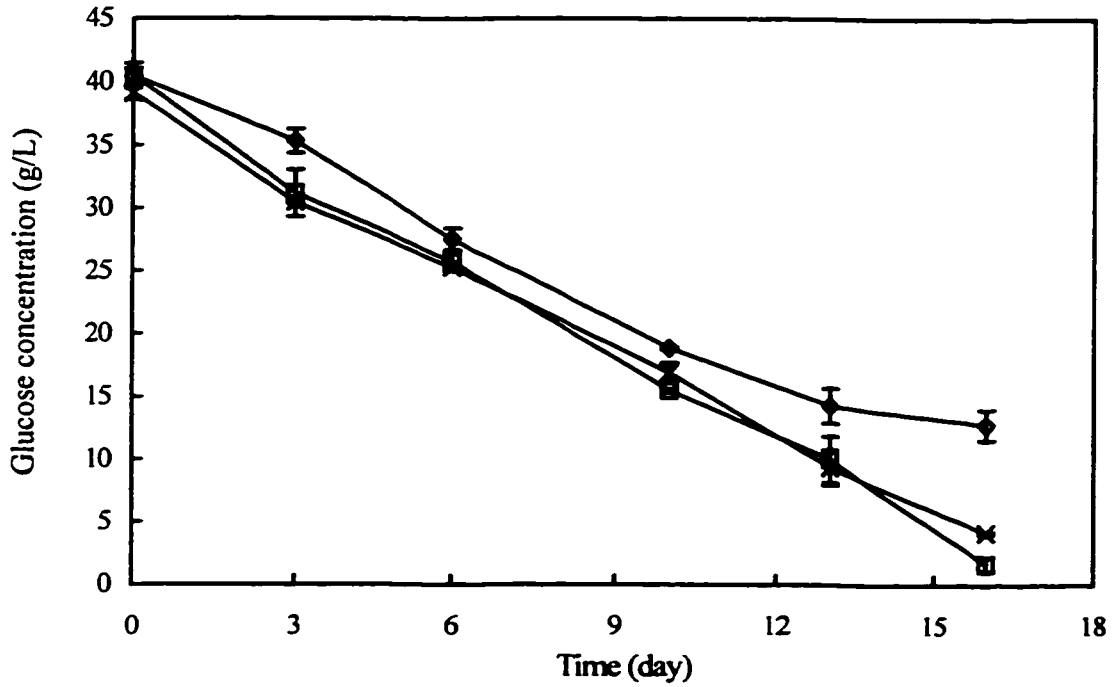


Figure 5.6 Effect of inoculum concentrationon glucose consumption during submerged process

5.1.4 Effect of Oxygen Concentration

Microorganisms can obtain energy by oxidative (respiratory) metabolism. Respiratory metabolism also results in conversion of organic compounds to carbon dioxide and water with energy being generated. The normal oxidant for respiration in fungi is oxygen (Carlile and Watkinson[2], 1994). Oxygen concentration plays an important role in microbial growth and formation of process products. To test the effect of oxygen concentration on enzyme production, different volumes of medium were used in Erlenmeyer flasks of the same size and shape for submerged processes. The difference in volume of medium would create various free air spaces above the medium in the flasks covered with foam plugs. Although the gas phase above the liquid contains similar concentration of oxygen (21%) in each flask at the beginning of the process, the contact surface becomes smaller when larger volume was used. Hence, the rate of oxygen diffused into the medium would be different. This resulted in difference in oxygen concentration in the liquid cultures.

The experimental results are shown in Figure 5.7. When the medium volume was decreased from 240 mL to 160 mL in a 500mL flask, oxygen concentration in the medium was expected to increase. The increase in oxygen concentration resulted in a higher enzyme production as shown in Figure 5.7. This could indicate that the fungus grew faster in the presence of higher oxygen concentration. However, with a further reduction in culture volume to 80 mL, the enzyme activity decreased. The maximum enzyme activity was around 53% of that found in the 160 mL culture. Al-Asheh (1993) found that oxygen had positive effect on biomass production in his study using *A.*

carbonarius for phytiase production, but only two different amounts of medium were tested.

From Figure 5.8, it is clear that glucose consumption was in agreement with the enzyme production. The fastest decrease in glucose concentration was observed in the 160 mL culture where the highest enzyme activity was reached, and a slower rate of consumption was found in the 240mL culture compared to that in the 160 mL culture. These results showed that when there was insufficient oxygen concentration in the liquid medium, negative effects were obvious on biomass and enzyme production. On the other hand, higher oxygen content might inhibit the production of enzymes. Therefore, there was an optimum oxygen concentration for the best growth of the fungus and for the production of the enzyme in the submerged process. The volume of 160 mL medium was found to be the optimum in this study.

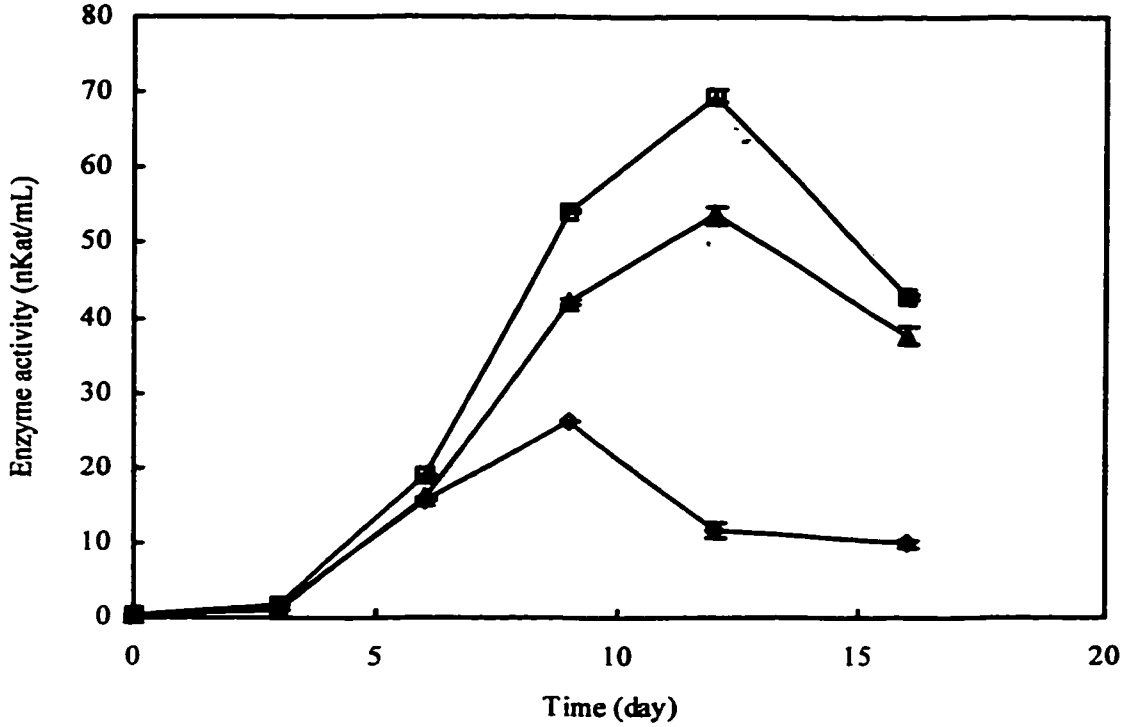


Figure 5.7 Effect of oxygen concentration on enzyme production during submerged process

80mL/500mL 160mL/500mL 240mL/500mL

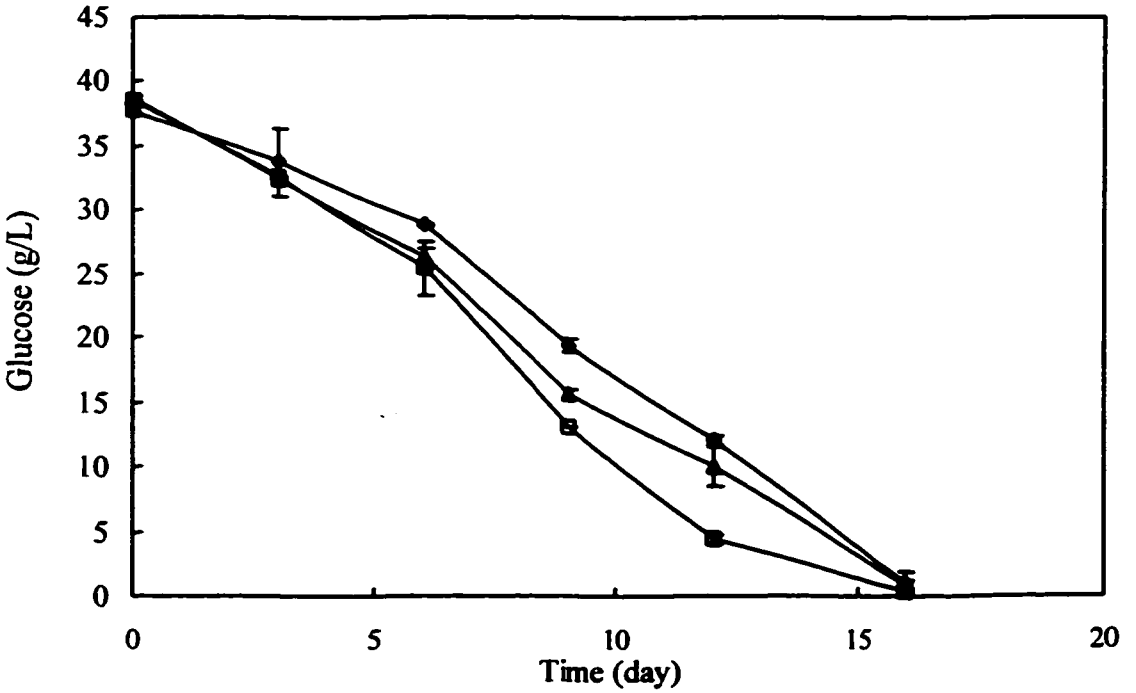


Figure 5.8 Effect of oxygen concentration on glucose consumption during submerged process

80mL/500mL 160mL/500mL 240mL/500mL

5.1.4 Effect of Inducers

Laccase from *P. ostreatus* is inducible as observed by many researchers (Leonowicz et al., 1978; Bollag and Leonowicz, 1984). In this study, five inducers were tested for their effects on enzyme production in submerged process. The results are shown in Figures 5.9 to 5.11. It was found that the most effective inducer was xylidine, which resulted in an increase in enzyme activity 1.8 times as compared to the control. Xylidine is a well-known inducer for laccase, and it has been studied by Bollag and Leonowicz (1984). They found that a three folds increase in specific enzyme activity was measured after the addition of xylidine into the submerged fermentation with *P. ostreatus (Fr. no. 13)*. However, the concentration was not given and could not be compared in this study. It should be noted that further study would be helpful to confirm the production of induced forms of laccase. Isolation and characterization of these induced forms of enzyme are necessary to have a better understanding of the enzymes produced by this strain of *P. ostreatus*.

The effects of other inducers are also compared (Table 5.2). Sinapine and sinapic acid had some induction effects but not comparable to xylidine. Little effect was found with caffeic acid on the production of enzyme. These results are quite different compared to laccase induction tested on other fungi with the same concentration of inducers. Koroljova-Skorobogat'ko et al. (1998) found that sinapic acid caused a 2.9 fold increase and caffeic acid had a 2.1 increase in enzyme produciton using *Coriolus hirsutus*. They also found that syringaldazine was the best laccase inducer tested in their study. At a very low concentration (0.11μM), it was able to increase the enzyme yield by 1000%. However, the results from in this study showed that at this low concentration,

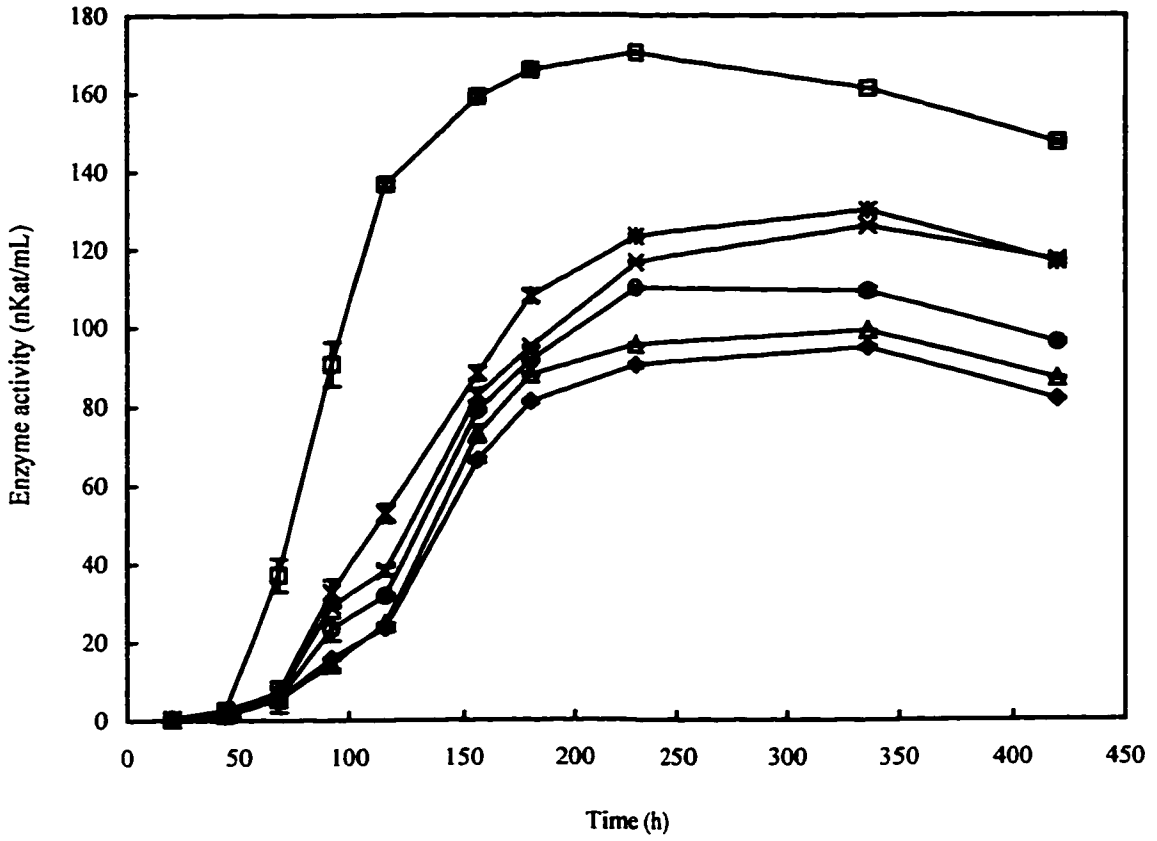


Figure 5.9 Effect of inducers on enzyme production during submerged process

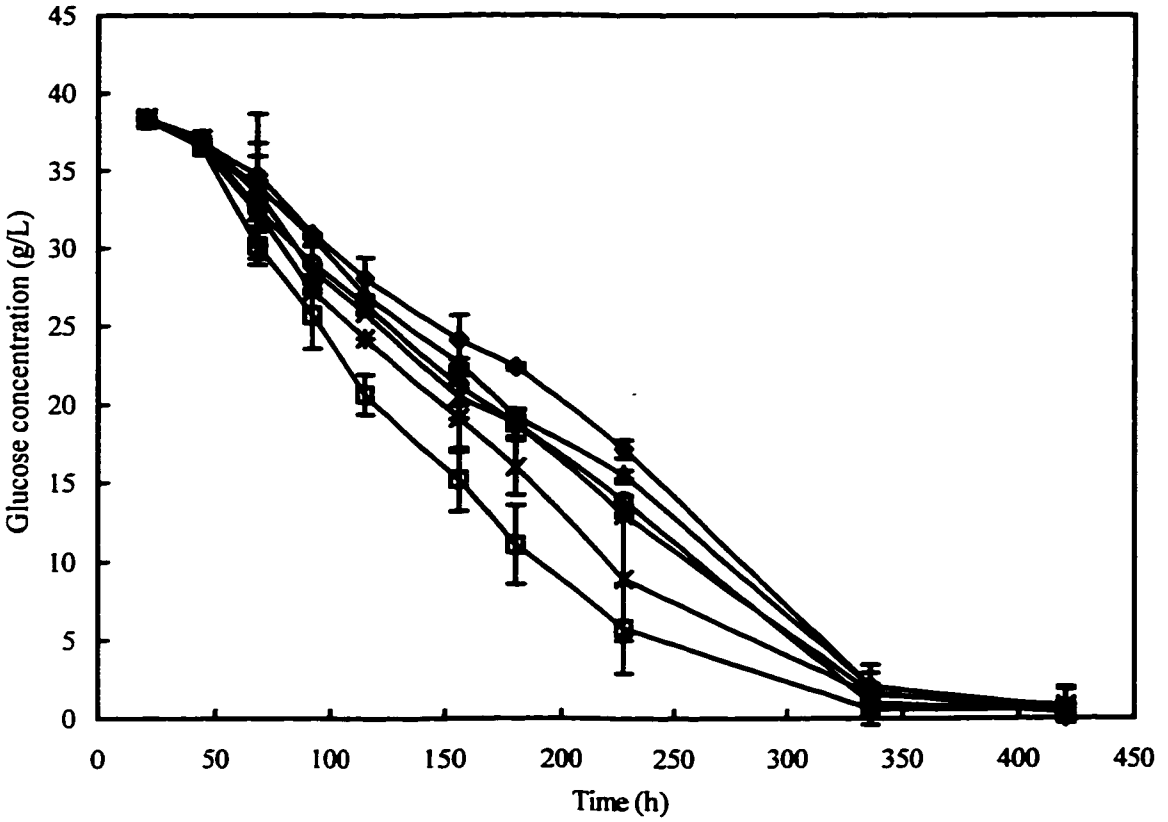


Figure 5.10 Effect of inducers on glucose consumption during submerged process

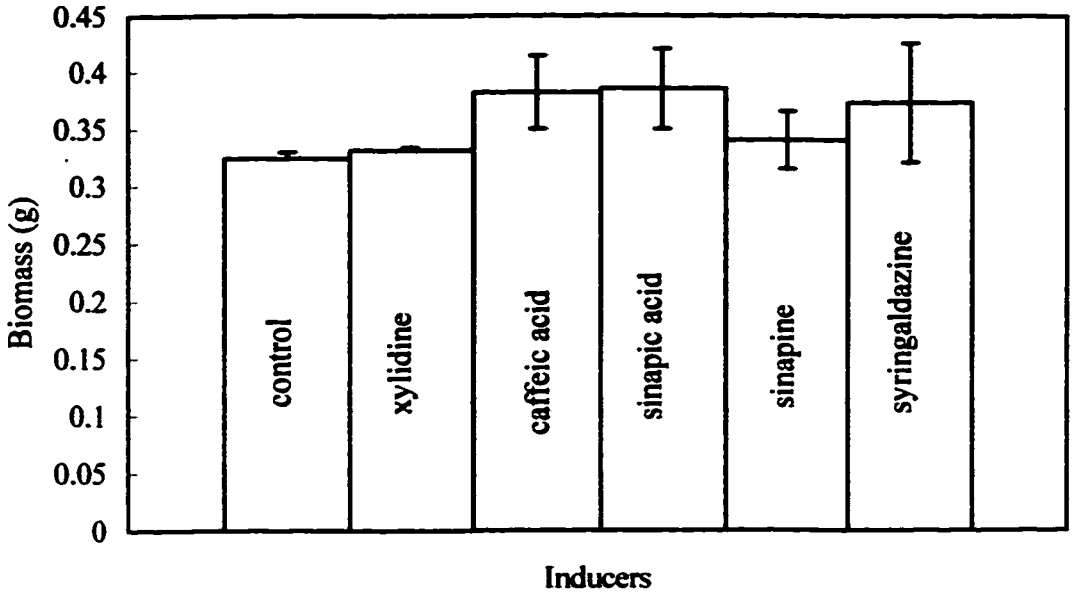


Figure 5.11 Effect of inducers on biomass production during submerged process

syringaldazine did not have such significant effect on the production of the enzyme (Figure 5.9). Therefore, the response of each fungus to the same inducers can be totally different.

A summary of the maximum activity from cultures with different inducers and their comparisons with the control are given in Table 5.2.

Table 5.2 Effect of inducers on enzyme production during submerged fermentation.

Name of inducer	Concentration of inducers (mM)	Maximum enzyme activity (nkat/ml)	Comparison to control (ratio)
Control	---	95	1.00
Xylidine	0.25	170	1.80
Sinapine	0.20	130	1.37
Sinapic acid	0.20	125	1.30
Caffeic acid	0.20	100	1.05
Syringaldazine	0.11×10^{-3}	110	1.15

Figure 5.10 shows the decrease of glucose concentration during the submerged processes. It is clear that the glucose consumption was in agreement with the enzyme production. The fastest decrease in sugar concentration was found in the cultures with xylidine added. The inducers also had effects on biomass production (Figure 5.11). Although xylidine was the most effective among the five inducers tested, it produced the least amount of biomass, nearly the same as in the control. The cultures with sinapine produced larger biomass compared to the ones with xylidine, but still lower than those with the other three inducers. The results from this experiment showed that some inducers only cause increase in enzyme production with little change in biomass production, while others, such as sinapic acid and caffeic acid, tend to increase the biomass growth and have a little or no effect on enzyme production.

Considering that sinapine and sinapic acid are the main components of phenolic compounds in CM, it can be expected that the addition of CM in submerged fermentation will have some positive effects on enzyme production.

5.1.5 Effect of CM Concentration

Canola meal can be used as a complex nutrient in submerged fermentation processes. As discussed earlier, CM has high contents of proteins and other nutritional components. Lacki (1997) summarized the composition of commercial canola meal. There are about 35-40% proteins, 27-30% hulls, 13% carbohydrates, 11% fiber, 8% moisture and 2% oil. There are also approximately 2% phytic acid and 2% phenolics. 50% of the carbohydrates is water soluble sugars, including 9% of monosaccharides and 91% water soluble polysaccharides. The composition of the meal pheolics was determined in Lacki's study (1997). It was found that free sinapic acid comprised 10% of

all phenolics and sinapine represented 71% of soluble and insoluble sinapic acid esters (SAE).

To examine the effect of CM on enzyme production, five tests with the synthetic medium and different concentrations of CM in submerged cultures were carried out. Glucose, soluble carbohydrates and enzyme activity were followed during these processes.

Figure 5.12 shows the enzyme activity during these processes. It can be seen that 3% of CM is an optimum concentration for the enzyme production. The maximum activity was around 1.7 times of that from the control culture, which was carried out without any CM. But the maximum occurred three days late compared to the control. This delay in maximum activity was also found in the cultures with other CM concentrations tested. After the maximum enzyme activity was reached, it started decreasing. The decrease seemed to be slower in the cultures with CM. This may be explained from the glucose and carbohydrates consumption curves (Figure 5.13 and 5.14, respectively). After nine days of the process, the glucose level was near to zero in the control culture. Without this main nutrient source, biomass starvation could have occurred and the production of enzyme would cease. Enzyme that had been produced earlier and released in the liquid culture would slowly loss its activity with time due to denaturation. On the other hand, in the cultures with CM, the glucose and the total soluble carbohydrates concentrations were almost five times higher when compared to those in the control. They helped the microorganisms continue growing for a few days more.

