OPTIMIZATION OF TEMPERATURE AND AGITATION RATE ON EXPRESSION OF RECOMBINANT XYLANASE IN *Kluyveromyces lactis* USING RESPONSE SURFACE METHODOLOGY

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A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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MAY 2009

I declare that this thesis entitled “Optimization of Temperature and Agitation Rate on Expression of Recombinant Xylanase in *Kluyceromyces lactis* Using Response Surface Methodology” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to my family members,
My fellow lecturers
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Xylanase has been used widely in industries today due to its ability in catalyzing various types of biochemical reactions. However, production cost of xylanase enzyme is very expensive. A research to reduce the cost by optimization of temperature and agitation rate on expression of recombinant xylanase in *K. lactis* using Response Surface Methodology (RSM) was successfully done. At the early stage of experiment, one factor at a time was employed to screen the best range for temperature and agitation rate. All the parameter ranges obtained were used in Response Surface Methodology (RSM). Response Surface Methodology in Design Expert version 7.1.6 software was used with Central Composite Design (CCD) mode. Thirteen sets of experiments with different parameter values were suggested by the software. The predicted optimum values for temperature, agitation rate and xylanase activities were 34.68^{o}C, 253 rpm and 9.198 U/ml respectively. One set of experiment was run using the optimized parameter and as a result, 9.221 U/ml xylanase activity was recorded. Before optimization, xylanase activity was only 5.221 U/ml and the activity was increased by 43.75% or 1.78-fold after optimization. The optimization also reduces the energy consumption as the temperature was reduced from 38^{o}C to 34.6^{o}C and agitation rate reduced from 280 to 253 rpm. As conclusion, this research is successful to increase recombinant xylanase production, reduce the energy consumption and also able to reduce the production cost.

ABSTRAK

Xylanase telah digunakan secara meluas dalam industri pada hari ini atas kebolehannya memangkinkan pelbagai jenis tindak balas biokimia. Walaubagaimanapun, kos penghasilan enzim xylanase sangat mahal. Satu kajian untuk merendahkan kos penghasilan dengan mengoptimumkan suhu dan kadar adukan terhadap ekspressi xylanase rekombinan dalam *K. lactis* menggunakan Kaedah Permukaan Tindak balas (RSM) telah berjaya dilakukan. Diawal peringkat eksperimen, kaedah satu faktor pada satu masa telah digunakan untuk menyaring julat suhu dan kadar adukan yang terbaik. Kesemua julat parameter yang diperolehi, digunakan dalam Kaedah Permukaan Tindak balas (RSM). Kaedah Permukaan Tindak balas (RSM) dalam perisian Design Expert versi 7.1.6 telah digunakan dengan mod Rekabentuk Komposit Pusat (CCD). Tiga belas set eksperimen berlainan nilai parameter telah dicadangkan oleh perisian ini. Nilai optimum yang diramalkan untuk suhu, kadar adukan dan aktiviti xylanase masing-masing 34.68^{o}C, 253 rpm dan 9.198 U/ml. Satu set eksperimen telah dijalankan bagi menguji parameter yang telah dioptimumkan dan sebagai keputusannya, 9.221 U/ml aktiviti enzim xylanase telah dicatatkan. Sebelum pengoptimuman, aktiviti xylanase cuma 5.221 U/ml dan aktivitinya meningkat 43.75% atau 1.78-kaliganda selepas pengoptimuman. Pengoptimuman juga menurunkan penggunaan tenaga seperti suhu telah direndahkan dari 38^{o}C kepada 34.6^{o}C dan kadar adukan dikurangkan dari 280 rpm kepada 253rpm. Sebagai konklusinya, kajian ini telah berjaya meningkatkan penghasilan xylanase rekombinan, mengurangkan penggunaan tenaga dan juga mampu menurunkan kos penghasilan.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
CCD	-	Central composite design
DNS	-	Dinitrosalicylic
g	-	Gram
g/L	-	Gram per liter
L	-	Liter
M	-	Molar
mg	-	Milligram
min	-	Minutes
mM	-	Milimolar
MW	-	Molecular weight
OD_{575}	-	Optical density at 575nm
OFAT	-	One Factor at a Time
OSX	-	Oat spelt xylan
RSM	-	Response surface Methodology
Rpm	-	Rotation per minutes
T	-	Temperature
U	-	Unit for enzyme activity
^{o}C	-	Degree Celcius
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Xylanase is the name given as the enzymes which deconstructs plant structural material by breaking down hemicelluloses, a major component of the plant cell wall (Isil and Nilufer, 2005). By degrade the linear polysaccharide β-1,4-xylan into xylose, β-1,4-xylanases present great potential in several biotechnological applications. They catalyze the hydrolysis of internal β-1,4-D-xylose units and was used in bread making, clarification of beer and juices as well as conversion of xylan-containing lignocellulosic materials to D-xylose, which can be converted to a variety of bioproducts with high aggregate value (Damasco *et al*., 2004). Xylanases are also believed to be essential in improving the nutritional quality of animal feed and in the recovery of textile fibers.