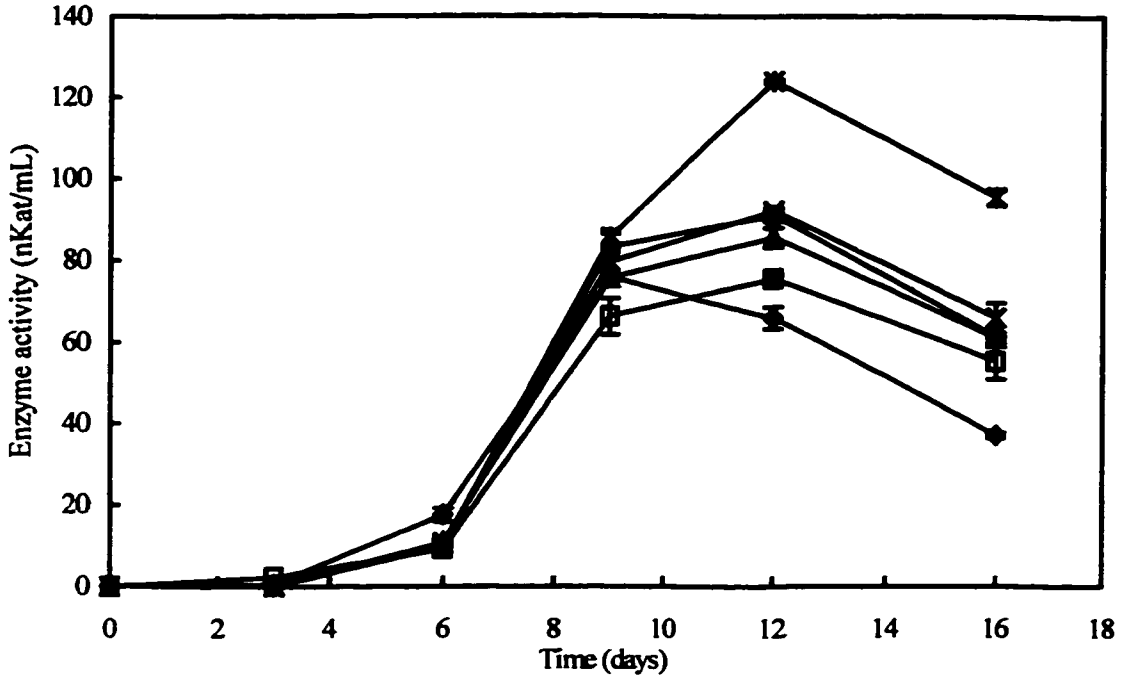


Figure 5.12 Effect of CM concentration on enzyme production during submerged process

0% 0.50% 1% 2% 3% 4%

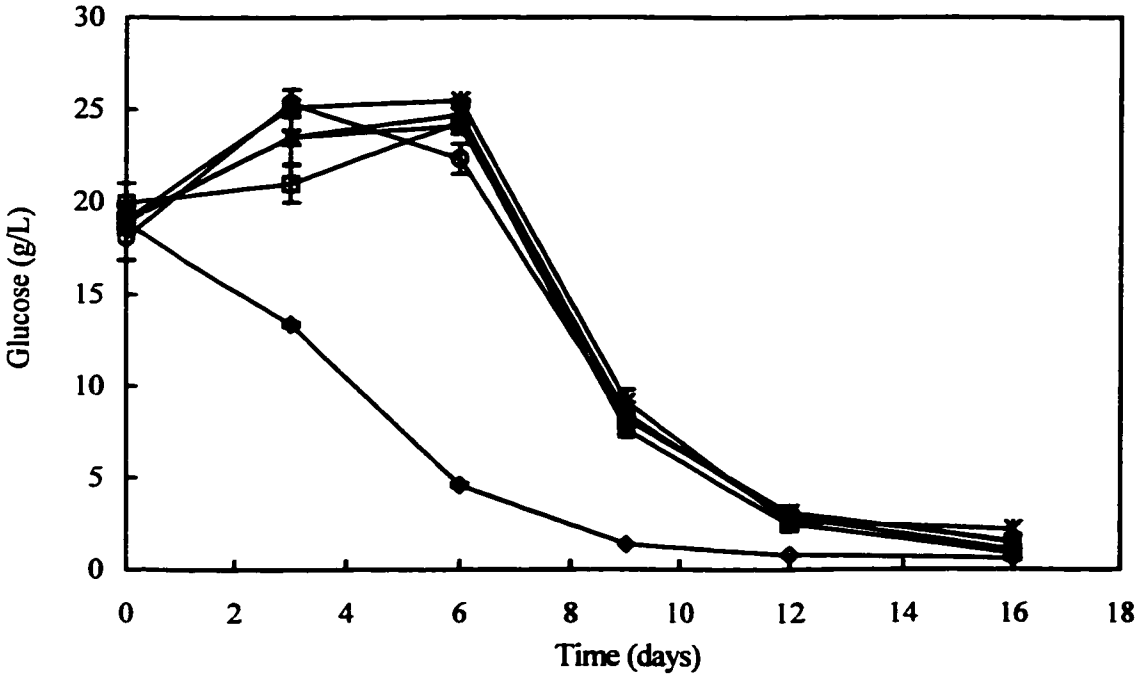


Figure 5.13 Effect of CM concentration on glucose concentration during submerged process

0% 0.5% 1.0% 2.0% 3.0% 4.0%

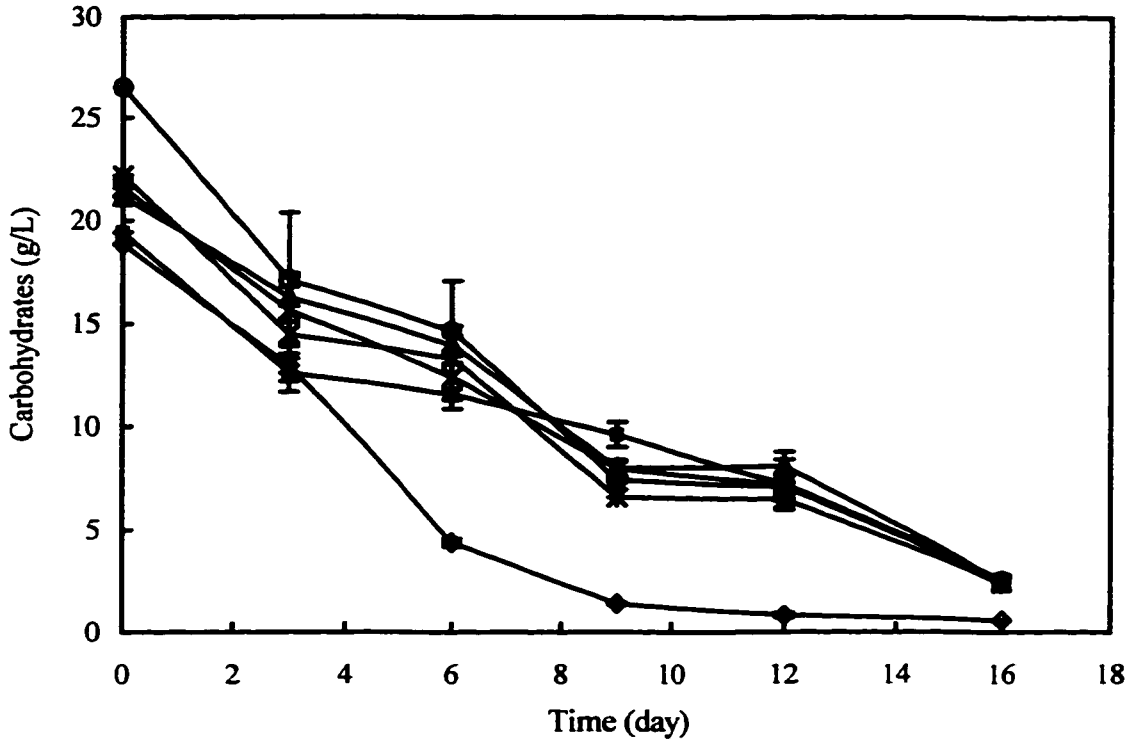


Figure 5.14 Effect of CM concentration on carbohydrates concentration during submerged process

The production of enzyme was increased with the addition of CM in the submerged process, and the concentration of this natural nutrient also played an important role, as can be seen from Figure 5.12. When there was more than 3% of CM added to the culture, the maximum enzyme activity decreased. One reason might be that a higher CM concentration created a less suitable environment for the growth of biomass and production of enzymes due to more difficult aeration in the viscous liquid. Also, there might be inhibition by the substrate at high concentrations. This was not found by Woods (1999) when he studied the effect of CM concentration on the production of PPO using *T. versicolor*. In his study, it was found that an increase of CM concentration from 3% to 5% did not make a significant difference in enzyme increase. He explained that, although CM contains some enzyme-stimulating compounds, once these compounds are present in certain levels, the increase in concentration would not cause the fungus to produce more enzymes. A different behavior was found with *P. ostreatus* in this study, significant inhibition effect was shown as discussed earlier.

It was noticed that glucose concentration increased for the first 6 days in the liquid cultures with CM (Figure 5.13). This could be due to a couple of reasons. First, soluble polysaccharides could have been depolymerized so that glucose was released. This reaction could have been catalyzed by enzymes such as cellobiase and amylase (Bailey and Ollis, 1986[8]), produced during the fermentation with *P. ostreatus*.

Additional glucose could also come from cellulose and hemicellulose hydrolysation. Enzyme, such as cellulase, is capable of degrading the insoluble carbohydrates. *P. ostreatus* is known to produce cellulase (Akhmedova, 1995). When

cellulose was hydrolyzed, the product was cellobiose and it can be further degraded to glucose by cellobiase, which can also be produced by *P. osteatus*.

At the beginning of the fermentation, the rate of total carbohydrate consumption was similar to the glucose consumption in the control culture (Figure 5.14), indicating that additional glucose was mainly from soluble polysaccharides. As the fermentation went on, total carbohydrate concentration decreased slower than the glucose in the control, which meant that some insoluble carbohydrates had been hydrolyzed and converted to glucose, such as cellulose and hemicellulose.

5.2 Laccase Characteristics

When enzymes are utilized, it is important to know their basic characteristics. To study the characteristics of enzymes produced by this fungus, enzyme preparation from submerged fermentation and three reaction substrates were used. The substrates were sinapic acid (SA), sinapine (SIN) and sinapaldahyde (SALD), which represent the main phenolic compounds in CM. Tests carried out included the effects of substrate concentration, pH, temperature of reaction mixture and preincubation time of enzyme preparation on the enzyme activity.

5.2.1 Effect of Substrate Concentration

The three substrates, SA, SIN and SALD, were dissolved in water and diluted to different concentrations. The effect of concentration of each substrate on the enzyme activity is shown in Figure 5.15. It should be noted that only the increasing parts of the experimental data were used for the calculations of Michaelis-Meten's parameters: the constant (K_m) and the maximum activity (v_m). Equation 3.4-2 and the method described by Bailey and Ollis (1986) (Chapter 3) were used for these calculations. The calculated K_m and v_m are summarized in Table 5.3 together with the optimum concentrations for enzyme activity.

The optimum SA concentration for enzyme activity was around 160 nmol/mL, and the activity was at similar level when the concentration was in the range of 150 to 250 nmol/mL. For SIN, the highest enzyme activity was attained when its concentration was around 140 nmol/mL. Enzyme activity did not change much when the concentration of this substrate was in the range 100 to 200 nmol/mL. The concentration of SALD was at 200 nmol/mL when the highest activity was reached. When the concentration of

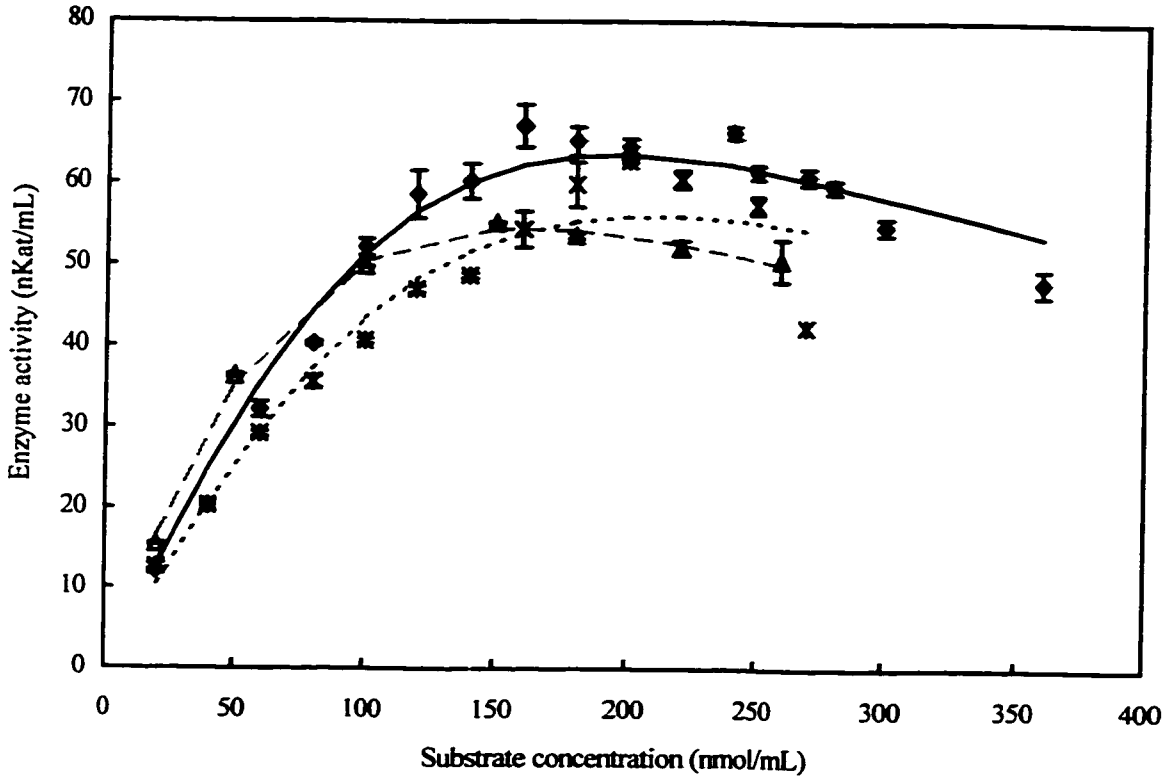


Figure 5.15 Effect of substrate concentration on enzyme activity; the symbols represent experimental data and lines represent model predictions (equation 3.4-3).

◇ Sinapic acid (SA) ——— Model-SA
△ Sinapine (SIN) – – – Model-SIN
✱ Sinapaldehyde (SALD) · · · · · Model-SALD

the substrate was higher, the enzyme started experiencing inhibition by the substrate and its activity decreased quickly. This decrease in enzyme activity was also found with the other two substrates, but with slower rates.

From Table 5.3, it is clear that similar v_m values were found with substrates SA and SALD, while a lower v_m was determined with SIN. Among the three substrates tested, K_m value was the highest for SALD, followed by SA and SIN. With similar maximum enzyme activity, a lower K_m for SA indicates that enzyme had higher affinity towards this substrate than SALD. Furthermore, from Figure 5.15, it is clear that the rates of increase in enzyme activity were similar with substrates SA and SIN, but inhibition occurred sooner with SIN, which resulted in low v_m and K_m.

The Michaelis-Menten equation was not used to model enzyme activity at different substrate concentrations, instead, a semi-empirical equation was used, equation 3.4-3. This equation was found to be more suitable when enzyme activity inhibition occurred at high substrate concentrations. The model equation fitted the data reasonably well and the predicted results are also shown in Figure 5.15.

Table 5.3 Optimum concentrations of substrates for enzyme activity, K_m and v_m values.

Substrate	Sinapic acid (SA)	Sinapine (SIN)	Sinapaldehyde (SALD)
Optimum concentrations (nmol/mL)	160	140	200
v_m (nKat/mL)	91.7	70.4	90.1
K_m (mmol/dm^3)	0.085	0.045	0.115

5.2.2 Effect of pH

pH also has large effect on enzyme activity as shown in Figure 5.16. The test of pH effect on enzyme activity was carried out using the optimum substrate concentrations from previous analyses. In this study, the optimum pH values were around 4.4, 5.0 and 4.7, using SA, SIN and SALD as the substrates, respectively. Bollag and Leonowicz (1984) reported a lower pH value of 3.8 as the optimum for laccase activity, when the enzyme produced by *P. ostreatus (Jacqu)* was tested using SA as the substrate. With laccase from a different fungus, *T. versicolor*, the optimum pH was also a little different. The highest enzyme activity was found to be around pH 4 (Lacki and Duvnjak, 1996a).

The optima for laccase activity with the other substrates were quite different from the values obtained by Lacki and Duvnjak (1996a) (Table 5.4). Enzyme from this work had higher optimum pH values, which meant that the catalyses were favored in a less acidic environment.

A model equation (Equation 3.4-8) used by Al-Asheh (1993) and the least square curve fitting were used to model the pH effect on enzyme activity (Figure 5.16). The lines in Figure 5.16 represent the model predictions and symbols are experimental data. The values of equilibrium constants, K_1 and K_2 (defined in equations 3.4-6,7), calculated from these data are summarized in Table 5.4 together with the optimum pH values. The model fitted the experimental data quite well.

In general, when the constants, K_1 and K_2, are small, large amount of enzyme are in the inactive forms by protonation and deprotonation of the active sites of enzyme (equations 3.4-6,7) and enzyme activity change would be more rapid with the change in pH. Both K_1 and K_2 were the smallest when SIN was used as the substrate compared to

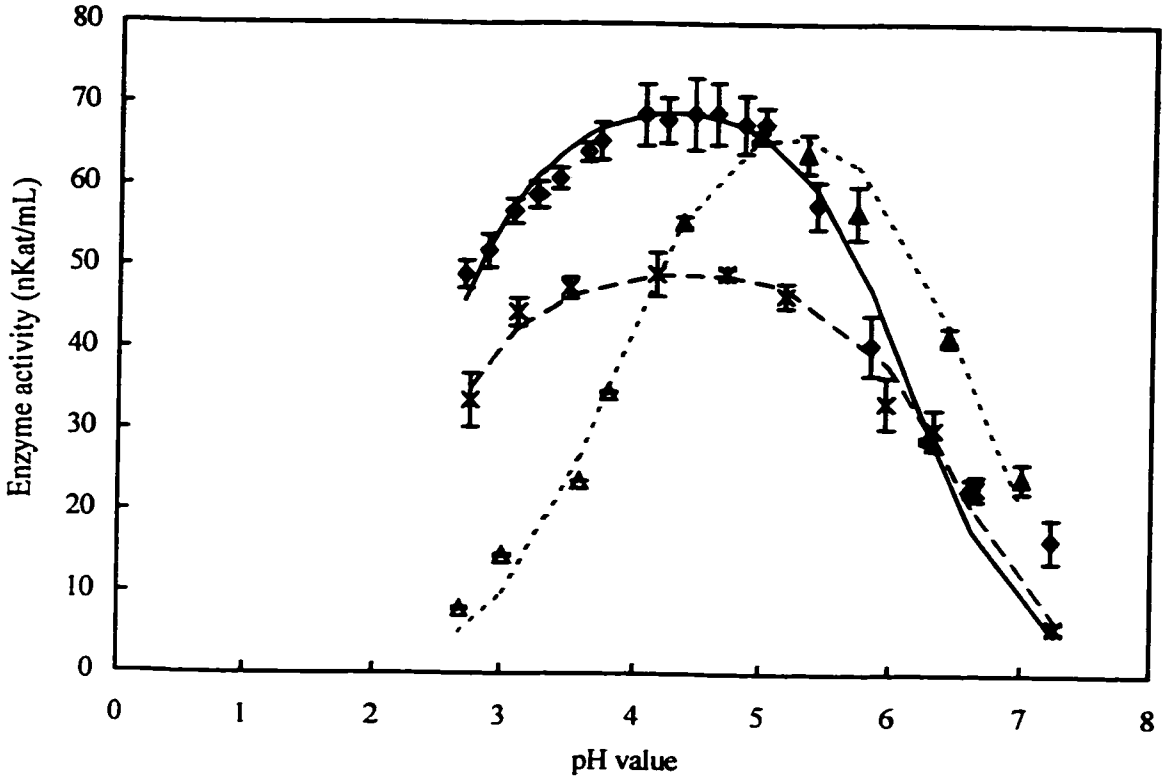


Figure 5.16 Effect of pH on enzyme activity; symbols represent experimental data and lines represent model predictions (equation 3.4-8)

◇ sinapic acid-SA ——— Model-SA
△ sinapine-SIN ····· Model-SIN
✱ sinapaldehyde-SALD — — — Model-SALD

the other two substrates tested. This would mean more rapid change in enzyme activity would occur with this substrate. This is shown by the experimental results (Figure 5.16), where the quickest decrease in enzyme activity was found after reaching the maximum with SIN. Enzyme activity in catalyzing SALD had a plateau of the maximum activity for a wide range of pH values between 3.3 to 5.0. For SA, the range of the maximum enzyme activity was a little narrower, from 3.7 to around 5. The optima of pH values found here were used for subsequent characterization analyses, effect of temperature and pre-incubation.

Table 5.4 Optimum pH values and equilibrium constants from model equation

Substrate name	Optimum pH value from this work	Optimum pH value from Lacki and Duvnjak (1996)	K_1 M	K_2 M
Sinapic acid	4.4	4.0	3.7×10^{-3}	7.19×10^{-7}
Sinapine	5.0	4.5	1.6×10^{-4}	2.45×10^{-7}
Sinapaldehyde	4.7	3.3	4.25×10^{-3}	3.44×10^{-7}

5.2.3 Effect of Temperature

Most enzymes are sensitive to temperature change. Their activities usually increase with temperature and reaches a maximum, further increase will result in denaturation of enzyme. Figure 5.17a shows the experimental results of temperature effect on enzyme activity measured using the three substrates. As it was expected, enzyme activity in each of the three catalyses increased with reaction temperature, then a decrease occurred at a certain point after it reached the maximum.

The amounts of change in enzyme activity seemed to be similar with all three substrates. The optima were all in the range of 313 to 328K. The maximum enzyme

activity did not change much when the temperature was in the range of 311 to 331K with SA as the substrate, a similar range was found with SALD. The range of temperature for maximum enzyme activity was a little larger with SIN, around 306 to 336K. Even at 343K, the activity was only 10% lower than the maximum. The enzymes produced by *P. ostreatus* are more thermally stable compared to many other enzymes. Denaturation of enzyme protein often begins to occur at 318 to 323K and is severe at 328K (Bailey and Ollis, 1986[7]). One of the physical mechanisms for this phenomenon is that as the temperature increases, the atoms in the enzyme molecule have greater energy and a greater tendency to move. Eventually, they acquire sufficient energy to overcome the weak interactions holding the globular protein structure together, and deactivation follows.

5.2.4 Effect of Pre-incubation Time

The thermo-stability of the enzyme is also an important characteristic. To determine the thermostability of the laccase secreted by *P. ostreatus*, the enzyme preparation was pre-incubated. Figure 5.17b shows the experimental results of remaining enzyme activity after the pre-incubation for specified amount of time at different temperatures. It was found that the enzyme was quite stable at 313K. Even after seven hours of pre-incubation, 65% of the enzyme activity remained. Deactivation occurred faster as the pre-incubation temperature increased. At 323K, it took three hours to deactivate the enzyme to 65%. At 333K, the decrease in activity was rapid. Less than 20% activity was left within one hour of pre-incubation.

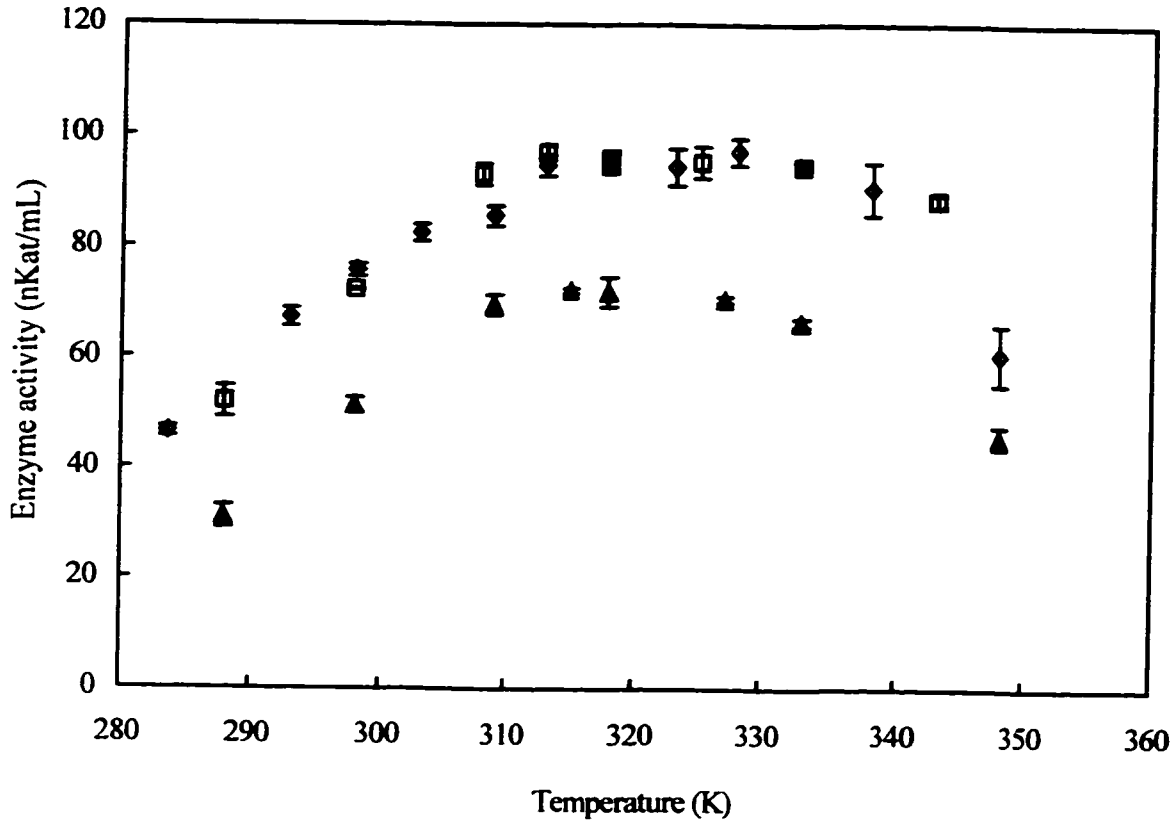


Figure 5.17a Effect of temperature on enzyme activity

◇ sinapic acid □ sinapine △ sinapaldehyde

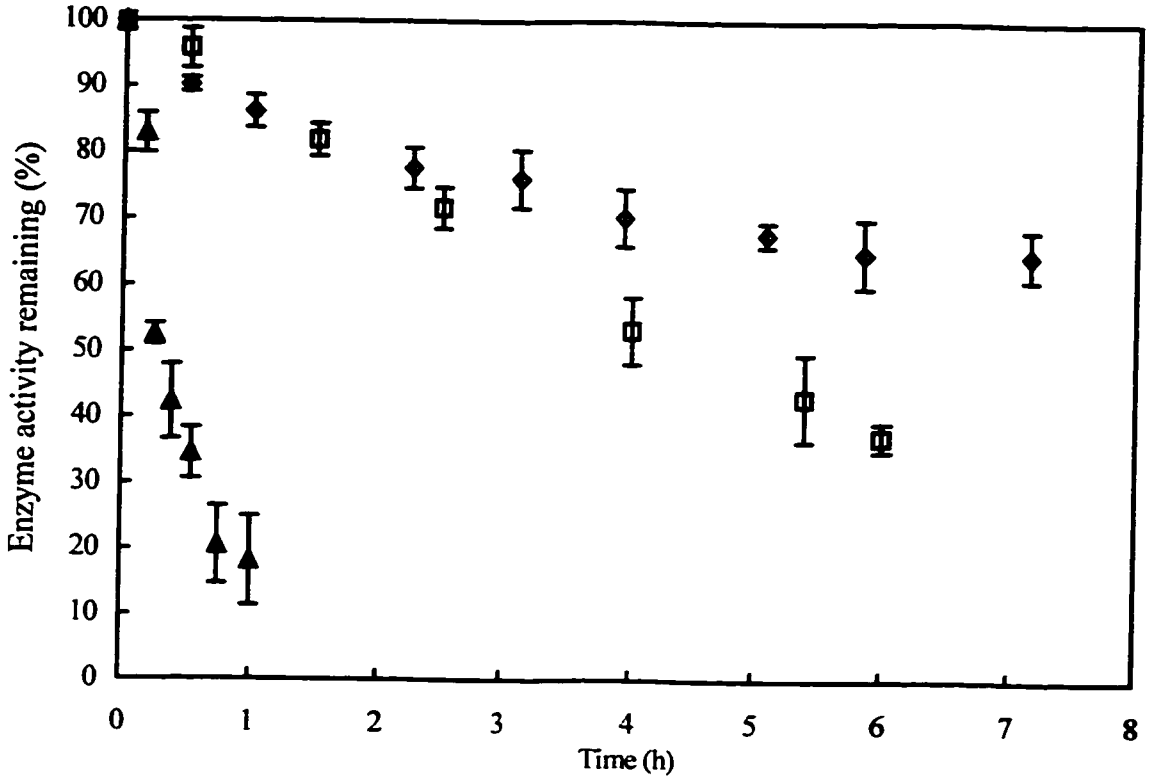


Figure 5.17 b Effect of pre-incubation time on enzyme activity using SA as the substrate

◇313K □323K △338K

5.3 Solid State Fermentation

P. ostreatus is capable of producing certain extracellular enzymes that can degrade phenolic compounds. The degradation of phenolics by this fungus is a complex enzymatic process. In the first part of this thesis, the production of laccase was studied using the submerged process. The enzyme preparation from the submerged process was used for the characterization of the enzymes. One of the objectives of this thesis was also to study the production of the same enzyme by *P. ostreatus* in solid state processes. In these processes, CM was used as the medium and the decrease of phenolics content in CM was followed. The effects of four process parameters on the SSF were studied to optimize the process conditions. The parameters included effect of moisture content in solid culture, amount of inoculum, inoculum homogenization time and canola meal particle size. Enzyme and biomass concentrations in solid cultures, as well as phenolics content were monitored during the fermentation processes.

5.3.1 Effect of Initial Moisture Content in Solid Culture

Moisture content in solid culture is important for the growth of microorganisms. Although solid state fermentation is close to the natural habitat for fungi to grow, the growth of biomass requires water. CM used in this study contained some moisture (10% or less), but it was far from being sufficient for the microorganisms to grow well. Therefore, addition of water to the solid culture was necessary. Solid state fermentation is usually carried out in moist substrates (Ridder et al., 1998; Alam et al., 1994) and the optimum of moisture content often varies largely, depending on the substrate, microorganisms, fermentation products and other factors.

For example, Ridder et al. (1998) studied the moisture effect on xylanase production on wheat bran using *T. longibrachiatum*. It was found that 55% moisture content resulted in the highest enzyme activity. Different results were obtained in another study of xylanase production. The optimized initial moisture level was 80% for *T. lanuginosus* and 50% for *T. augrantiacus* in solid state fermentation on lignocellulose material, including wheat bran (Alam et al., 1996). Therefore, large difference in the optimum exists when different fungi are used.

To investigate the effect of moisture content on enzyme production and degradation of phenolic compounds in CM by *P. ostreatus*, different amounts of water were added to make the moisture content of solid culture in the range from 50% to 75%. Moisture content above 75% could not be absorbed by CM and would become free water at the bottom of the flask leading to non-homogenous culture conditions.

The biomass and enzyme concentrations, as well as SAE content in CM during the fermentation process were measured and are shown in Figure 5.18, 5.19 and 5.20. It can be seen that the growth of microorganisms depended largely on the initial moisture content. The slowest biomass growth was in the medium with 50% moisture. As the initial moisture content increased, biomass growth also increased. The best growth was found in the 70% moisture fermentation for the first 15 days (Figure 5.18). By observing the growth during the fermentation, it was noticed that the solid culture became dry and hard after a few days in the cultures with 50% moisture content. This made it difficult for fungi to penetrate through CM for more nutrients. With an increase in moisture content, swelling of the substrates could occur (Al-Asheh, 1993), and this facilitated its utilization by microorganisms. However, further increase in addition of water, at 75% moisture

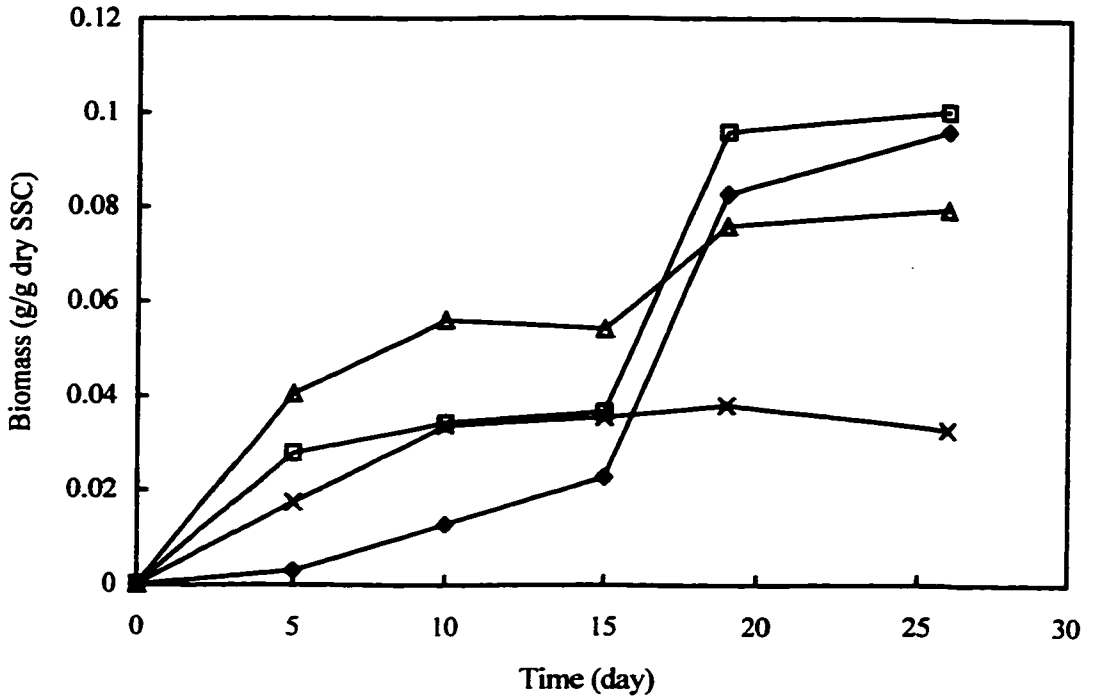
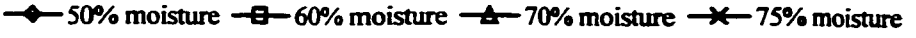


Figure 5.18 Effect of moisture content on biomass production during solid state process



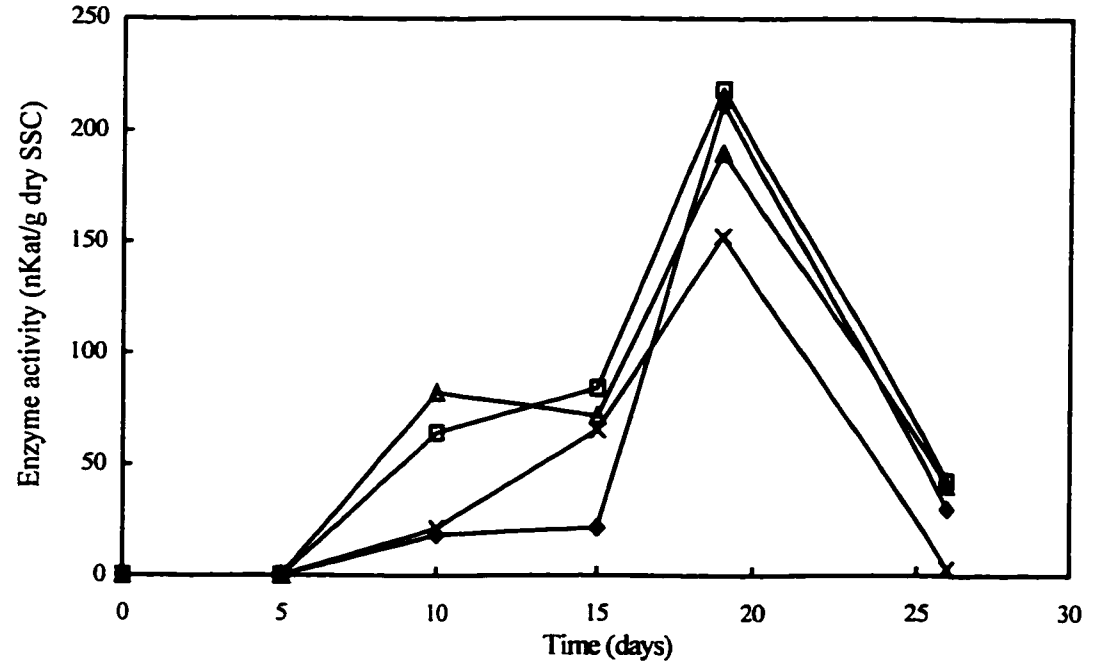
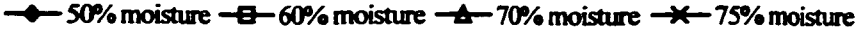


Figure 5.19 Effect of moisture content on enzyme production during solid state process



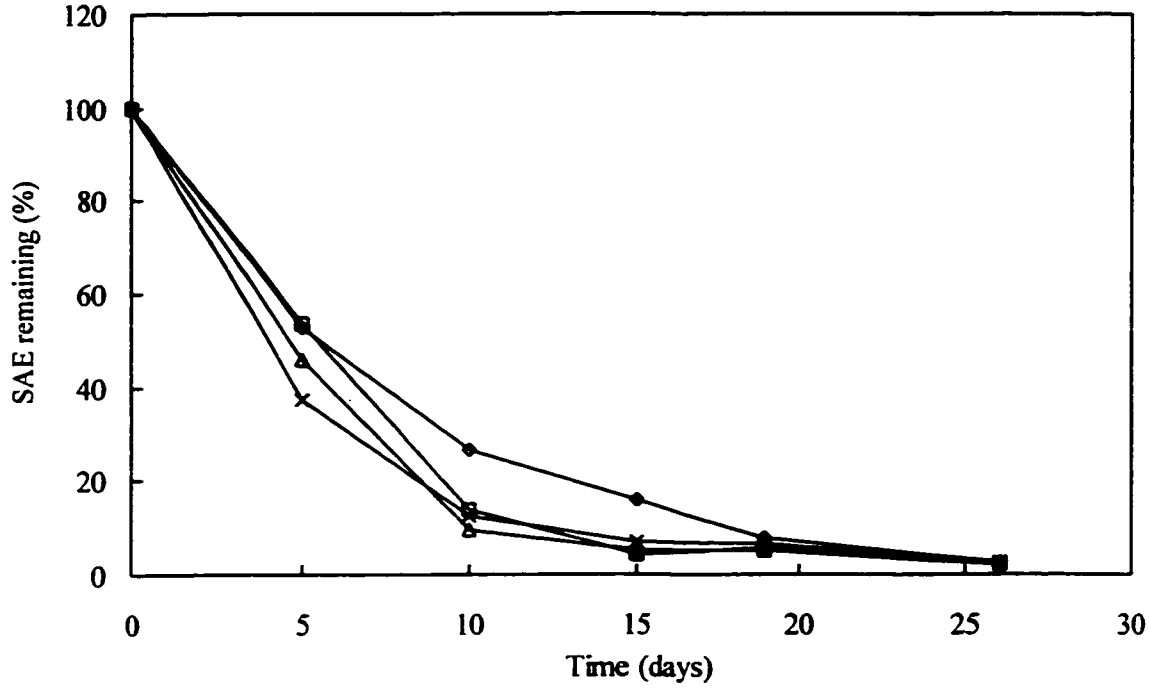


Figure 5.20 Effect of moisture content on SAE degradation during solid state process

content, the growth was less than that from 70% moisture cultures. This could be due to difficulty in oxygen transfer when there was too much water in the solid culture.

As the fermentation went on, better growth was found in the culture with lower moisture content. The highest biomass production was obtained in the culture with 60% moisture content around day 19. There was no obvious change in biomass after that in most of the media indicating the exhaustion of nutrients.

The highest enzyme activity was produced in the 60% moisture culture (Figure 5.19). This is in agreement with the biomass production. Therefore, the optimum moisture content for biomass and enzyme production was around 60%.

The decrease in SAE content appeared to be the most rapid in culture with 75% moisture content (Figure 5.20). Although less biomass and enzyme were produced in this culture compared to the others, there was probably more phenolics dissolved in the liquid phase of the solid culture and could be more easily utilized or degraded by the fungus. With 50% initial moisture content, the decrease of SAE in the solid culture was the slowest at the beginning, because of the late growth and formation of enzyme, but there was no difference after 18 days indicating enough enzymes present in each culture for the degradation of SAE.

The optimum moisture content for solid state fermentation depends on the objective of the study. High initial moisture content at 75% facilitated the degradation of SAE, while relatively low moisture content resulted in better production of enzyme and biomass.

Modeling

Biomass and enzyme productions, as well as SAE degradation, have been predicted by model equations based on the logistic law (Chapter 3). By fixing initial conditions and fitting experimental data, equation parameters, X_m, μ_m, K_v and K_s, were determined (Table 5.5).

For modeling biomass with different initial moisture contents, equations 3.1-6 and 3.1-7 were used. The predicted results are shown together with experimental data in Figure 5.21. The model equation was not able to fit all data well. The best fit was for biomass concentration in cultures with 75% moisture content. In this process, biomass growth seemed to go into exponential phase directly. Similar growth was found in cultures with 70% initial moisture content, while a lag phase occurred in the cultures with 50% and 60% moisture. The model is known to cover exponential and stationary phase (Sangsurask et al., 1996), and therefore was not suitable in the cases of biomass growths in lower initial moisture contents. Experimental errors were obvious in the measurements on day 15 in the cultures with 60% and 70% initial moisture contents. These points were excluded when the model equation was used to fit the data.

Equation 3.2-3 was used to predict the increasing phase of enzyme production and the results are shown in Figure 5.22 with the experimental data. Parameter of the modeling equation, K_v was determined when X_m and μ_m from biomass modeling equation were fixed (Table 5.5). Modeling enzyme activity using logistic law at different moisture content was found to be suitable only during the increase phase of the activity. The decrease of enzyme activity could not be modeled by this model equation. The fitting was reasonable and better fit was found for 70% and 75% moisture contents.

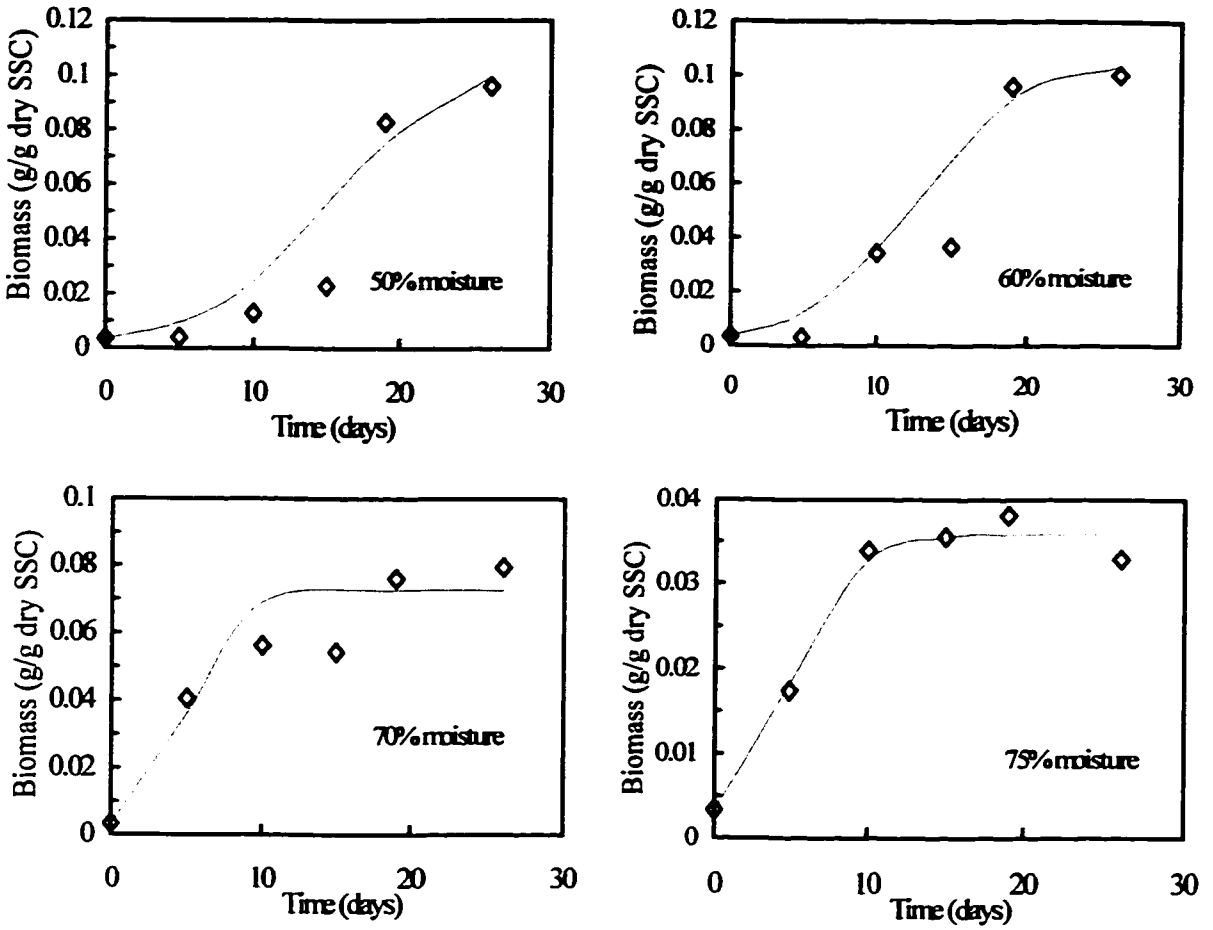


Figure 5.21 Modeling of biomass production in SSC with various initial moisture contents; symbols are experimental results, lines are model predictions (equation 3.1-6).

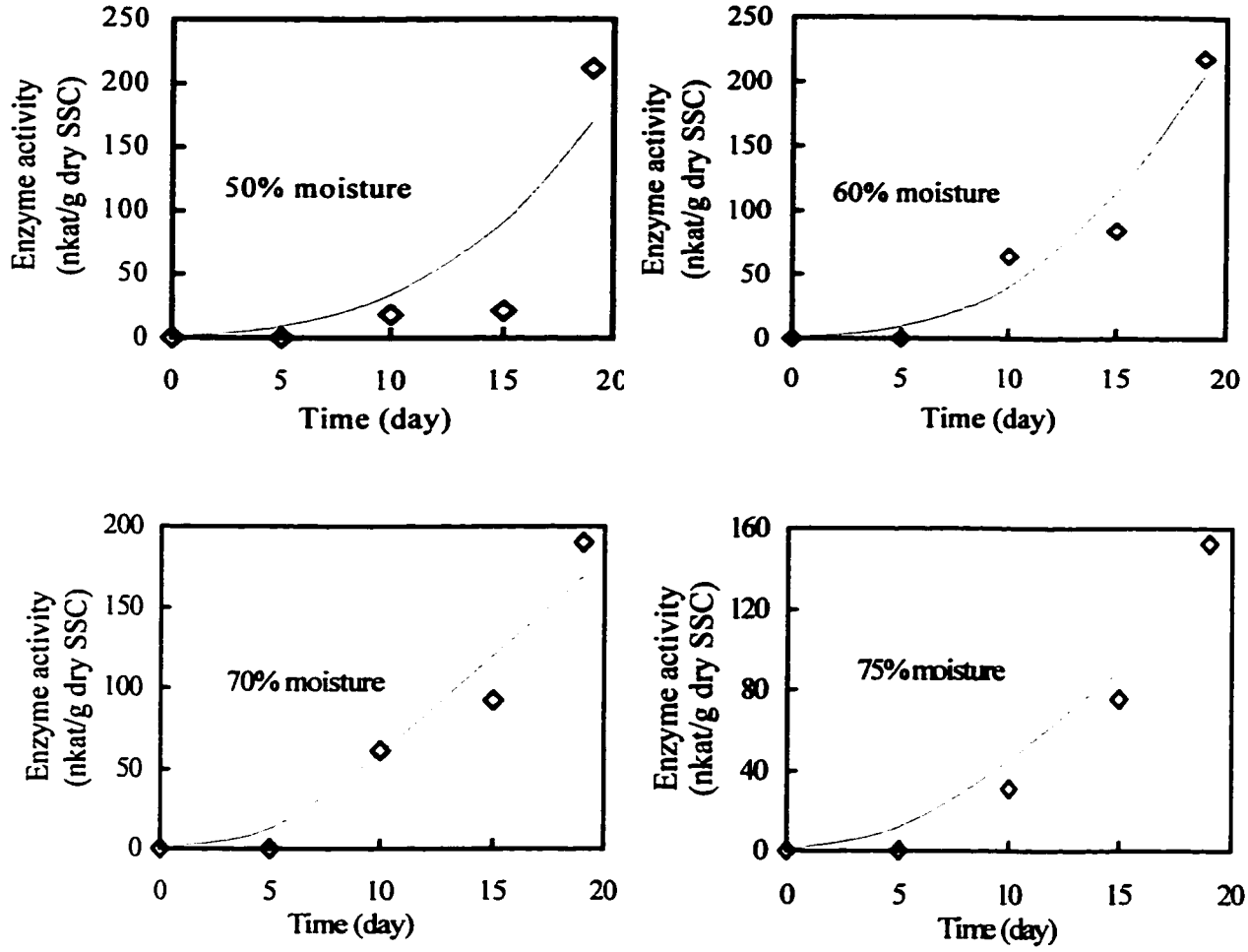


Figure 5.22 Modeling of enzyme production in SSC with various initial moisture contents; symbols represent experimental data and lines represent model predictions (equation 3.2-3).

SAE remaining in the SSC was predicted using equation 3.3-4 and compared with the experimental data. Figure 5.23 shows the remaining SAE as percentage of original content vs. time. In general, the experimental results were well predicted by the model equation. The model equation was able to describe the degradation of SAE better when biomass modeling was better, especially in the initial phase of the process.