In Malaysia, the oil palm industry produces about 90 million tonnes of lignocellulosic biomass i.e. oil palm biomass (OPB) each year of which about 40 million tonnes are the empty fruit bunches (EFB), oil palm trunks (OPT) and oil palm fronds (OPF). This lignocellulic biomass can be degrade into simple sugar. Thus, the sugar can be feedstock for producing bioethanol. Xylanase can be used to degrade the xylan in palm lignocellulic to form xylose, a simpler sugar that can be feed stock for ethanol production. (Kabbashi *et al*., 2007)

For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms that produce high levels of this enzyme. Several strains of the fungus like *Trichoderma reesei* secrete high levels of xylanases, which are very active and stable at elevated temperatures (Gessesse, 1998). Beside the productive microorganism, their growth condition such as pH, temperature, shear stress, aeration, agitation and presence of inhibitors and cofactors, must be concern to ensure the microorganism produce optimum quantity of xylanase.

1.2 Problem Statement

Malaysia palm oil industry produce large amount of lignocellulic biomass which are consist of empty fruit bunch, palm oil trunks and palm oil fronds. The large amount of biomass waste just being dispose because of limited application. For instance, the application of EFB are become fertilizer and burnt to heat up steam boiler in palm oil mill (Kabbashi *et al*., 2007). Nowadays, scientists around the world are working to produce second generation of bioethanol. Second generation of bioethanol is bioethanol from biomass waste rather than cereal or food. Second generation bioethanol surely can solve the food crisis.

However, to produce second generation of bioethanol from cellulose biomass, xylanase enzyme must be utilized to catalyze the conversion of cellulose into simpler sugar before it can proceeds with fermentation. Utilization of enzyme will lead to high production cost. A more efficient and economically viable process is essential to reduce the cost of xylanase production (Mazeau *et al*., 1999). Therefore, investigation of the optimization conditions on expression of xylanase should be concerned in order to improve its productivity process, maximize the yield and reducing the production cost.

1.3 Objective

The aim of this study is to determine the optimum temperature and agitation rate toward expression of recombinant xylanase in *Kluyveromyces lactis* using Response Surface Methodology (RSM).

1.4 Scope of the study

In this research, there are two type of parameters will be investigate. They are temperature and agitation rate. The scopes of this study are as follows:

a) To screen the best range of temperature and agitation rate on production of recombinant xylanase
b) To optimize the combination of temperature and agitation rate on production of recombinant xylanase using Response Surface Methodology (RSM)

CHAPTER 2

LITERATURE REVIEW

2.1 Xylan

Xylan is one of the major components of the hemicellulose. β-1,4-Xylans are found mainly in secondary walls of plants and can represent up to 35% of the total dry weight in certain plants **(**Grange *et al*., 2001**)**. Unlike cellulose, xylan is a complex polymer consisting of a β-1,4-linked xylopyranoside backbone substituted with side chains. Hydrolysis of the xylan backbone is catalyzed by endo-β-1,4-xylanases and β-D-xylosidases (Xiong, 2004). They represent 20 to 30% (dry weight) of wood and up to 50% (dry weight) of some cereal seeds. In the current trend for a complex and effective utilization of biomass, increasing attention has been paid during the last few years to the exploitation of xylans as biopolymer resources (Mazeau *et al*., 1999) Xylan can be enzymatically hydrolyzed to xylose and converted into economically valuable products such as xylulose, xylitol and ethanol (Jiang *et al.*, 2004). Interest in the enzymology of xylan hydrolysis has recently increased because of its great potential in industrial application, such as in biobleaching, paper making and in the food as well as animal feed industries (Yang *et al*., 1988). Figure 2.1 shows the structure of xylane.