Table 5.5 Parameters from modeling equations for the effect of moisture content during solid state process

Moisture content (%)	X_m (g/g dry SSC)	μ_m (day^{-1})	K_v (nKat/g /day)	K_s (g dry SSC/g /day)
50	0.1089	0.2244	307.7	15.14
60	0.1058	0.2775	275.5	16.58
70	0.0724	0.6060	168.1	9.651
75	0.0359	0.4632	252.3	19.29

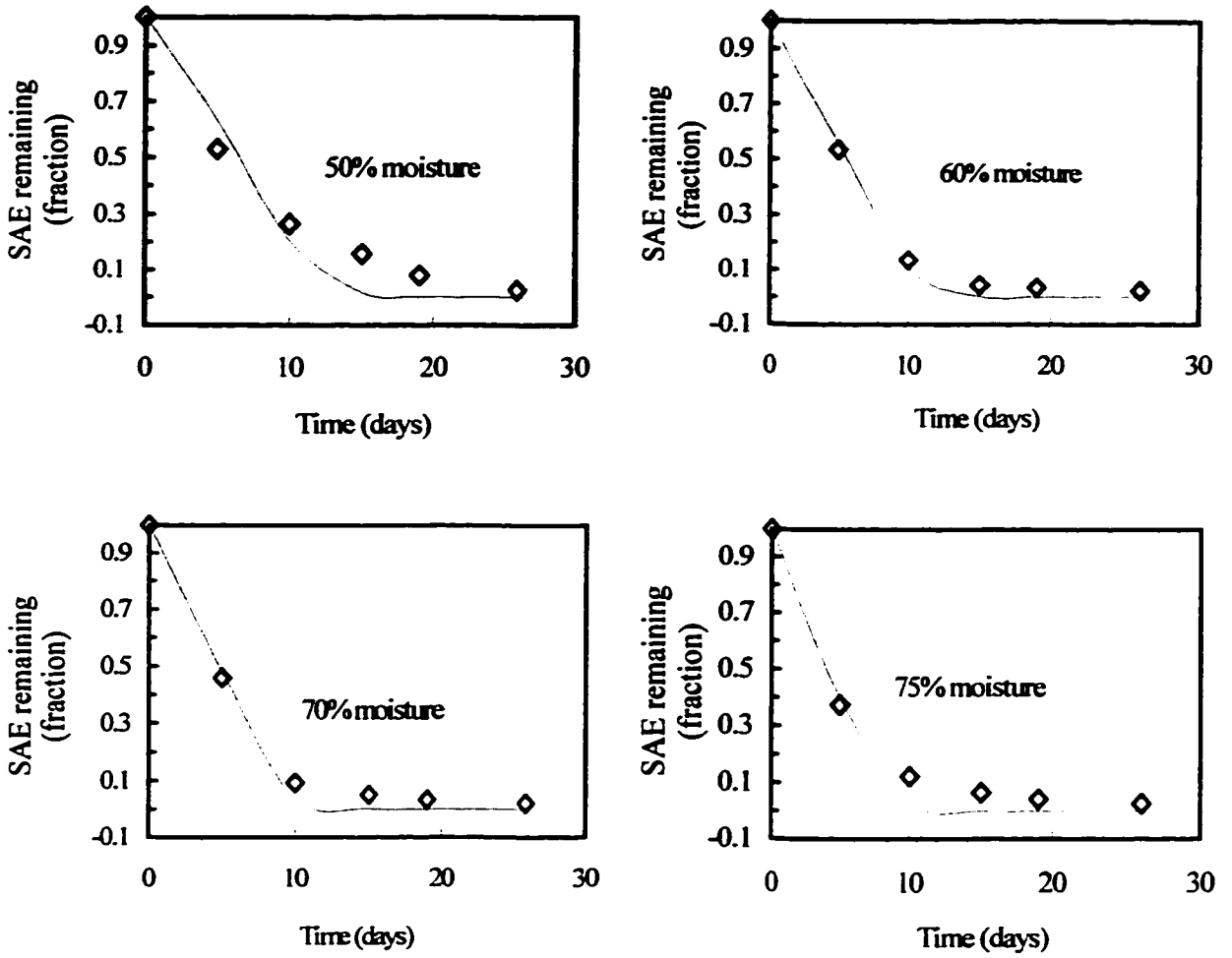


Figure 5.23 Modeling of SAE degradation in SSC with various initial moisture contents; symbols represent experimental data and lines are model predictions (equation 3.3-4).

5.3.2 Effect of Inoculum Concentration

Inoculation of solid state culture is usually more difficult compared to liquid medium. There is more risk of contamination while stirring to obtain a homogenous culture. Transferring and stirring could also damage certain amount of microorganisms. Too little of inoculum would result in a slow growth and too much could be wasteful. Therefore, it is important to find out how much inoculum would be sufficient for the success of a solid state fermentation process.

The effects of inoculum concentration on the biomass and enzyme production, as well as SAE degradation were studied. Tests with five different inoculum concentrations were carried out. The experimental results are shown in Figures 5.24 to 5.26. With an increase in inoculum concentration, the initial growth rate of biomass also increased, as expected. In the cultures with inoculum concentration of 4.4 mg/g and 5.5 mg/g, the growth went into the exponential phase directly, reached a maximum biomass concentration and maintained at the same level after day 10 (Figure 5.24). On the other hand, the growth of biomass with lower inoculum concentrations was much slower for the first 15 days, then rapid growth occurred between day 15 and day 20. These results indicated that large amount of inoculum can shorten or eliminate the lag phase of biomass growth.

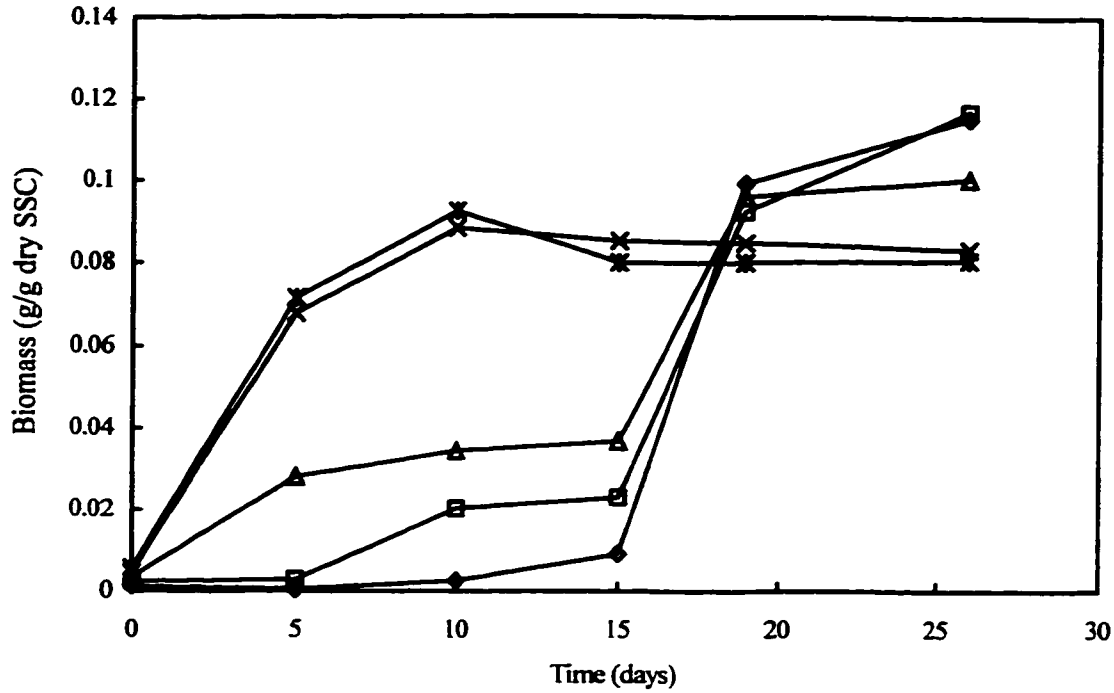
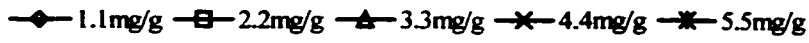


Figure 5.24 Effect of inoculum concentration on biomass production during solid state process



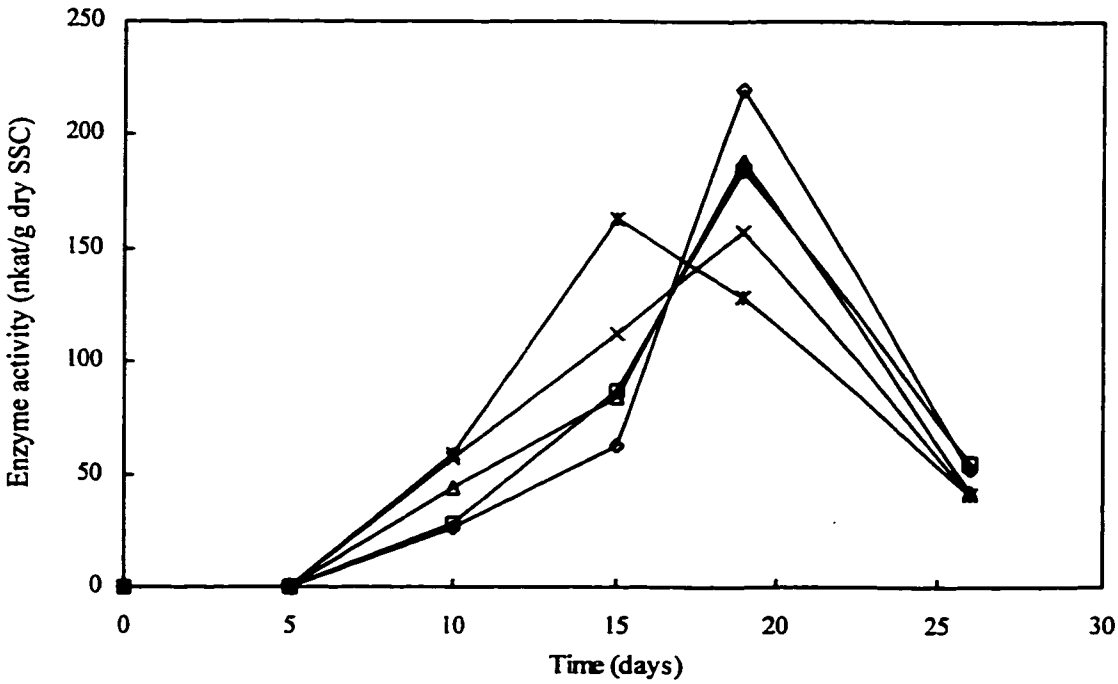
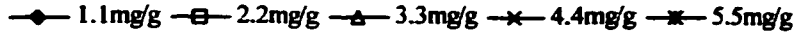


Figure 5.25 Effect of inoculum concentration on enzyme production during solid state process



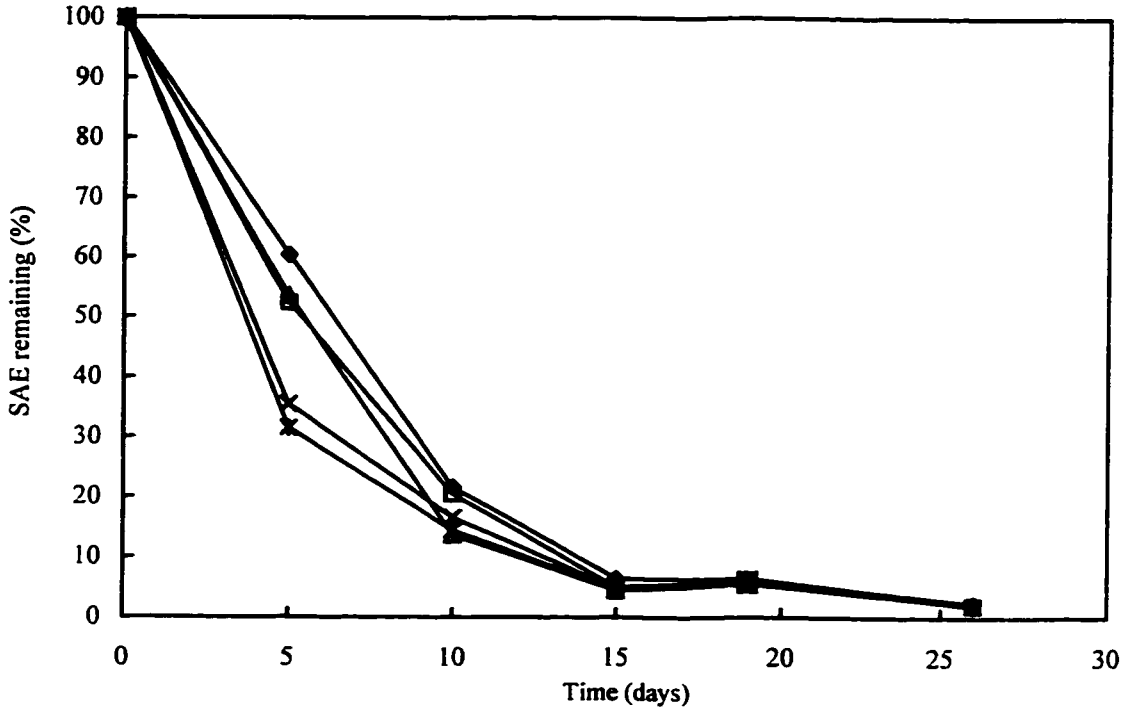


Figure 5.26 Effect of inoculum concentration on SAE degradation during solid state process

Enzyme activity was detectable after day 5 in the solid cultures (Figure 5.25). With higher initial inoculum concentration, enzyme production was also higher, and this is in agreement with biomass growth for the first 15 days. However, the trend was reversed after day 15. Later growth of biomass in the culture with the lowest inoculum concentration tested (1.1mg/g) helped the production of enzyme, and the maximum enzyme activity was found in this culture.

The initial SAE decrease was the fastest in the culture with the highest inoculum concentration tested (Figure 5.26). Although no enzyme activity was detected in the liquid extract from the solid cultures on day five, it was possible for enzyme to be bounded to the CM particle surface and could not be detected. This was confirmed by treating CM, which was not inoculated, in the same way as that inoculated. It was found that less than 10% of SAE content was decreased. This may indicate that the enzyme was produced in the inoculated cultures before day 5, which resulted in a decrease of SAE much more than 10%. It seems that a large amount of enzyme was not required to effectively degrade SAE compounds in CM. SAE contents remaining after 15 days were around 5% in all solid cultures.

Similar trend in the effect of inoculum on enzyme production was found by Woods (1999), with the fungus *T. versicor* growing in solid state process using CM as the medium. It was found that after the process started for eight days, higher enzyme activity level was found in culture with lower inoculum size.

Modeling of biomass with different inoculum concentration during the SSF is shown in Figure 5.27. Some experimental errors are obvious when data are compared with the model predictions. Points on day 15 for the three lower inoculum concentration

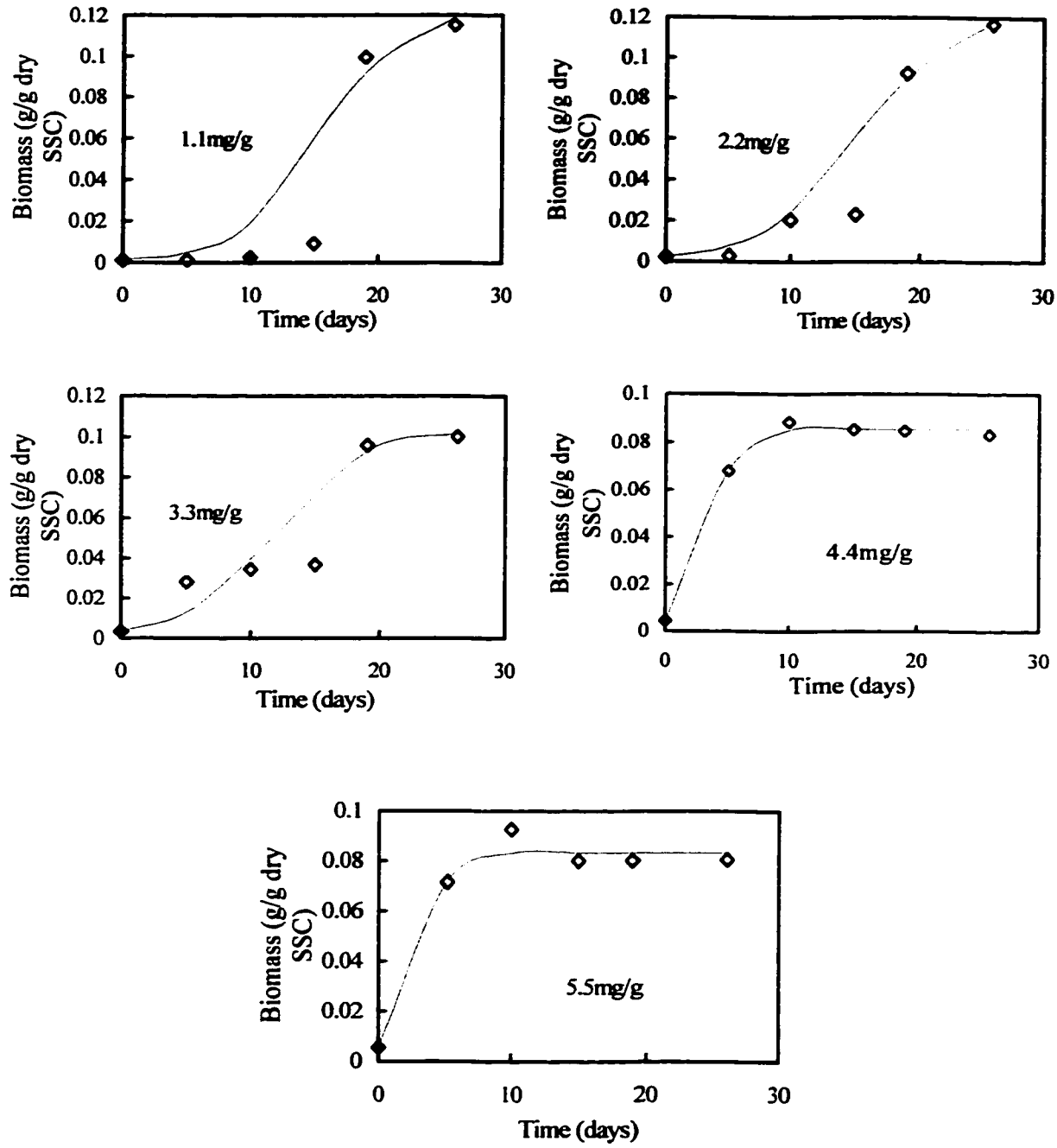


Figure 5.27 Modeling of biomass in the SSC with various initial inoculum concentrations; symbols represent experimental data and lines are model predictions (equation 3.1-6).

cultures were excluded when data were fitted for modeling. The parameters determined are in Table 5.6. When there was small amount of inoculum, the obvious lag phase could not be fitted using these equations. Better fitting was found for cultures with higher inoculum concentrations, which had little lag phase for biomass growth.

Enzyme activity was modeled using equation 3.2-3. The predicted results and experimental data are shown in Figure 5.28. Reasonable fitting was found for most of the experimental data, except for the largest inoculum concentration tested. With large amount of initial biomass, the initial growth was expected to be fast, which would result in higher enzyme production. However, enzyme production on day 5 was not detectable in all cultures, which made the fitting more difficult.

In modeling SAE content remaining in the solid culture, equation 3.3-4 was found to fit most experimental data well. The predicted results and experimental data are shown in Figure 5.29. The experimental data were fitted well in most cases for the beginning of the process. The slow decrease at the end from the experimental data could be due to inaccuracy in measurements.

Table 5.6 Parameters from modeling equations for the effect of inoculum concentration.

Inoculum concentration (g/g dry SSC)	X_m (g/g dry SSC)	μ_m day^{-1}	K_v (nKat/g /day)	K_s (g dry SSC/g /day)
0.0011	0.1241	0.3031	377.8	29.80
0.0022	0.1256	0.2604	305.5	23.15
0.0033	0.1035	0.2939	227.5	15.74
0.0044	0.0854	0.8549	114.5	6.17
0.0055	0.0832	0.8967	144.5	6.10

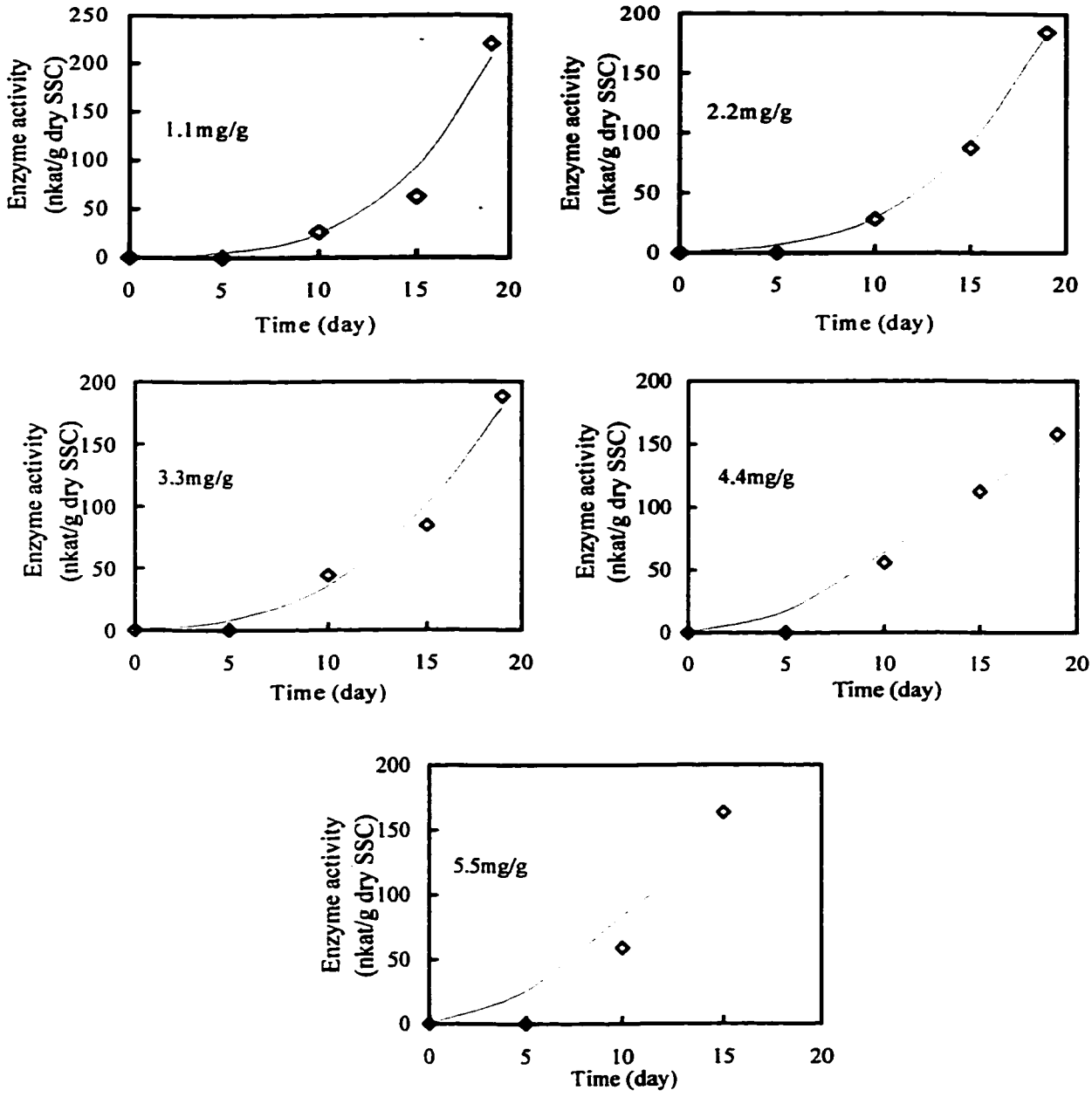


Figure 5.28 Modeling of enzyme production in SSC with various initial inoculum concentrations; symbols represent experimental data and lines are model predictions (equation 3.2-3).

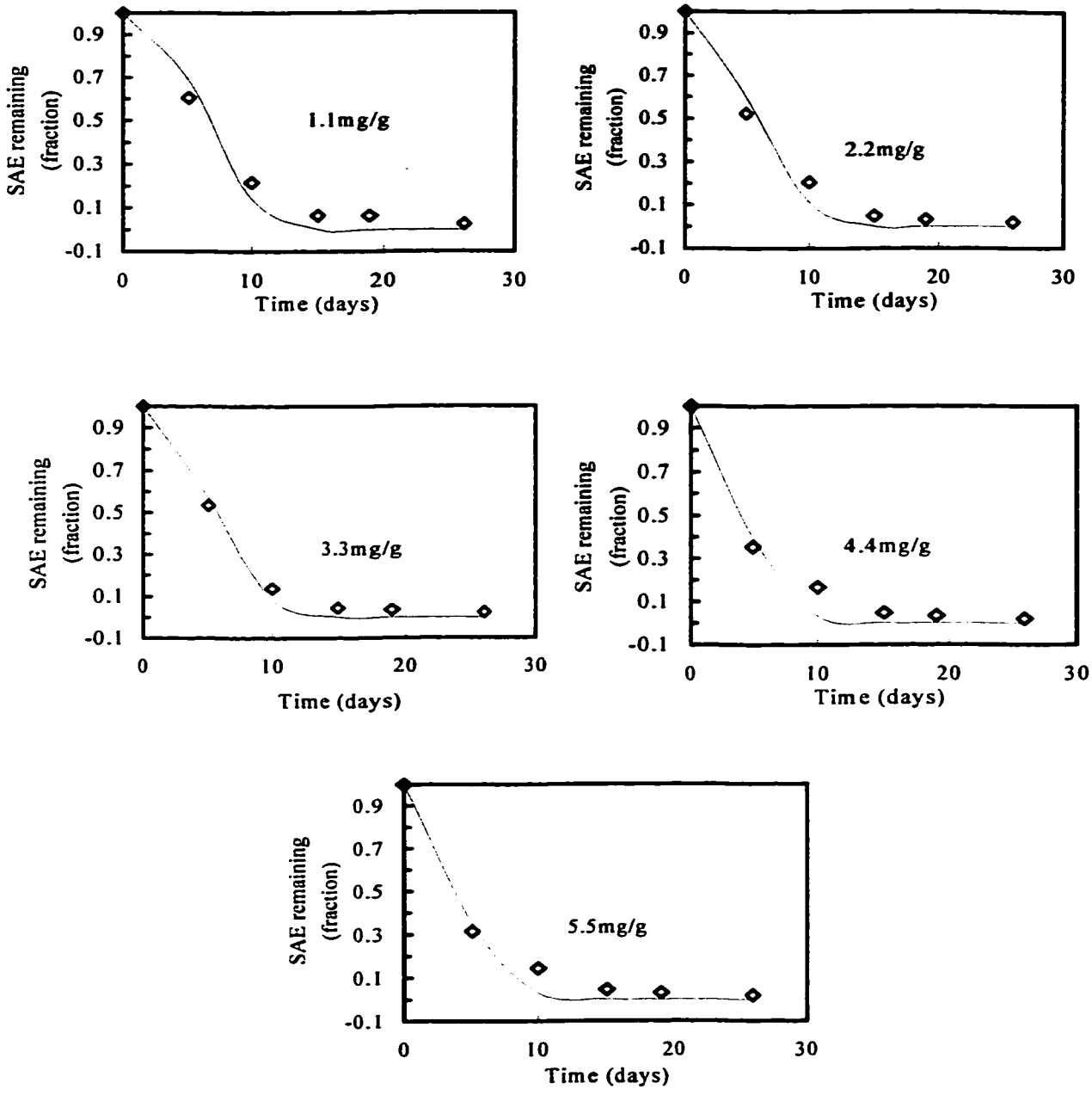


Figure 5.29 Modeling of SAE degradation in the SSC with various initial inoculum concentrations; symbols represent experimental data and lines represent model predictions (equation 3.3-4).

5.3.3 Effect of Homogenization Time

Inoculum prepared from liquid culture was in pellet form with different sizes, usually in the range of a few mm in diameter, but bigger pieces could also occur. In order to obtain a culture with uniformly dispersed biomass at the start of the fermentation, inoculum was homogenized prior to inoculation. It was assumed that longer homogenization time would result in smaller pieces of inoculum. Five processes were carried out with different homogenization time for inoculum preparation. The results are shown in Figures 5.30 to 5.32, with blending time ranging from 15 to 200 seconds.

It was found that an increase of time from 15 seconds to 30 seconds for homogenization helped the growth of biomass and enzyme production, probably due to a more uniformly dispersed biomass in the culture. Results also indicated that as the time increased, homogenization could have also damaged some of the fungal mycelia and resulted in a slower growth at the beginning of the fermentation (Figure 5.30). However, the trend of biomass production was reversed after day 12. The cultures inoculated with the longest time of inoculum homogenization had the highest biomass concentration. This was because smaller pieces of inoculum would give more growing points, and a larger amount of biomass could be in direct contact with the nutrients in CM.

The trend in enzyme production (Figure 5.31) was similar as for biomass. The highest enzyme activity was obtained in the culture with the most homogenized inoculum. Ebune et al. (1995) reported a similar trend in the effect of homogenization on enzyme production during SSF with *Aspergillus ficuum* in CM. They found that both the level and the rate of phytase production were lower in initial stages of SSF when

inoculum was homogenized for longer periods of time. However, after 36h of the process, an increase in phytase production was noticed with an increase in homogenization time.

The change of inoculum homogenization time did not have large influence on the rate of SAE degradation (Figure 5.32). This may indicate that enzyme produced at early stage was sufficient to catalyze the degradation rapidly.

Modeling of biomass using equation 3.1-6 is shown in Figure 5.33. Solid lines represent model equations obtained using data in the increasing phase only, while dotted lines are model predictions determined using all experimental data. It can be seen that this model is more suitable for the increasing phase of biomass growth.

Modeling of enzyme production using 3.2-3 is shown in Figure 5.34. Only the increasing phase was modeled, because the decreasing phase could not be fitted using this model. The model equation is not able to describe the enzyme production well. There could be experimental error in enzyme activities measured on day 10. The activity seemed to be low in all cultures sampled.

Modeling of SAE degradation using equation 3.3-4 is shown in Figure 5.35. The predicted results are well in agreement with the experimental data at early stages of the process. All parameters from these model equations, K_m, ν, K_v and K_s, are summarized in Table 5.7.

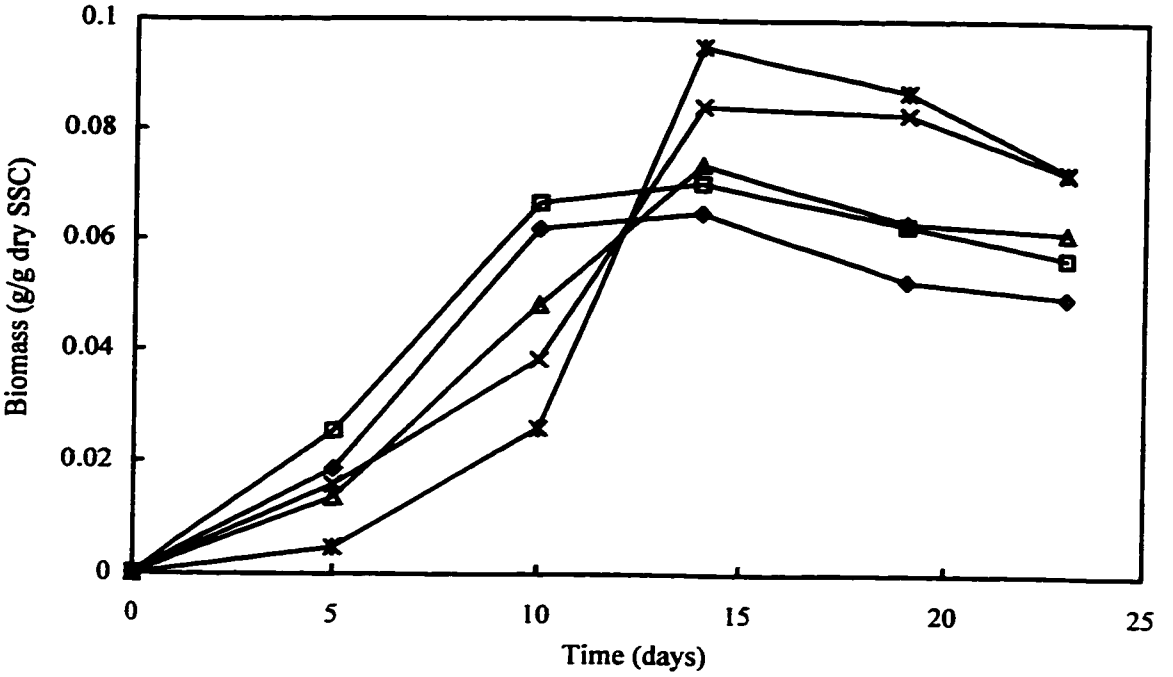
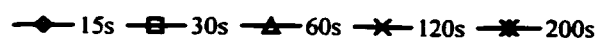


Figure 30. Effect of homogenization time of innoculum on biomass production during solid state process



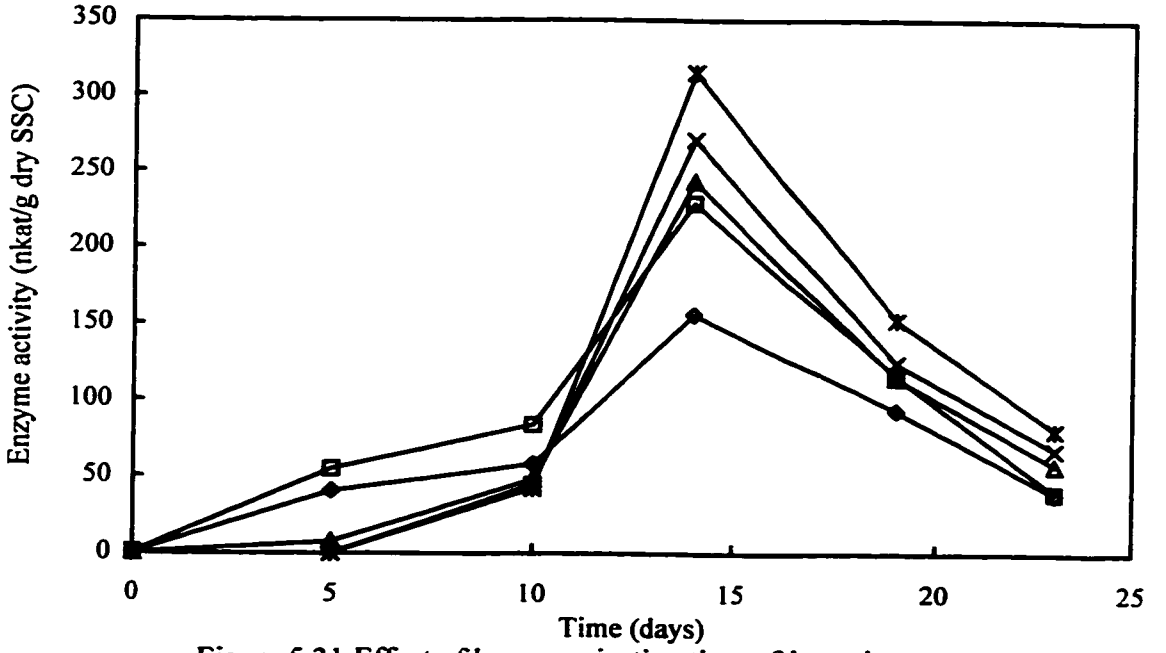
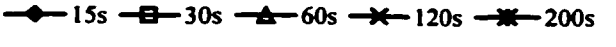


Figure 5.31 Effect of homogenization time of inoculum on enzyme production during solid state process



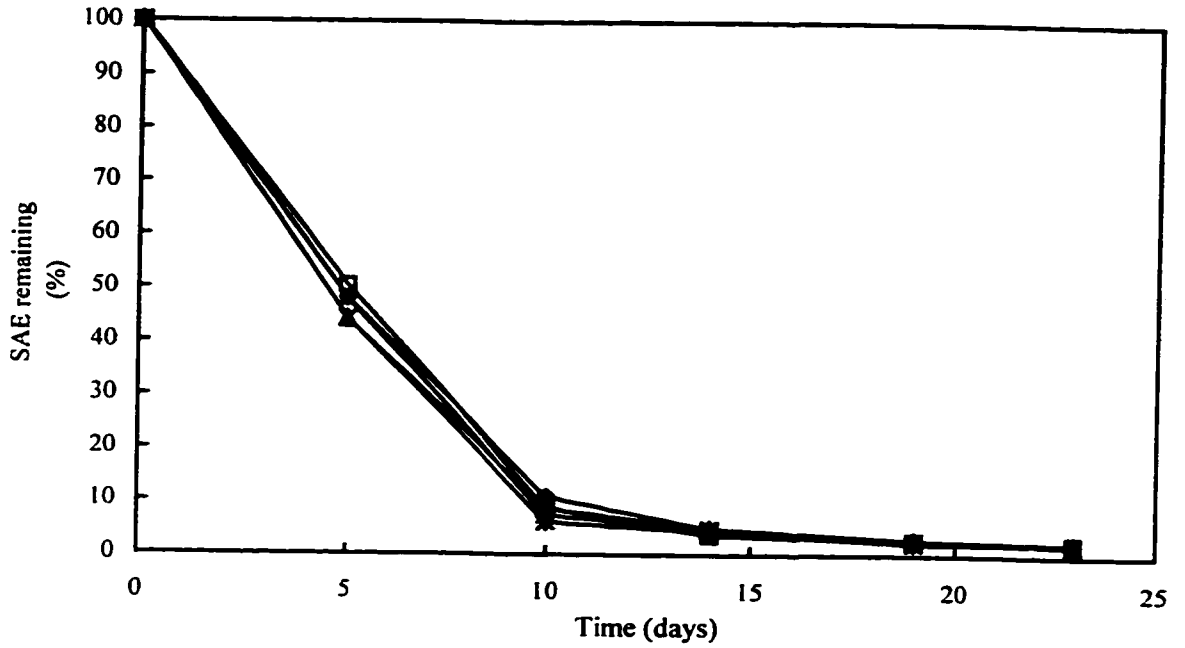


Figure 5.32 Effect of homogenization time of inoculum on SAE degradation during solid state process

Table 5.7 Parameters from modeling equation with different homogenization time.

Homogenization time (s)	X_m (g/g dry SSC)	μ_m (day^{-1})	K_v (nKat/g /day)	K_s (g dry SSC/g /day)
15	0.0580	0.5138	299.6	12.06
30	0.0650	0.5482	388.8	10.30
60	0.0668	0.4039	487.1	17.70
120	0.0824	0.3527	538.9	17.81
200	0.0877	0.3303	682.1	21.12

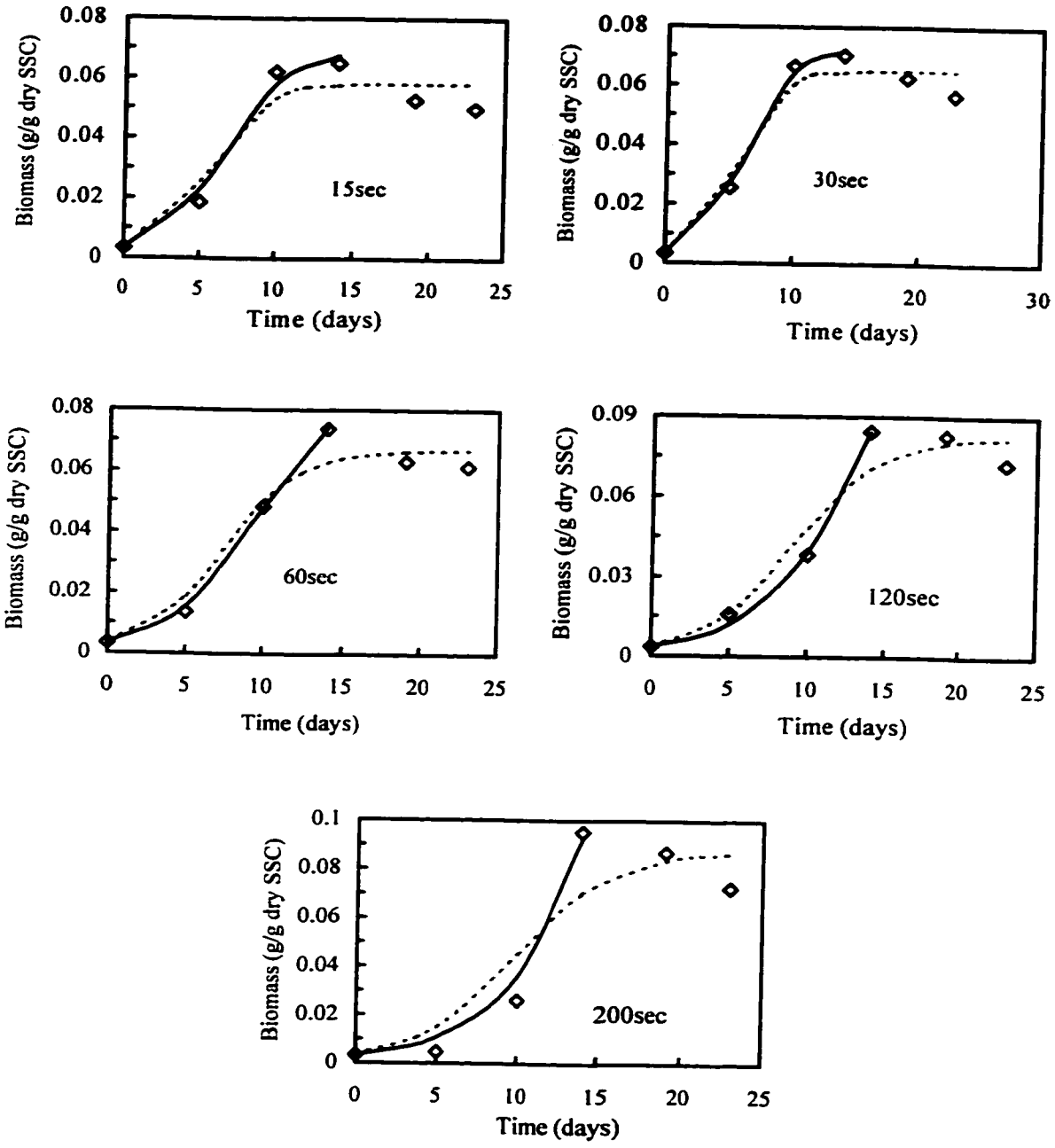


Figure 5.33 Modeling of biomass production in the SSC with various homogenization times of inoculum; symbols represent experimental data and lines represent model predictions (equation 3.1-6).

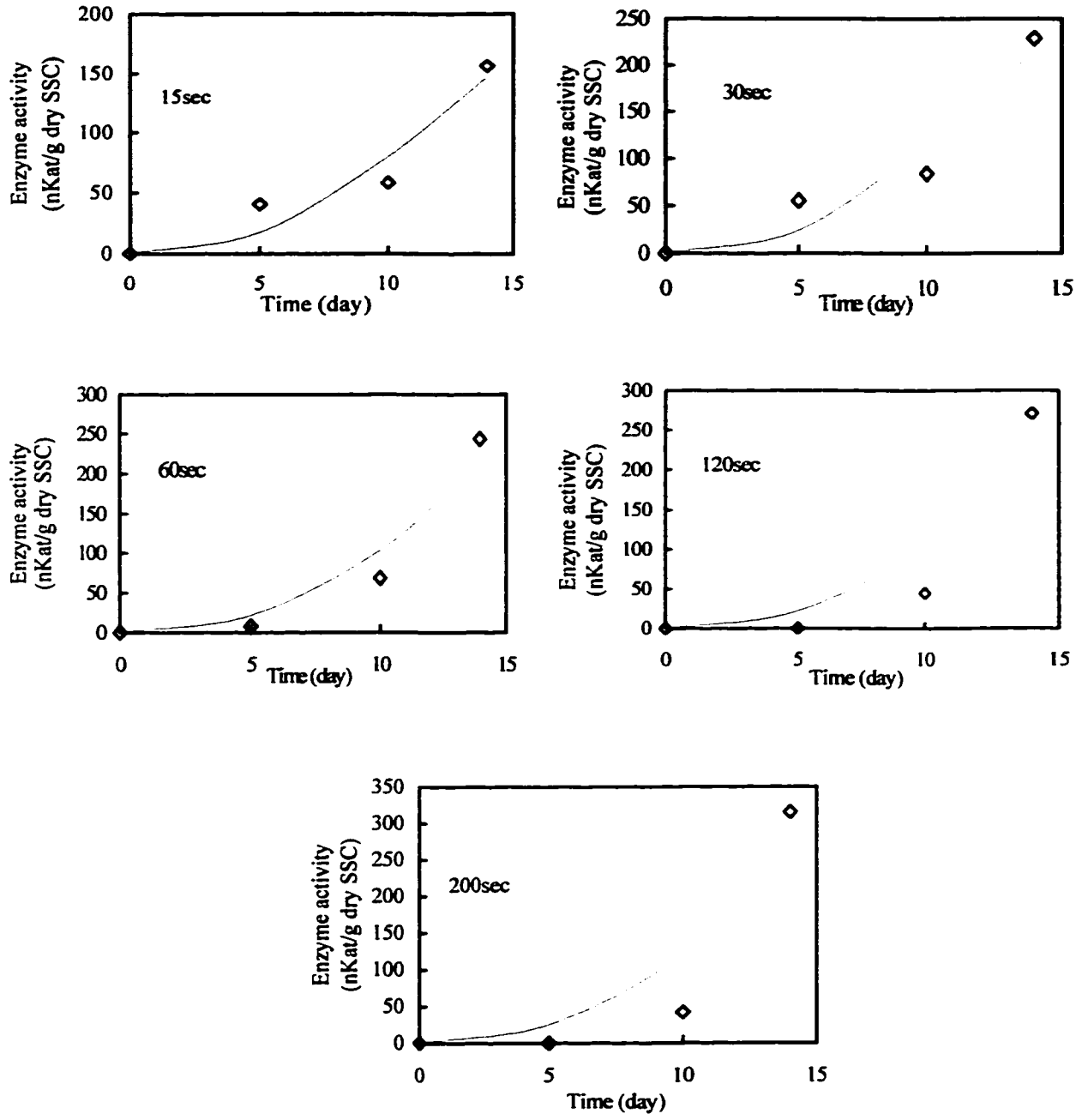


Figure 5.34. Modeling of enzyme production in the SSC with various homogenization times of inoculum; symbols represent experimental data and lines represent model predictions (equation 3.2-3).

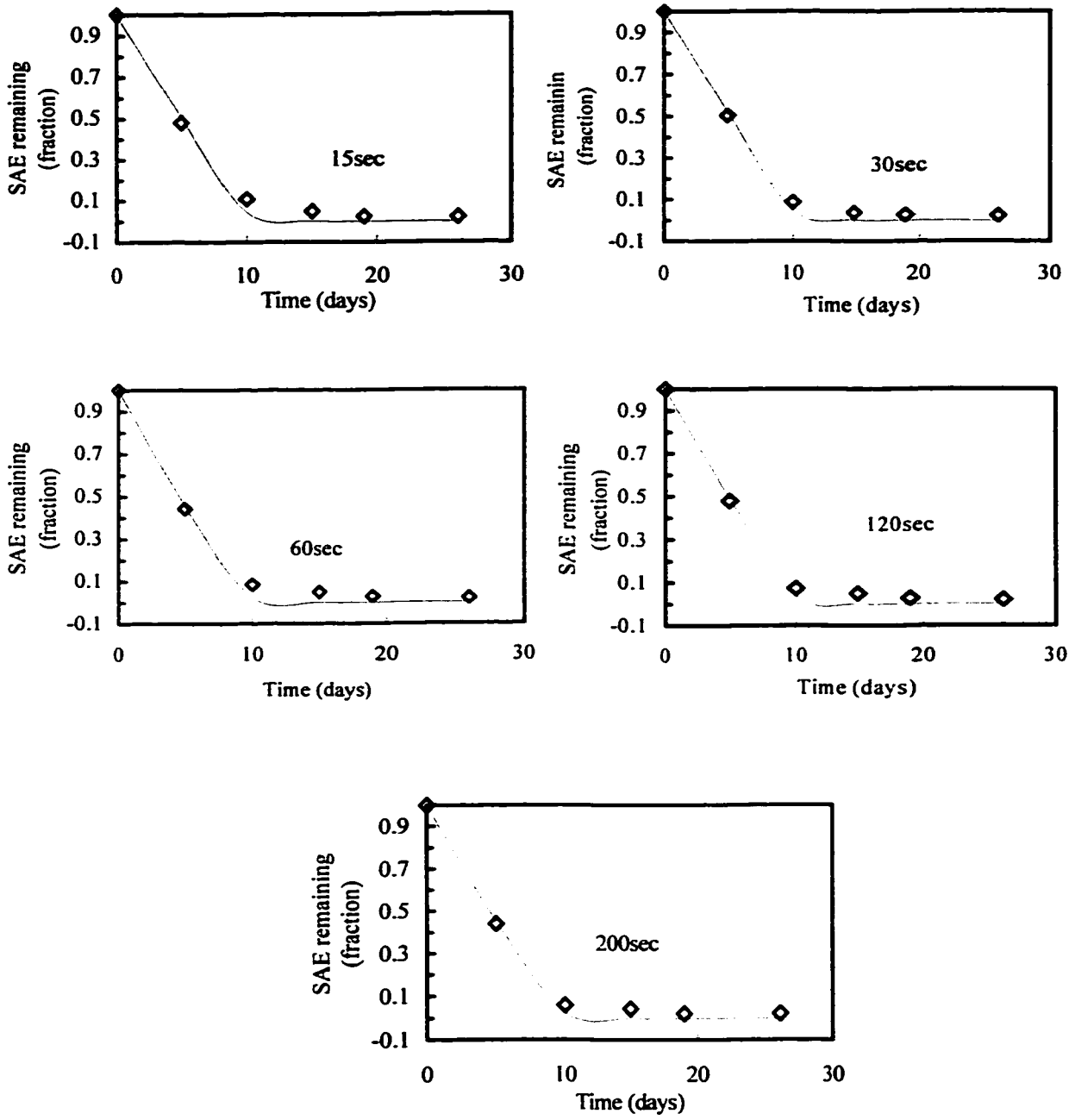


Figure 5.35. Modeling of SAE degradation in the SSC with various homogenization times of inoculum; symbols represent experimental data and lines represent model predictions (equation 3.3-4).

5.3.4 Effect of Particle Size of Canola Meal

Commercial canola meal consists of particles with different sizes. To study whether particle size has any effect on the growth of the fungus and production of enzyme, six tests were carried out. A certain amount of canola meal (500g) was screened using six meshes with different sizes and the fractions of meal with each range of size are displayed in Table 5.8.

Table 5.8 Mass fractions of canola meal in different sizes

Particle sizes	Mass fraction of meal
S1 < 0.18mm	0.091
0.18mm < S2 < 0.425mm	0.242
0.425mm < S3 < 0.710mm	0.240
0.710mm < S4 < 1.0mm	0.190
1.0mm < S5 < 1.4mm	0.182
S6 > 1.4mm	0.055

The experimental results of biomass growth are shown in Figure 5.36. From the biomass growth curves, it is clear that the growth was favored in CM with larger particle sizes. The highest biomass production was obtained in the fraction with the largest particle size (S6> 1.4mm) of CM tested. This could be due to better oxygen transfer through cultures with larger particles. Similar results were found by Sarikaya et al. (1997), when they tested the growth of *P. ostreatus* on three sizes of particles of rapeseed meal, where the biomass was assayed by measuring the diameter of mycelia growth. It was believed that larger interparticle spaces associated with the larger particle size material allowed better penetration of the mycelia as well as enhanced distribution concentration of oxygen.

However, Al-Asheh (1993) found that larger particle size of substrate could have negative effect on biomass growth when he tested the growth of *Aspergillus carbonarius* on CM. The best growth was found in particles with size 1.0 mm to 1.4 mm. A decrease in biomass production was noticed in canola meal with particles larger than 1.4 mm. He believed the reason was that with larger particles of substrate, total surface area decreased. This would counter balance the effect of better oxygen transfer. These two factors together would result in an optimum for the growth of microorganisms. The growth of *P. ostreatus* on CM did not indicate any decrease in biomass growth when particle size of substrate was larger than 1.4 mm. The growth was probably more influenced by oxygen transfer than any other factors in this process.

The highest maximum enzyme activity was found in the culture with the largest particles (Figure 5.37), in agreement with the biomass growth. The maximum activities occurred around day 15, when the highest biomass concentration was reached. The initial rate of SAE decrease was higher in the culture with the largest particle size tested (Figure 5.38), but the contents were at similar level after day 14. The rapid decrease in SAE content indicated that the enzyme produced was effective in degrading phenolic compounds even at small amounts at the beginning stage of the fermentation.

Modeling of biomass using equation 3.1-6 is shown in Figure 5.39. The parameters, X_m and μ_m, are summarized in Table 5.9. Some experimental errors were suspected, such as the data points on day 5 and 10 for S1 < 0.18mm cultures and the result on day 5 for S6>1.4mm culture. It can be seen that the model is suitable in fitting exponential and stationary phases of biomass growth. It was also noticed that the

stationary phase occurred sooner in the larger particle size of CM cultures compared to smaller particle size CM cultures.

Modeling of enzyme activity using equation 3.2-3 is shown in Figure 5.40. The parameter K_v is in Table 5.9. Only the increasing phase of the enzyme production was fitted with the logistic model. It can be seen that the equation is not able to fit the data well. Enzyme activities on day 10 are lower than predicted in all cultures except in culture with S1<0.18mm. This could be due to experimental error in enzyme measurements or the extraction of enzyme from solid cultures.

Modeling of SAE degradation is shown in Figure 5.41. The parameter K_s is given in Table 5.9. Figure 5.41 shows that the model fits the experimental results quite well.

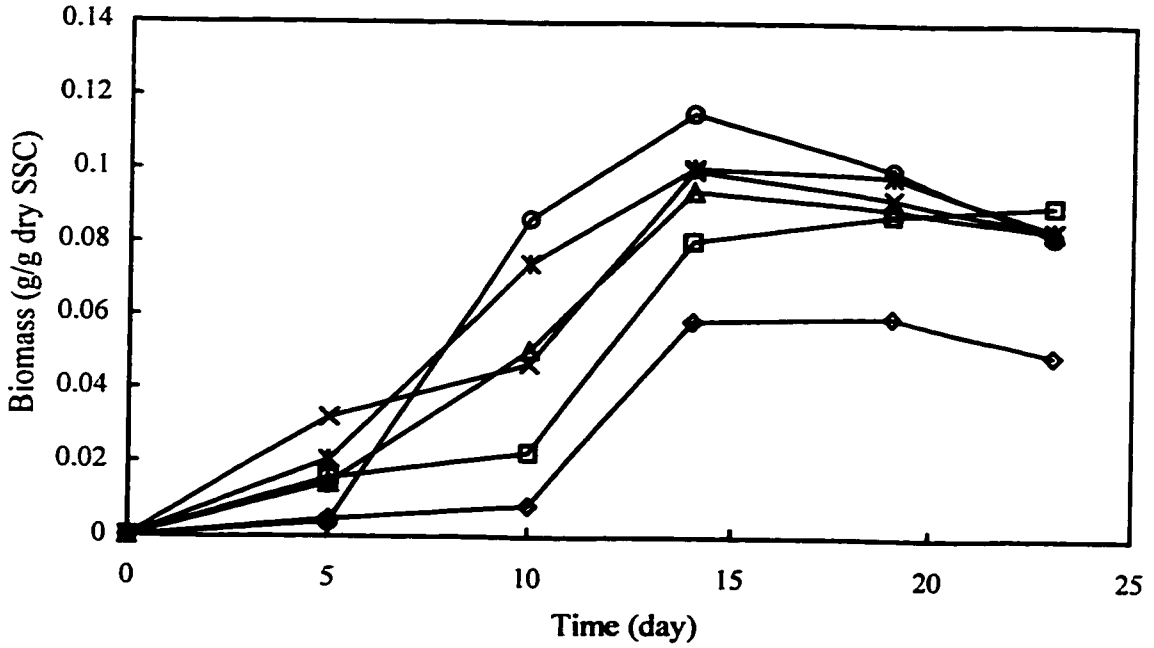
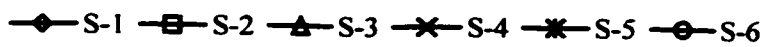


Figure 5.36 Effect of particle size of CM on biomass production during solid state process



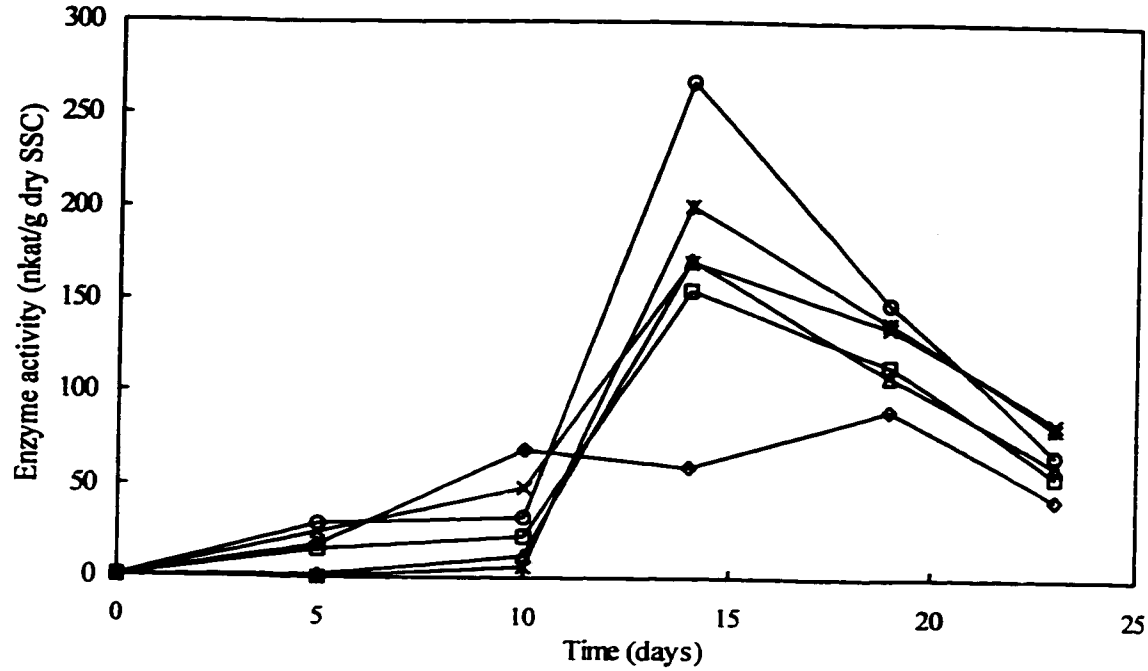
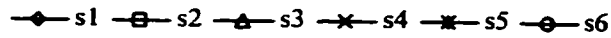


Figure 5.37 Effect of particle size of CM on enzyme production during solid state process



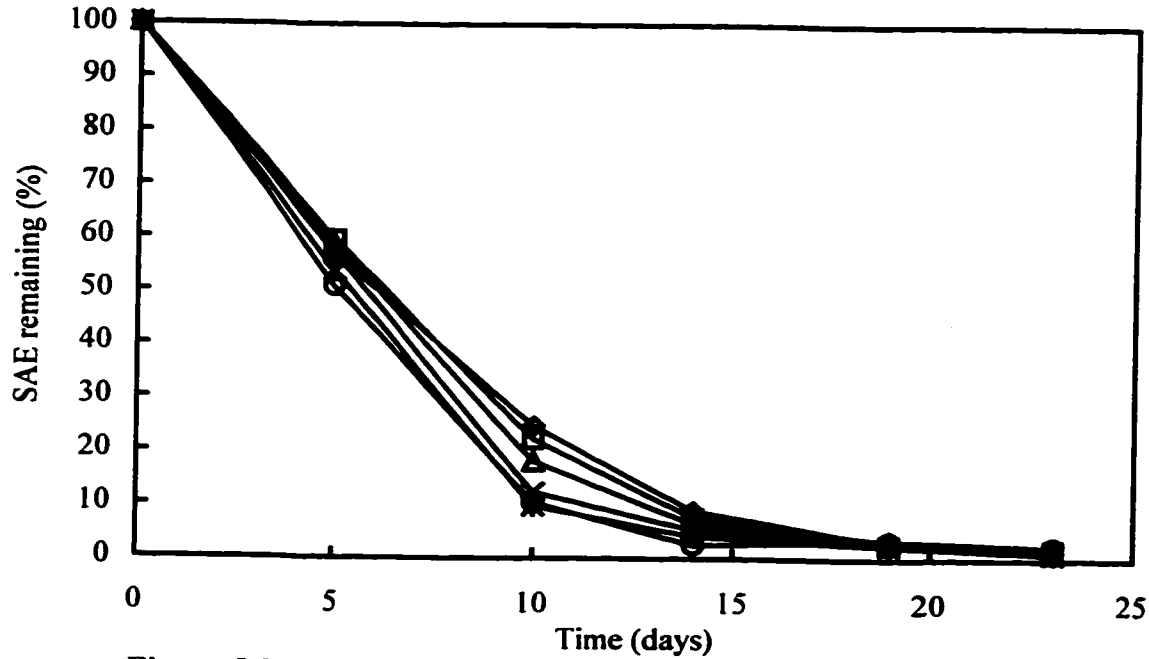


Figure 5.38 Effect of particle size of CM on SAE degradation during solid state process

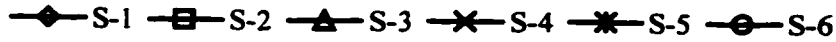


Table 5.9 Parameters from modeling equations at different canola meal size.

Canola meal size	X_m (g/g dry SSC)	μ_m (day^{-1})	K_v (nKat/g /day)	K_s (g dry SSC/g /day)
S1	0.0557	0.1609	404.0	21.18
S2	0.0511	0.2312	670.5	16.57
S3	0.0477	0.3019	516.6	13.79
S4	0.0494	0.3126	543.3	15.29
S5	0.0482	0.3766	482.6	15.28
S6	0.0541	0.3675	652.9	16.26

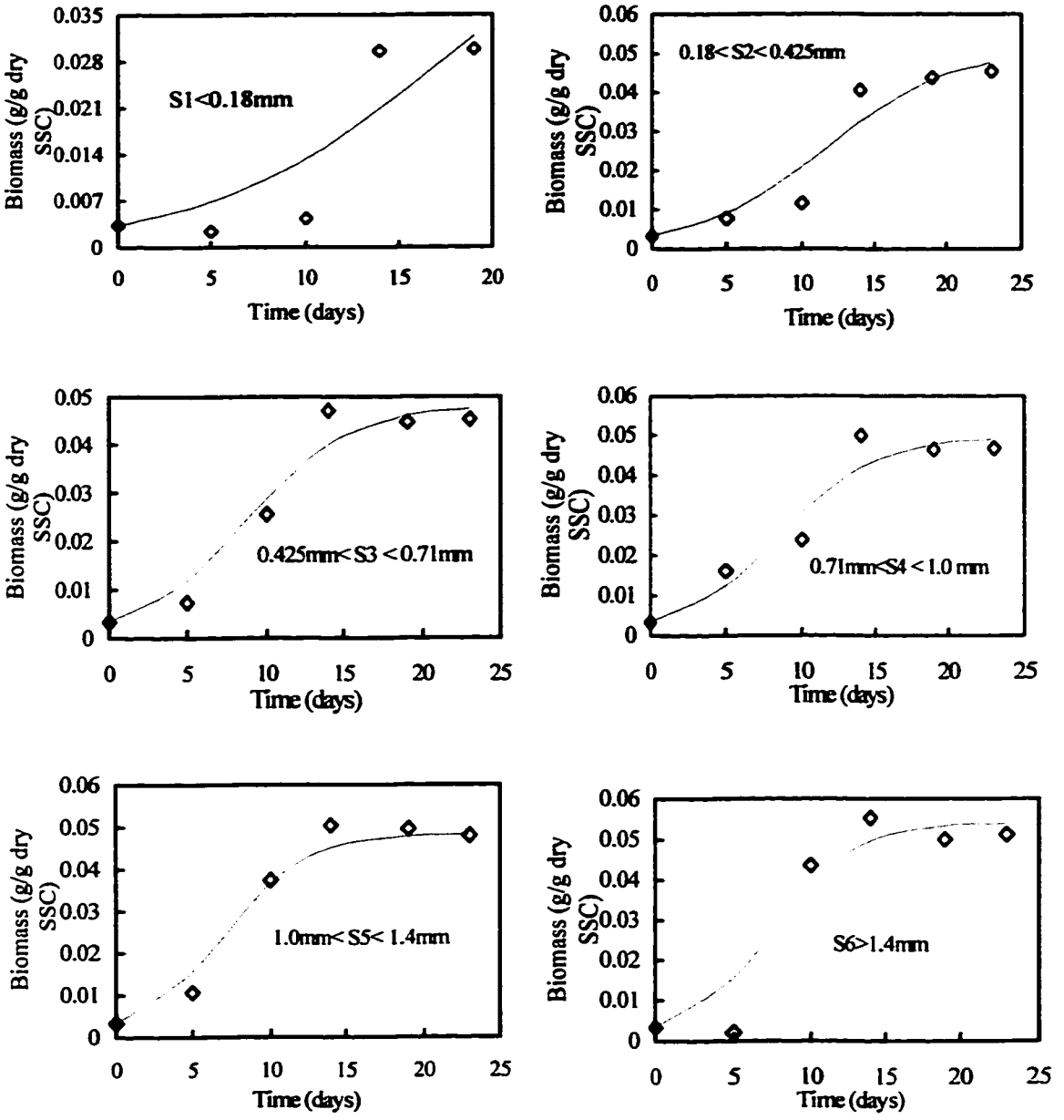


Figure 5.39 Modeling of biomass production in the SSC with various particle sizes of canola meal; symbols represent experimental data and lines represent model predictions (equation 3.1-6).

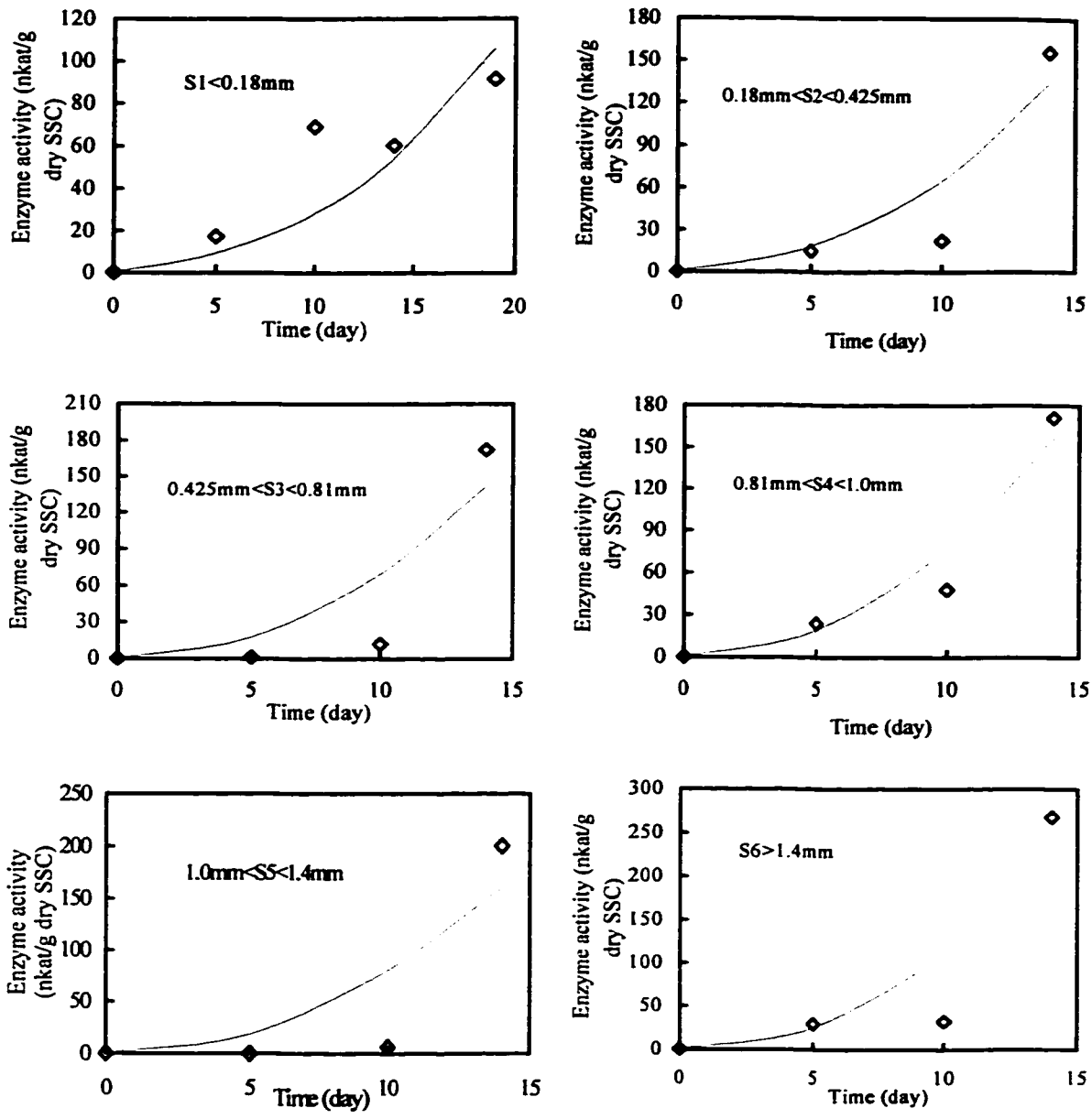


Figure 5.40 Modeling of enzyme production in the SSC with various particle sizes of canola meal; symbols represent experimental data and lines represent model predictions (equation 3.2-3).

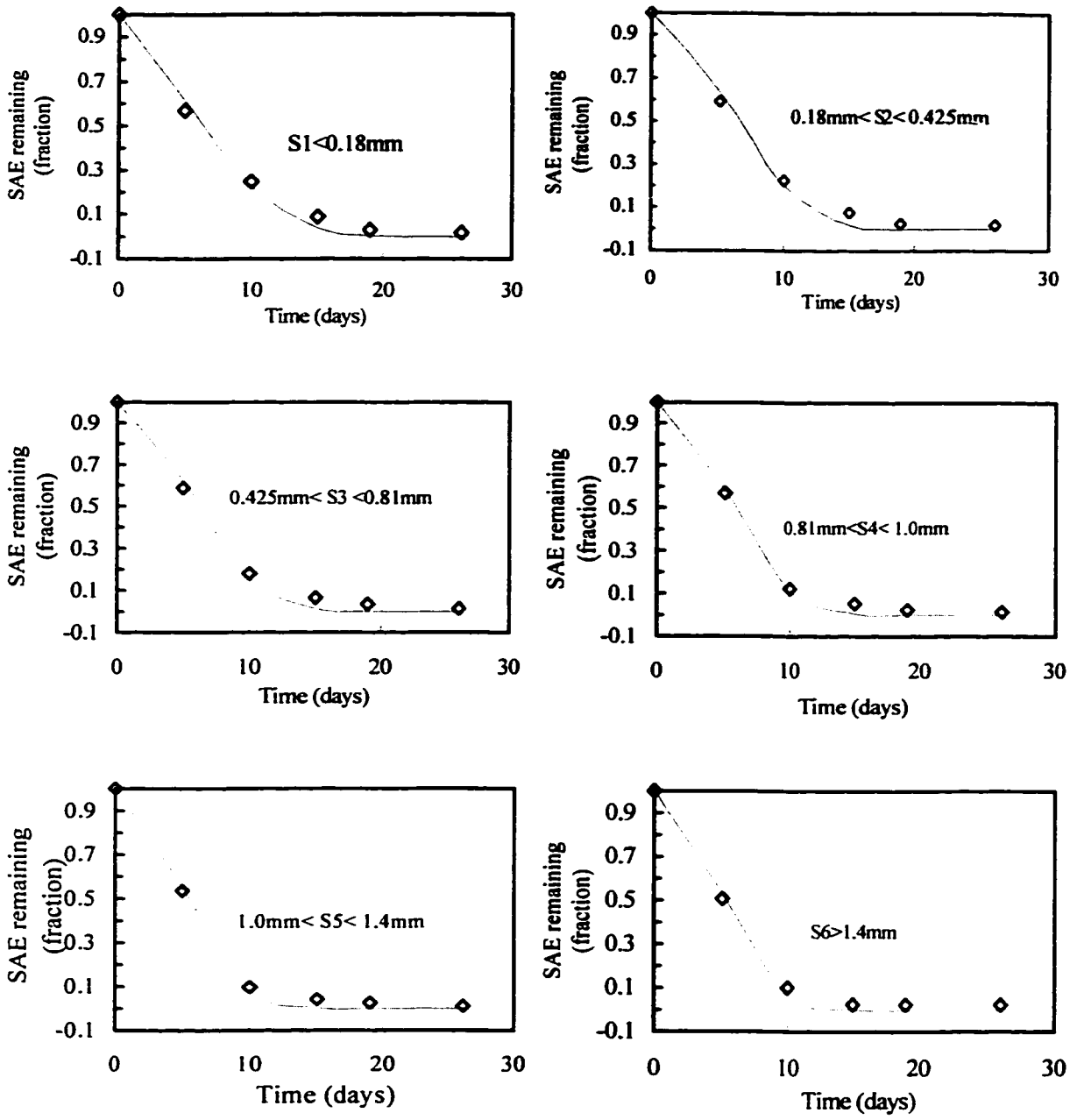


Figure 5.41 Modeling of SAE degradation in the SSC with various particle sizes of canola meal; symbols represent experimental data and lines represent model predictions (equation 3.3-4).

5.3.5 Effect of surfactants on SSF

Certain surfactants are known to affect enzyme production and the production of other fermentation products. Dombrovskaya and Kostyshin (1996) studied the effects of Tween 80 and Tween 40 on the ligninolytic enzyme complexes of white-rot fungi *Pleurotus floridae*, and found a 1.8-fold increase in laccase activity using both surfactants. Triton X-100 was reported (Lee et al., 1996) to increase enzymatic saccarification of cellulosic hydrolyzates during ethanol fermentation, but had negative effects on enzyme production. Al-Asheh and Duvnjak (1995) studied the effects of three surfactants, Tween 80, sodium oleate and Triton X-100, on solid state fermentation for the production of phytase. It was found that maximum enzyme formation occurred in the culture containing sodium oleate. Tween 80 caused increases in both biomass and enzyme production, but Triton X-100 had negative effects on both. Amtual et al. (1988) explained why some surfactants could affect the production of enzyme and other fermentation products. They stated that surfactant increased the permeability of the bacterial cell wall. This facilitated the release of enzyme into the medium and hindered the immobilization of the enzyme on the substrate by reducing the strength of adsorption.

Sodium oleate, Tween 80 and Triton X-100 (concentrations of 0.5% in medium) were tested for their effects on enzyme, biomass production, and SAE degradation in this study. The analyses were carried out after 15 days of fermentation. This was usually the time when the highest enzyme activity occurred in SSF using *P. ostreatus*. The results of enzyme production are shown in Figure 5.42. The highest enzyme activity increase was found in cultures containing sodium oleate (40% increase), followed by Triton X-100 (31% increase) and the least increase was with Tween 80 (7% increase) (Figure 5.42).

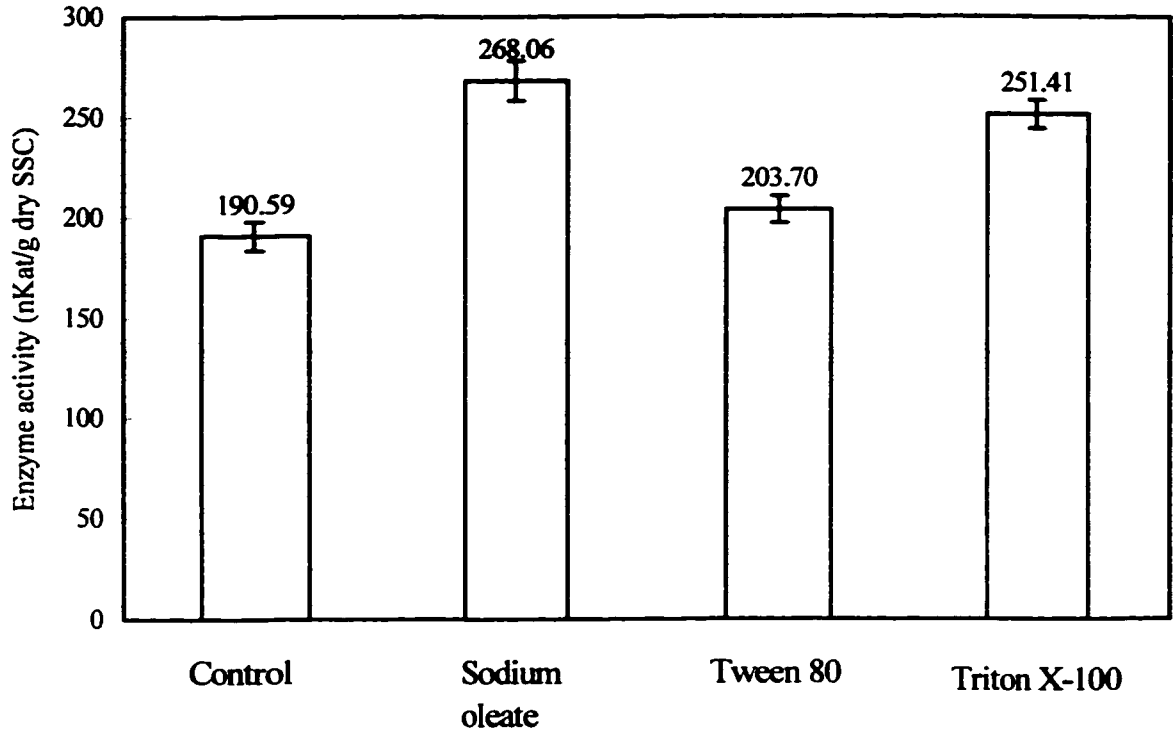


Figure 5.42 Effect of surfactants on enzyme production during solid state process

Similar trend was found in biomass production (Figure 5.43), the increase was also the highest when sodium oleate was added.

The SAE remaining after 15 days was the least in the cultures with sodium oleate (Figure 5.44). It was about 2% less than the SAE content in the control cultures. The SAE contents in the cultures with other surfactants were similar to that of the control.

To study the effect of concentration of surfactant on the fermentation, five media with concentrations of sodium oleate ranging from 0.1% to 2% were prepared. The results are shown in Figures 5.45 to 5.47. With the increase in sodium oleate concentration, the

biomass and enzyme productions also increased (Figure 5.45). At 0.5% concentration, a 23% increase in biomass was found, but higher concentration did not change biomass production. Enzyme activity seemed to be the highest with a concentration of 1% and more sodium oleate did not improve the production. Similar result was found by Al-Asheh and Duvnjak (1995) for the production of phytase during SSF process. An optimum sodium oleate concentration was found to be 1% for the enzyme production.

Faster SAE decrease was found with the increase in sodium oleate concentration. However, when there was 0.5% or more sodium oleate added, the SAE contents in the cultures after 15 days were similar (Figure 5.46). Therefore, when surfactants are used to promote enzyme production, an optimum concentration should be found to minimize the cost of the process.

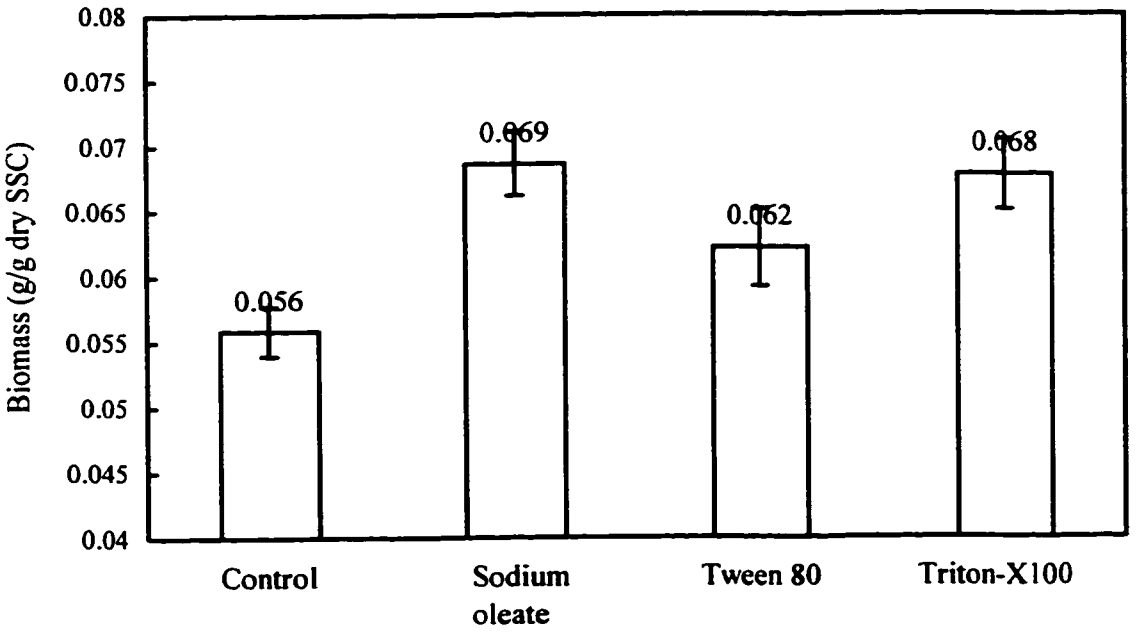


Figure 5.43 Effect of surfactants on biomass production during solid state process

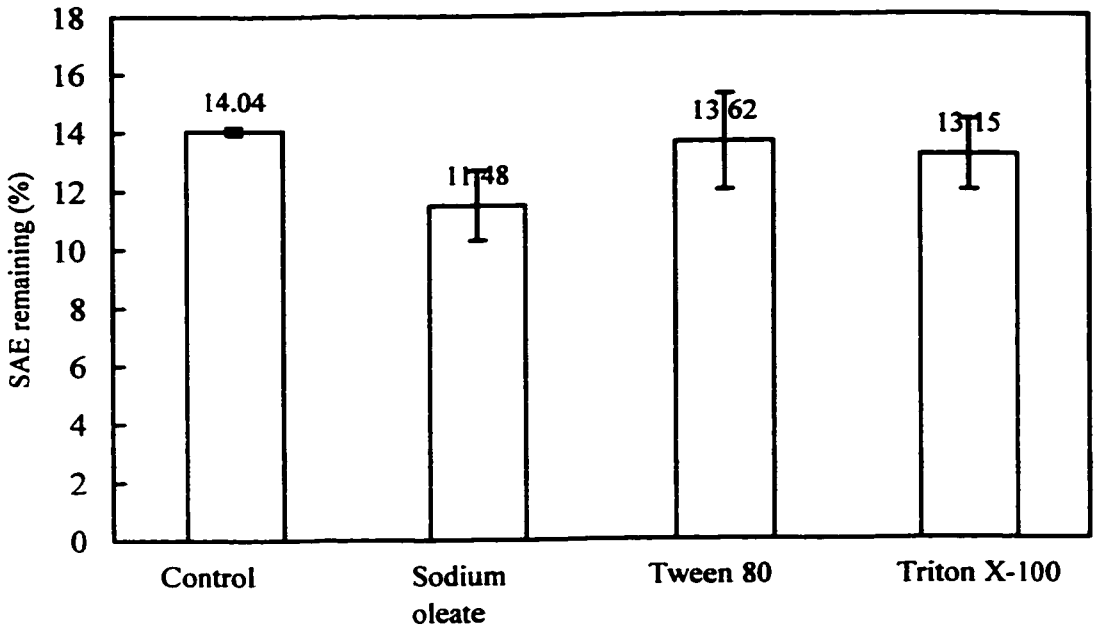


Figure 5.44 Effect of surfactants on SAE degradation during solid state process

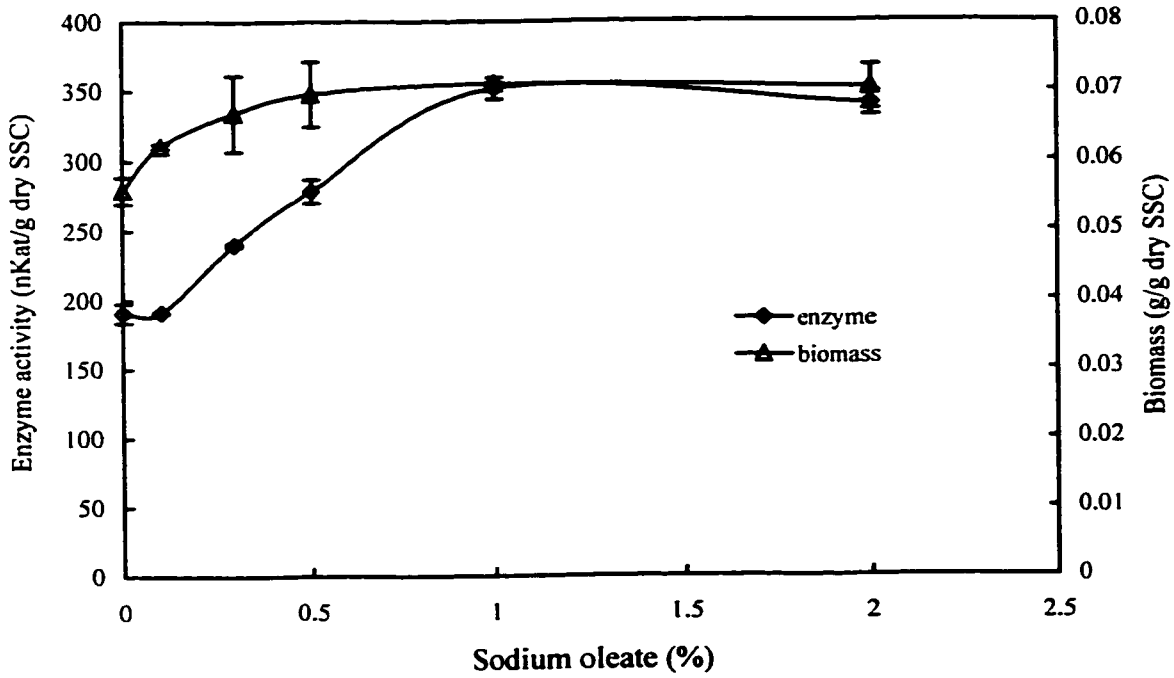


Figure 5.45 Effect of sodium oleate concentration on biomass and enzyme produciton during solid state process

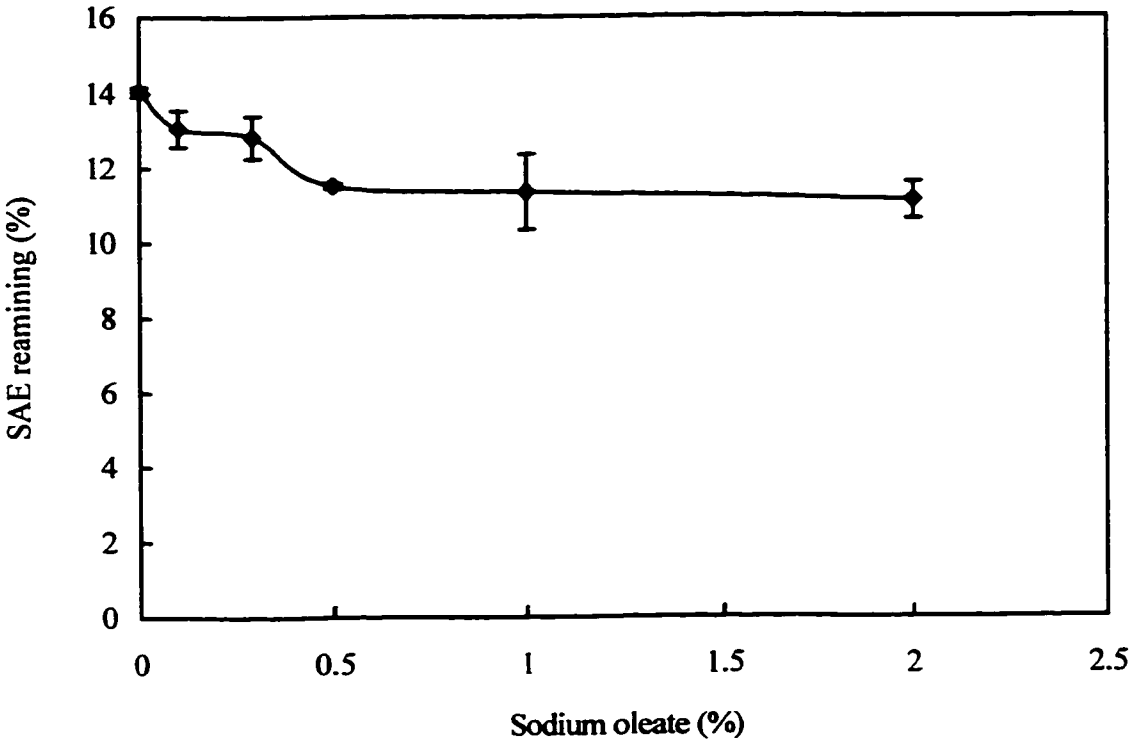


Figure 5.46 Effect of sodium oleate on SAE degradation in SSC during solid state process

Chapter 6

Conclusions

This section summarizes the findings from this research.

- The white-rot fungus *P. ostreatus* is able to produce extracellular enzymes during both submerged and solid state processes that degrade phenolic compounds.
- Glucose concentration in submerged fermentation culture affected both the growth of biomass and production of enzymes. A 4% initial glucose concentration was found to be the optimum for enzyme production, and the enzyme productivity was around 570nKat/g day.
- An increase in inoculum concentration for a submerged process seemed to have a positive effect on enzyme production during the first few days of the process. However, the maximum enzyme production was found in the culture with the lowest inoculum tested.
- High oxygen concentration could inhibit the production of enzyme in submerged cultures. An optimum medium volume was found in this study to be around 160 mL in a 500mL Erlenmeyer flask.
- Among the inducers tested, 2,5-xylidine was the most effective. It resulted in an increase in the maximum enzyme production about 1.8-folds. However, xylidine did not have an obvious effect on biomass production when compared to the control.
- When CM was used as a supplemental nutrient in the submerged process, enzyme production was increased. There was an optimum of CM concentration for the enzyme formation, at about 3%.

- Some characteristics of the enzymes were studied. It was found that enzyme was inhibited at high concentrations of all three substrates tested. The optimum pH values were 4.4, 5.0 and 4.7, using SA, SIN and SALD as the substrate, respectively. Enzyme activity was found to be at the maximum in the range of 313K to 333K, using any of the substrate.
- In studying the solid state process, the effect of moisture content in the SSC was investigated. It was found that the optimum for biomass production and enzyme formation was around 60%. However, the rate of degradation of phenolic compounds increased with the increase in moisture content.
- Small amount of inoculum resulted in a long lag phase of biomass growth during the solid state process. However, the rapid growth in later stage of the process increased the production of enzyme. The rate of SAE degradation was the fastest in the culture with the highest inoculum concentration.
- The increase in homogenization time of inoculum had positive effect on enzyme production. However, no significant difference was found in the rate of SAE degradation among the various homogenization times tested.
- Larger particle size of CM favored the growth of biomass and enzyme production, as well as SAE degradation in the SSC. The maximum biomass and enzyme concentrations were found in the SSC with the largest particle size of CM tested.
- Modeling has been attempted for biomass and enzyme production, as well as SAE degradation for the effects of operating parameters during solid state process. The modeling equations were based on a logistic law for biomass growth kinetics. The equation used to describe biomass production was not able to fit all of the

experimental data well. Better fitting was found when there was no or a short lag phase in the growth. Only the increasing phase of the enzyme production could be modeled based on this logistic law. Modeling of SAE degradation was reasonable, with better fitting at the beginning of the process.

- Three surfactants, Triton X-100, Tween 80 and sodium oleate, were tested for their effects on enzyme production and SAE degradation. Addition of sodium oleate resulted in the highest increase in enzyme production, at about 40%. A concentration of around 1% sodium oleate was optimum for enzyme production and SAE degradation.
- In general, submerged process was easier to carry out and to be monitored compared to solid state process. On the other hand, the use of CM as a nutrient source during solids state process helped the production of enzymes, and more importantly, phenolics in CM could be degraded during the process without other procedures. The degradation of SAE was found to be significant, and a 95% reduction in SAE content was measured in most of the SSC after 15 days of the process.

Chapter 7

Recommendations

1. Further study can be done to improve enzyme production in submerged cultures by optimizing other operating parameters, such as temperature, pH, size and shape of flasks.
2. Difficulties in sample analyses for solid state cultures could have contributed to experimental errors in this study. Other methods for biomass concentration analysis can be tested for a simpler procedure and more accurate results. A less time consuming method would also allow replicated process to be carried out for comparison.
3. Tests can also be carried out to see how much enzyme can be bound to the particle surface of the solid substrate, or CM.
4. The degradation of phenolic compounds during the solid state process was effective, but the process was long. It would be interesting to see how fast the degradation would be if enzyme preparation, from either submerged or solid state cultures, was added to CM.
5. The ability to produce enzymes by different strains of *P. ostreatus* might be very different. Screening for a better strain for enzyme production might be valuable for further studies.
6. In this study, research was carried out in lab scale. Further studies are necessary if the process would be developed for larger scale applications.
7. Finally, although the fungus *P. ostreatus* was found to be effective in degrading phenolic compounds with the enzymes it produces, the end-products of enzymatic

treatments are still to be examined. Whether this enzymatic treatment has improved the nutritional values of CM, and if it has any negative effects on the animals when treated CM is used in the feeds, can be interesting topics for further research and studies.

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Appendix A

Calibration Curves

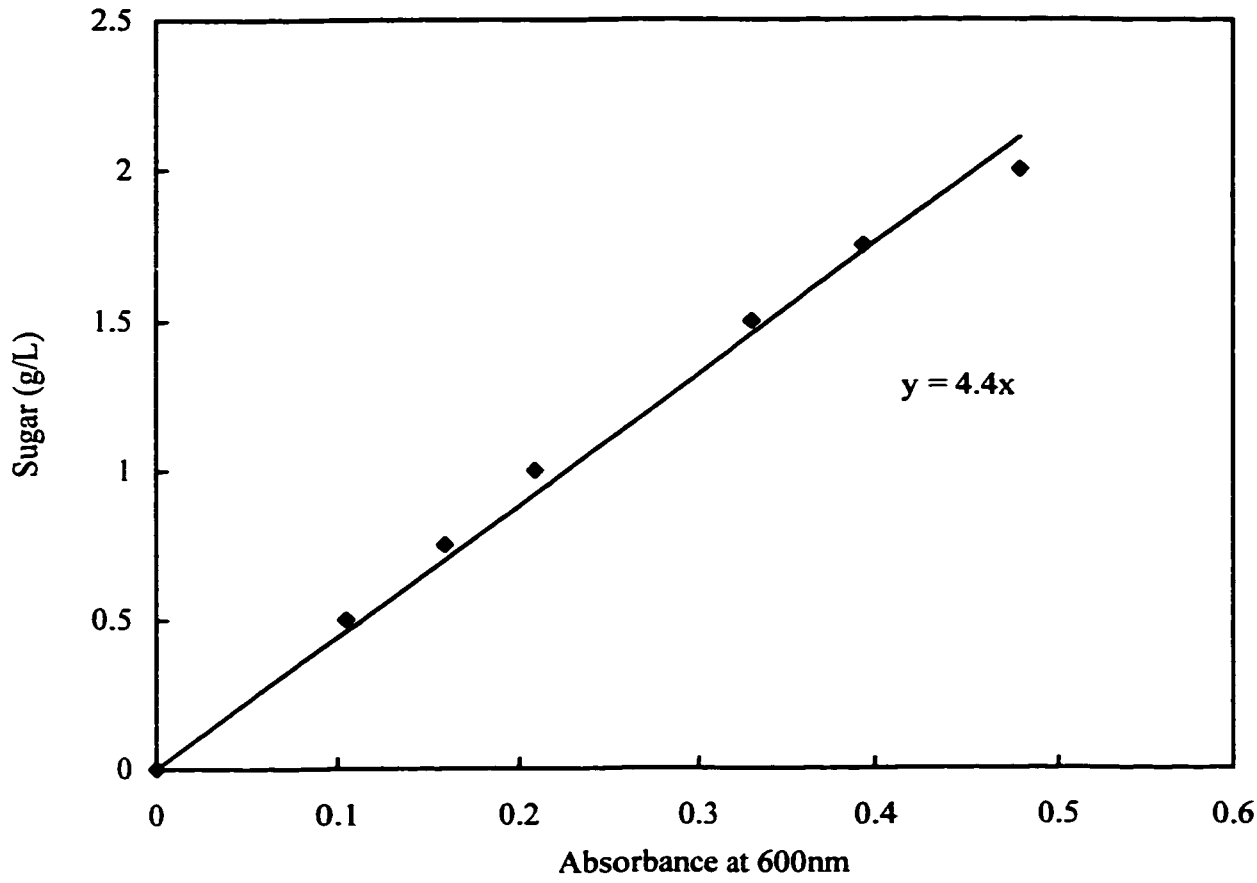

Figure A.1 Calibration curve for the analysis of reducing sugar concentration

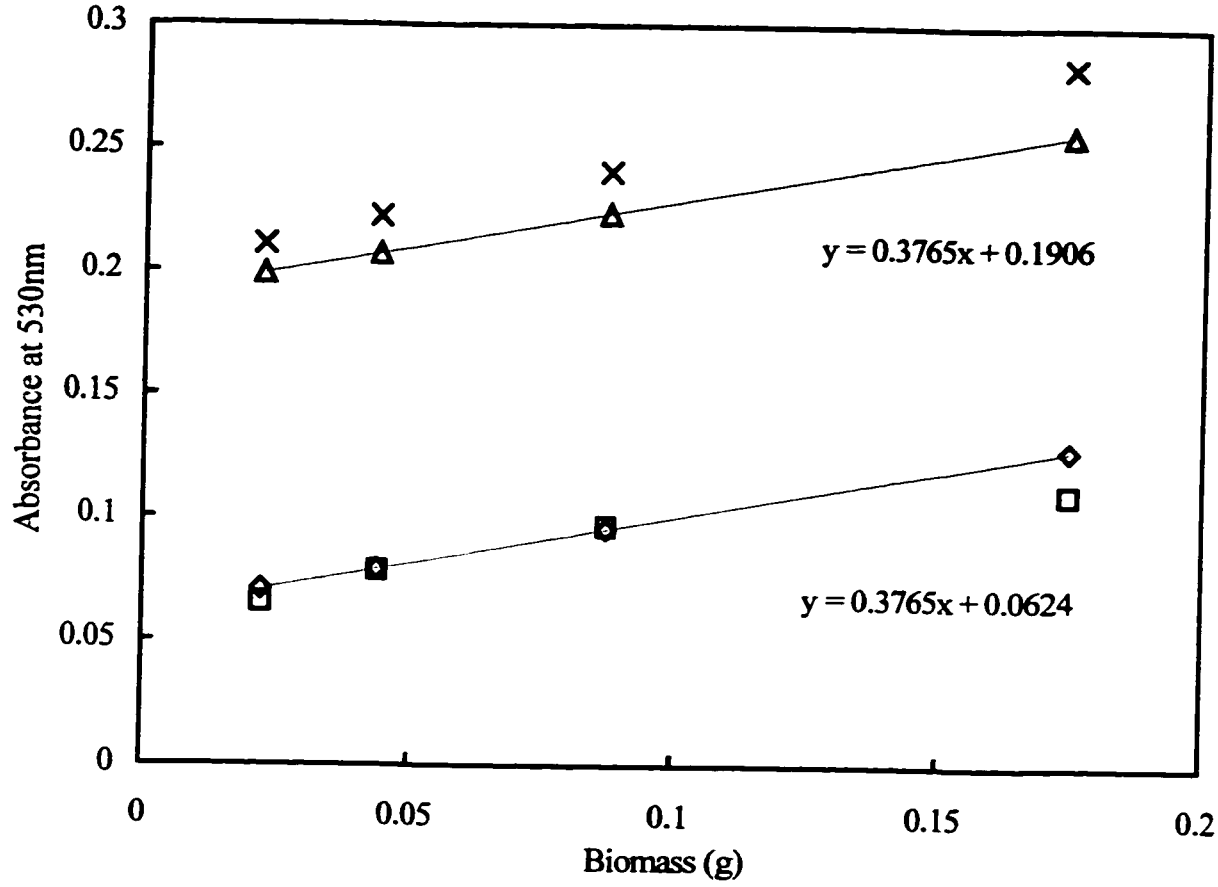

Figure A.2 Calibration curve for the analysis of biomass concentration in solid culture

◇ 0.5gCM+biomass, theo. □ 0.5gCM+biomass, exp.
△ 1.0gCM+biomass, theo. × 1.0gCM+biomass, exp.
—— Linear (0.5gCM+biomass, theo.) —— Linear (1.0gCM+biomass, theo.)

Calculations for biomass concentration:

$$Biomass = \frac{Absorbance - Y_{cm}}{0.3765}$$

$$Y_{cm} = \frac{X - 0.5}{0.5}(0.1906 - 0.0624)$$

where X is the weight of dry solid sample determined using moisture analysis.

Appendix B

Experimental Data

Table B.1 Effect of initial glucose concentration on biomass production during submerged process

Initial glucose concentration	Time (day)					
	0	3	5	7	9	12
	Biomass (g/mL)					
1%	0.0026	0.0030	0.0112	0.0094	0.0070	0.0062
2%	0.0026	0.0035	0.0109	0.0139	0.0119	0.0101
4%	0.0026	0.0027	0.0101	0.0173	0.0162	0.0156
6%	0.0026	0.0028	0.0103	0.0169	0.0186	0.0167

Table B.2 Effect of initial glucose concentration on glucose consumption during submerged process

Initial glucose concentration	Time (day)					
	0	3	5	7	9	12
	Glucose (g/L)					
1%	10.00	5.70	0.15	0.14	0.06	0.04
2%	20.00	11.06	6.28	0.92	0.12	0.07
4%	40.00	22.61	14.34	8.07	2.57	0.52
6%	60.00	31.95	18.28	12.76	7.31	2.11

Table B.3 Effect of initial glucose concentration on enzyme production during submerged process (syringaldazine as substrate)

Initial glucose concentration	Time (day)					
	0	3	5	7	9	12
	Enzyme activity (nKat/mL)					
1%	0.00	0.81	1.97	35.08	46.49	40.97
2%	0.00	0.89	0.41	35.42	66.53	64.34
4%	0.00	0.50	2.53	26.12	88.59	86.42
6%	0.00	0.45	0.40	25.50	57.50	52.45

Table B.4 Effect of initial glucose concentration on enzyme production during submerged process (ferulic acid as substrate)

Initial glucose concentration	Time (day)					
	0	3	5	7	9	12
	Enzyme activity (nKat/mL)					
1%	0	0.0366333	0.6960333	6.7771667	9.06675	8.1509167
2%	0	0.05495	0.1465333	7.1618167	11.411283	10.5395
4%	0	0.0732667	0.8425667	4.7108667	13.206317	12.5286
6%	0	0.05495	0.0366333	3.9891	11.502867	10.6603

Table B.5 Effect of inoculum concentrationon enzyme production during submerged process (syringaldazine as substrate)

Inoculum	Time (day)					
concentration	0	3	6	10	13	16
(mg/mL)	Enzyme activity (nKat/mL)					
1.5	0.50	3.28	40.46	50.20	32.15	14.93
3	0.61	24.09	66.78	81.87	66.41	42.41
6	1.04	34.28	62.68	75.15	58.79	42.71
	Error (nKat/mL)					
1.5	0.04	0.24	1.03	1.25	1.32	0.80
3	0.11	1.31	1.62	0.97	0.73	1.35
6	0.15	1.81	1.68	1.21	1.42	2.09

Table B.6 Effect of inoculum concentration on glucose consumption during submerged process

Inoculum	Time (day)					
concentration	0	3	6	10	13	16
(mg/mL)	Glucose (g/L)					
1.5	40.41	35.31	27.48	18.81	14.28	12.79
3	40.47	31.15	25.71	15.47	10.01	1.64
6	39.09	30.42	25.26	16.85	9.36	4.14
	Error (g/L)					
1.5	0.66	0.96	0.87	0.07	1.39	1.22
3	0.97	1.89	0.77	0.16	1.85	0.55
6	0.48	0.10	0.04	0.80	1.41	0.08

Table B.7 Effect of oxygen concentrationon on enzyme production during submerged process (syringaldazine as substrate)

Medium	Time (day)					
volume	0	3	6	9	12	16
(mL/500mL)	Enzyme activity (nKat/mL)					
80	0.23	1.34	15.68	26.32	11.63	9.89
160	0.19	1.73	18.78	54.24	69.52	42.74
240	0.19	1.00	16.05	42.04	53.65	37.61
	Error (nKat/mL)					
80	0.01	0.21	0.14	0.03	0.98	0.48
160	0.02	0.03	0.17	0.10	0.72	0.34
240	0.03	0.09	0.03	0.35	1.27	1.08

Table B.8 Effect of oxygen concentration on glucose consumption during submerged process

Medium	Time (day)					
volume	0	3	6	9	12	16
(mL/500mL)	Glucose (g/L)					
80	37.74	33.72	28.79	19.37	12.02	1.10
160	38.49	32.61	25.41	13.03	4.44	0.27
240	38.71	32.37	26.25	15.62	10.08	0.84
	Error (g/L)					
80	0.37	2.66	0.02	0.52	0.46	0.75
160	0.11	0.04	2.09	0.01	0.28	0.01
240	0.24	0.05	0.76	0.33	1.67	0.36

Table B.9 Effect of inducers on enzyme production during submerged process (syringaldazine as substrate)

Inducers	Time (h)					
	20	44	68	92	116	156
	Enzyme activity (nKat/mL)					
control	0.35	1.24	5.51	15.80	23.65	66.34
xylidine	0.25	2.54	37.21	90.64	136.72	159.09
caffeic acid	0.39	1.38	5.35	14.28	24.56	73.01
sinapic acid	0.30	1.76	6.22	29.20	38.29	82.78
sinapine	0.42	2.63	6.99	32.82	52.88	88.24
syringaldazine	0.39	1.36	5.26	23.38	31.90	78.93

180	228	336	420
Enzyme activity (nKat/mL)			
81.08	90.38	94.90	81.69
165.93	170.46	161.35	147.36
87.90	95.50	99.23	86.88
94.91	116.66	126.00	117.11
107.92	123.30	130.04	116.59
91.52	110.11	109.34	96.06

Table B.10 Effect of inducers on glucose consumption during submerged process

Inducers	Time (h)					
	20	44	68	92	116	156
	Glucose (g/L)					
control	38.28	36.81	34.74	30.88	28.06	24.10
xylidine	38.28	36.54	30.14	25.67	20.66	15.18
caffeic acid	38.36	36.79	34.00	30.66	26.98	22.61
sinapic acid	38.32	37.10	33.53	28.53	25.84	20.47
sinapine	38.46	36.58	32.27	27.20	24.22	19.16
syringaldazine	38.39	37.14	32.45	28.99	26.39	21.19

180	228	336	420
Glucose (g/L)			
22.40	17.19	2.11	0.60
11.08	5.77	0.62	0.61
19.21	15.53	2.07	0.68
18.80	13.00	1.70	0.37
15.99	8.93	1.54	0.82
18.77	13.87	1.01	0.46

Table B.11 Effect of inducers on biomass production during submerged process

Inducers	sample 1	sample 2	average	error
	Biomass (g)			
control	0.32	0.33	0.32	0.01
xylidine	0.33	0.33	0.33	0.00
caffeic acid	0.41	0.35	0.38	0.03
sinapic acid	0.35	0.42	0.39	0.04
sinapine	0.32	0.37	0.34	0.03
syringaldazine	0.32	0.43	0.37	0.05

Table B.12 Effect of CM on enzyme production during submbered process (syringaldazine as substrate)

CM	Time (day)					
concentration	0	3	6	9	12	16
	Enzyme activity (nKat/mL)					
0.0%	0	0.47	17.56	76.00	65.97	37.11
0.5%	0	2.28	9.17	66.35	75.56	55.36
1.0%	0	0.26	10.77	75.77	85.57	61.09
2.0%	0	0.16	10.63	79.59	92.02	66.00
3.0%	0	0.00	9.69	85.48	123.87	95.30
4.0%	0	0.00	9.78	83.23	90.86	61.35
	Error (nKat/mL)					
0.0%	0	0.01	1.64	0.37	2.66	0.62
0.5%	0	0.07	1.01	4.47	1.06	4.46
1.0%	0	0.00	0.15	0.69	2.41	0.23
2.0%	0	0.01	1.13	3.28	0.77	3.60
3.0%	0	0.00	1.08	1.63	0.48	1.84
4.0%	0	0.00	0.78	3.13	1.35	0.43

Table B.13 Effect of CM on reducing sugar concentration during submerged process

CM	Time (day)					
concentration	0	3	6	9	12	16
	Glucose (g/L)					
0.0%	18.83	13.37	4.58	1.38	0.79	0.61
0.5%	19.91	21.00	24.30	7.60	2.48	0.89
1.0%	18.93	23.51	24.09	8.44	2.83	1.10
2.0%	19.01	23.50	24.69	8.37	3.07	1.49
3.0%	19.00	25.14	25.46	9.13	2.60	2.20
4.0%	18.05	25.35	22.32	8.18	3.09	1.53
	Error (g/L)					
0.0%	0	0.21	0.12	0.10	0.03	0.00
0.5%	0	0.07	1.00	0.04	0.16	0.02
1.0%	0	2.09	1.44	0.02	0.67	0.21
2.0%	0	0.73	0.35	0.58	0.11	0.09
3.0%	0	0.16	0.01	0.09	0.67	0.01
4.0%	0	1.17	0.74	0.81	0.03	0.04

Table B.14 Effect of CM on hydrolyzable carbohydrates concentration during submerged process

CM	Time (day)					
concentration	0	3	6	9	12	16
	Carbohydrates (g/L)					
0.0%	18.86	12.96	4.39	1.39	0.82	0.58
0.5%	19.40	12.61	11.55	9.61	7.22	2.60
1.0%	21.17	16.28	13.97	8.00	8.08	2.47
2.0%	21.60	15.66	12.39	7.97	7.20	2.40
3.0%	22.21	14.50	13.32	6.58	6.47	2.40
4.0%	26.51	17.13	14.66	7.43	7.08	2.53
	Error (g/L)					
0.0%	0.00	0.36	0.18	0.04	0.12	0.01
0.5%	0.00	0.93	0.26	0.60	0.51	0.01
1.0%	0.00	4.10	3.13	0.37	0.72	0.03
2.0%	0.00	1.65	1.05	0.33	1.21	0.34
3.0%	0.00	0.59	0.22	0.00	0.08	0.23
4.0%	0.00	0.32	0.23	0.09	0.22	0.11

Table B.15 Effect of substrate concentration on enzyme activity (sinapic acid as substrate)

Substrate	Experimental	Model	Error
concentration	Enzyme activity (nKat/mL)		
(nmol/mL)			
20	12.12	12.81	0.19
40	20.25	24.85	0.57
60	32.18	35.50	0.95
80	40.24	44.37	0.25
100	52.13	51.34	1.02
120	58.65	56.49	2.92
140	60.26	60.02	2.12
160	67.03	62.18	2.62
180	65.17	63.25	1.78
200	64.49	63.49	1.02
240	66.27	62.26	0.76
250	61.44	61.72	0.85
270	60.94	60.44	1.01
280	59.67	59.74	0.79
300	54.84	58.24	1.02
360	47.86	53.45	1.65

Table B.16 Effect of substrate concentration on enzyme activity (sinapine and sinapaldehyde as substrates)

Substrate concentration (nmol/mL)	Experimental Enzyme activity (nKat/mL) (sinapine as substrate)	Model	Error	Substrate concentration (nmol/mL)	Experimental Enzyme activity (nKat/mL) sinapaldehyde as substrate	Model	Error
20	15.45	16.74	0.11	20	12.59	10.56	0.11
50	36.31	35.04	0.23	40	20.29	20.54	0.42
100	49.73	50.38	0.11	60	29.20	29.51	0.64
150	55.14	54.45	0.11	80	35.57	37.16	0.32
180	53.57	54.24	0.34	100	40.59	43.36	0.74
220	52.10	52.51	0.90	120	46.95	48.14	0.32
260	50.63	50.03	2.59	140	48.65	51.60	0.42
				160	54.38	53.93	0.32
				180	59.89	55.31	2.23
				200	62.79	55.94	2.76
				220	60.53	55.98	0.21
				250	57.42	55.25	1.06
				270	42.36	54.40	1.06

Table B.17 Effect of pH values on enzyme activity (sinapic acid as substrate)

pH values	Experimental enzyme activity (nKat/mL)	Model	error
2.70	49.00	45.82	1.65
2.88	51.88	52.04	2.03
3.07	56.79	57.42	1.52
3.24	58.82	61.13	1.65
3.39	60.85	63.63	1.27
3.61	64.07	66.19	1.14
3.71	65.42	67.00	2.28
4.05	68.72	68.60	3.80
4.22	68.05	68.87	2.79
4.44	68.81	68.78	4.44
4.63	68.89	68.29	3.81
4.85	67.54	67.15	3.55
5.01	67.54	65.81	2.03
5.42	57.64	59.51	2.92
5.84	40.45	47.29	3.68
6.28	28.86	29.89	0.38
6.62	23.02	17.72	1.02
7.23	16.50	5.36	2.67

Table B.18 Effect of pH values on enzyme activity
(sinapine and sinapaldehyde as substrates)

pH values	Experimental	Model	Error	pH values	Experimental	Model	Error
	Enzyme activity (sinapine) nKat/mL				Enzyme activity (sinapine) nKat/mL		
2.68	8.01	5.08	0.11	2.75	33.59	35.28	3.29
3.01	14.55	10.05	0.11	3.11	44.41	42.30	1.70
3.57	23.68	26.60	0.00	3.48	47.38	46.37	1.27
3.79	34.62	35.40	0.11	4.15	49.08	48.98	2.65
4.36	55.48	55.76	0.68	4.72	49.15	48.93	0.64
4.97	66.08	65.39	0.45	5.18	46.53	47.48	1.48
5.34	63.94	65.84	2.37	5.96	33.38	38.07	3.18
5.73	56.72	62.32	3.27	6.32	30.12	29.10	2.44
6.43	41.50	42.85	1.13	6.65	22.91	19.71	1.48
7	24.13	20.63	1.80	7.26	5.80	6.89	0.64

Table B.19 Effect of temperature on enzyme activity

Substrate:	sinapic acid			sinapine			sinapaldehyde	
T (C)	Enzyme activity		T (C)	Enzyme activity		T (C)	Enzyme activity	
	nKat/mL	Error		nKat/mL	Error		nKat/mL	Error
10.60	46.46	0.89	15.00	51.99	2.82	15.00	31.18	2.01
20.00	67.20	1.65	25.00	72.29	0.11	25.00	51.34	1.39
25.10	75.75	1.14	35.00	93.04	1.92	36.00	69.48	1.81
30.00	82.52	1.52	40.00	96.98	1.35	42.00	72.13	0.44
36.00	85.56	1.78	45.00	95.29	1.47	45.00	71.84	2.69
40.00	94.62	1.90	52.00	95.29	2.82	54.00	70.54	0.56
45.00	95.30	2.03	60.00	94.50	0.45	60.00	66.33	0.75
50.00	94.37	3.30	70.00	88.41	1.13	75.00	45.75	1.92
55.00	97.16	2.41						
65.00	90.47	4.70						
75.00	60.60	5.33						

Table B.20 Effect of pre-incubation time on enzyme activity
(sinapic acid as substrate)

	at 313K			at 323K			at 338K	
Pre-incubation time (h)	Remaining activity(%)	Error %	Pre-incubation time (h)	Remaining activity%	Error %	Pre-incubation time (h)	Remaining activity %	Error %
0.00	100.00	1.32	0.00	100.00	1.32	0.00	100.00	1.53
0.50	90.21	1.06	0.50	95.77	2.98	0.17	82.89	3.02
1.00	86.16	2.46	1.50	81.83	2.47	0.25	52.56	1.54
2.25	77.59	3.06	2.50	71.60	3.10	0.38	42.24	5.65
3.13	76.01	4.35	4.00	53.17	5.06	0.54	34.48	3.86
3.93	70.19	4.28	5.40	43.03	6.60	0.75	20.55	5.90
5.08	67.72	1.79	6.00	37.13	2.18	1.00	18.17	6.81
5.85	64.89	5.12						
7.17	64.74	3.71						

Table B.21 Effect of moisture content on biomass production during solid state process

Moisture	Time (day)					
content	0	5	10	15	19	26
	Biomass (g/g dry SSC)					
50%	0.0033	0.003	0.013	0.023	0.083	0.096
60%	0.0033	0.028	0.034	0.037	0.096	0.100
70%	0.0033	0.040	0.056	0.054	0.076	0.079
75%	0.0033	0.017	0.034	0.036	0.038	0.033

Table B.22 Effect of moisture content on enzyme production during solid state process (syringaldezine as substrate)

Moisture	Time (day)					
content	0	5	10	15	19	26
	Enzyme activity (nKat/mL)					
50%	0	0	18.2	21.1	211.7	29.7
60%	0	0	64.2	84.1	218.1	42.5
70%	0	0	82.5	71.5	189.8	40.4
75%	0	0	21.0	65.5	152.3	2.8

Table B.23 Effect of moisture content on SAE degradation during solid state process

Moisture	Time (day)					
content	0	5	10	15	19	26
	SAE remaining (%)					
50%	100	52.9	26.5	15.8	7.8	2.5
60%	100	53.4	13.6	4.4	5.5	2.2
70%	100	45.8	9.3	5.0	5.2	2.0
75%	100	37.4	12.3	6.7	6.4	2.6

Table B.24 Effect of inoculum concentration on biomass production during solid state process

Inoculum	Time (day)					
concentration	0	5	10	15	19	26
(g/g dry CM)	Biomass (g/g dry SSC)					
0.0011	0.0011	0.0003	0.0024	0.0091	0.0992	0.1150
0.0022	0.0022	0.0027	0.0202	0.0230	0.0924	0.1167
0.0033	0.0033	0.0280	0.0343	0.0367	0.0959	0.1002
0.0044	0.0044	0.0678	0.0884	0.0852	0.0847	0.0831
0.0055	0.0055	0.0715	0.0925	0.0800	0.0801	0.0805

Table B.25 Effect of inoculum concentration on enzyme production during solid state process (syringaldezine as substrate)

Inoculum	Time (day)					
concentration	0	5	10	15	19	26
(g/g dry CM)	Enzyme activity (nKat/mL)					
0.0011	0	0	26.42	62.29	220.16	52.88
0.0022	0	0	27.95	87.25	184.66	54.99
0.0033	0	0	44.15	84.15	188.11	42.51
0.0044	0	0	56.66	112.40	157.71	41.52
0.0055	0	0	58.79	163.21	127.93	41.31

Table B.26 Effect of inoculum concentration on SAE degradation during solid state process

Inoculum	Time (day)					
concentration	0	5	10	15	19	26
(g/g dry CM)	SAE remaining (%)					
0.0011	100	60.3	21.6	6.4	6.3	2.4
0.0022	100	52.3	20.5	4.9	6.1	2.0
0.0033	100	53.4	13.6	4.4	5.5	2.2
0.0044	100	35.5	16.5	4.7	6.4	2.0
0.0055	100	31.4	14.4	4.6	6.1	1.9

Table B.27 Effect of homogenization time of inoculum on biomass production during solid state process

Homogenization	Time (day)					
time	0	5	10	14	19	23
(s)	Biomass (g/g dry SSC)					
15	0.0033	0.0186	0.0619	0.0649	0.0526	0.0498
30	0.0033	0.0255	0.0666	0.0703	0.0626	0.0568
60	0.0033	0.0135	0.0483	0.0737	0.0631	0.0615
120	0.0033	0.0158	0.0383	0.0844	0.0829	0.0722
200	0.0033	0.0047	0.0260	0.0952	0.0870	0.0725

Table B.28 Effect of homogenization time of inoculum on enzyme production during solid state process (syringaldezine as substrate)

Homogenization	Time (day)					
time	0	5	10	14	19	23
(s)	Enzyme activity (nKat/g dry SSC)					
15	0	40.5	58.1	156.0	93.2	38.7
30	0	54.8	83.9	228.9	116.1	39.6
60	0	7.4	47.9	243.4	114.7	57.5
120	0	0.0	44.6	270.8	124.4	67.7
200	0	0.0	42.2	315.2	152.4	80.7

Table B.29 Effect of homogenization time of inoculum on SAE degradation during solid state process

Homogenization	Time (day)					
time	0	5	10	15	19	26
(s)	SAE remaining (%)					
15	100	47.9	10.8	4.8	2.3	2.4
30	100	50.4	8.7	3.6	2.7	2.3
60	100	44.1	8.5	4.9	2.8	2.1
120	100	47.8	7.3	4.8	2.7	1.9
200	100	44.1	6.0	4.3	2.2	2.1

Table B.30 Effect of particle size of CM on biomass production during solid state process

Particle size	Time (day)					
of CM	0	5	10	14	19	23
	Biomass (g/g dry SSC)					
S1	0.0033	0.0047	0.0084	0.0586	0.0598	0.0495
S2	0.0033	0.0158	0.0227	0.0805	0.0878	0.0904
S3	0.0033	0.0144	0.0506	0.0941	0.0895	0.0836
S4	0.0033	0.0322	0.0468	0.0998	0.0923	0.0837
S5	0.0033	0.0206	0.0743	0.1004	0.0986	0.0839
S6	0.0033	0.0040	0.0865	0.1152	0.1000	0.0822

Table B.31 Effect of particle size of CM on enzyme production during solid state process (syringaldezine as substrate)

Particle size	Time (day)					
of CM	0	5	10	14	19	23
	Enzyme activity (nKat/g dry SSC)					
S1	0	17.3	68.9	60.4	91.4	42.6
S2	0	14.7	22.3	156.0	115.2	55.4
S3	0	1.2	12.0	172.1	109.3	61.5
S4	0	23.8	48.0	171.0	135.9	84.0
S5	0	0.1	6.0	200.5	138.5	81.8
S6	0	29.4	32.5	267.4	149.4	66.5

Table B.32 Effect of particle size of CM on SAE degradation during solid state process

Particle size	Time (day)					
of CM	0	5	10	15	19	26
	SAE remaining (%)					
S1	100	56.8	24.7	8.8	2.8	1.6
S2	100	59.5	22.1	7.6	2.7	2.0
S3	100	58.7	18.0	6.4	3.4	1.3
S4	100	57.0	12.2	5.3	2.5	1.4
S5	100	53.6	9.7	4.1	2.5	1.0
S6	100	50.8	10.4	2.5	3.4	2.3

Table B.33 Effect of surfactants on enzyme production, biomass production and SAE degradation during solid state process

Surfactants 0.50%	enzyme activity (nKat/g dry SSC) syringaldazine	Error	Biomass (g/g dry SSC)	Error	SAE degradation (% remaining)	Error
Control	190.6	7.1	0.0558	0.0019	14.04	0.13
Na-Oleate	268.1	9.9	0.0686	0.0025	11.48	1.19
Tween80	203.7	6.7	0.0622	0.0030	13.62	1.63
Triton100	251.4	6.9	0.0677	0.0027	13.15	1.19

Table B.34 Effect of sodium oleate concentration on enzyme production, biomass production and SAE degradation during solid state process

Sodium oleate concentration	Enzyme activity (nKat/g dry SSC)	Error	Biomass (g/g dry SSC)	Error	SAE degradation (% remaining)	Error
0.00%	190.6	7.1	0.0558	0.002	14.0	0.1
0.10%	191.1	0.4	0.0621	0.000	13.1	0.5
0.30%	239.9	1.2	0.0668	0.005	12.8	0.6
0.50%	278.3	8.4	0.0695	0.005	11.5	0.1
1.00%	351.6	7.6	0.0710	0.000	11.4	1.0
2.00%	340.2	8.4	0.0704	0.003	11.1	0.5

Table B.35 Experimental data for calibration curve in Figure A.1

Absorbance	g/L
0.000	0.00
0.105	0.50
0.159	0.75
0.209	1.00
0.331	1.50
0.393	1.75
0.479	2.00

Table B.36 Experimental data for calibration curves in Figure A.2

	wet	Absorbance	
CM weight	biomass	Theoretical	Experimental
0.50	0.25	0.071	0.065
0.50	0.50	0.079	0.078
0.50	1.00	0.095	0.096
0.50	2.00	0.128	0.110
1.00	0.25	0.199	0.211
1.00	0.50	0.207	0.222
1.00	1.00	0.223	0.240
1.00	2.00	0.256	0.284

Absorbance for 0.5 g of CM = 0.0624
Absorbance for 1.0 g of CM = 0.1906
Absorbance for pure Biomass 0.3765x, where x is g of biomass