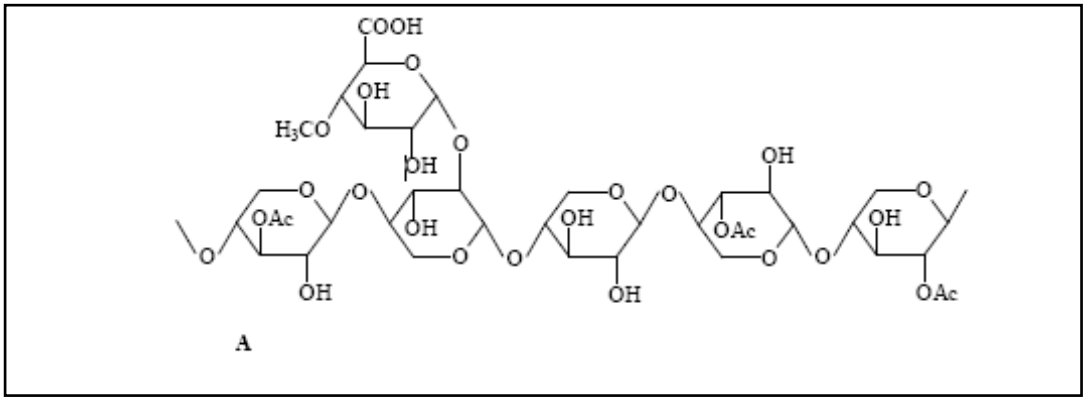

Figure 2.1: Xylan structure

2.2 Xylanase

Many bacterial and fungal species can produce a mixture of xylanase, β-xylosidase and accessory side-group cleaving enzymes in order to utilize xylan, a complex polymer which is the major component of hemicellulose in the plant cell wall. Xylan found in nature consists of a β-1,4-linked xylopyranose backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Enzymatic hydrolysis of xylan to xylose is catalyzed by endo-1,4-β-xylanase and β-xylosidase, the former randomly hydrolyzing xylan to xylooligomers and the latter producing xylose from xylooligomers. The side chain groups are liberated by α-L-arabinofuranosidase, α-D-glucoronidase, α-galactosidase and acetyl xylan esterase. β-xylosidase shows high activity toward xylobiose but no activity toward xylan. However, some xylanases may also have an ability to hydrolyze xylooligomers to xylose, especially in the cross-linked enzyme crystal form (Xiong, 2004).

2.2.1 Classification of xylanase

Xylanases can be classified at least three ways. The first, as suggested by Wong *et al*. (2003) is based on molecular weight and isoelectric point (pI). They are either high or low molecular weight and have either a high (basic) or low (acidic) isoelectric point. Information for this sort of classification is readily obtained during purification and initial characterization. The second is based on crystal structure. This can be derived indirectly by a determination of DNA sequence. Xylanases can be structurally classified into family F or (now known as glycosidase family 10) and family G (now known as family 11) (Jeffries *et al*., 1996). The third classification is based on kinetic properties, substrate specificity or product profiles (Collin *et al*., 2005).

Family 10 xylanases occasionally exhibit endocellulase activity. They generally have a higher molecular weight and they occasionally will possess a cellulose binding domain. Members of family 10 will act on both PNP-xylobiose and PNP-cellobiose. However, the overall catalytic efficiency on PNP-xylobioside is about 50 times higher. This suggests that family 10 enzymes act mainly on xylan. The family 10 catalytic domain is a cylindrical β/b barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the β-barrel. There are five xylopyranose binding sites. Catalytic domains of these enzymes belong to a "super family" that includes Family A cellulases, β-glucosidase, β-galactosidase, β-(1-3)-glucanases and β-(1-3, 1-4)-glucanases. Family 10 xylanases have relatively high molecular weights and they tend to form oligosaccharides with a low degree of polymerization (DP) (Jeffries *et al*., 1996).

Family 11 catalytic domains consist of principally of-pleated sheets formed into a two-layered trough that surrounds the catalytic site. Protruding down into the trough and located toward one side of the protein is a long loop terminating in an isoleucine. Tösrrönen and Rouvinden (1995) have likened the trough to the palm and fingers and the loop to the thumb of a right hand. The positions of many amino acids are essentially identical in Family 11 xylanases from bacterial (e.g. *Bacillus circulans)* or fungal (e.g. *Trichoderma harzianum)* origins. The *Trichoderma*

enzyme, however, is more complex. *Trichoderma reesei* produces two Family 11 xylanases: Xyn1 and Xyn2. Xyn1 has an acidic isoelectric point 5.5, possesses a smaller, tighter groove than Xyn2 and a lower pH optimum (Baily *et al*., 1995). It also exhibits a 15-fold higher turnover number. Xyn2 has a basic isoelectric point 9.0, a more open structure and a wider pH range. Xyn2 tends to produce larger oligosaccharides. Both Xyn1 and Xyn2 release xylobiose in the retained and configuration (Viikari *et al*, 1995).

2.2.2 Application of xylanase

Xylanase has been widely used in industrial and agricultural application. It is used in confectionery, cereals and starch, animal feeds, textiles, cellulose and paper (Saddler *et al*, 2008)

2.2.2.1 Degradation of Non Starch Polysaccharides (NSP)

The poultry feed mainly consist of mainly non starch polysaccharides (NSP) from plant origin. The digestive system of poultry birds has insufficient fiber digesting enzymes hence the absorption of all the nutrients from feed is not observed. The enzymes present in SEB feed xylanase will degrade this NSP material and will liberate ready source of energy in the form of reducing sugars which is absorbed easily from the intestine of the birds and helps in increasing the weight of the bird (Saddler *et al.*, 2008).

2.2.2.2 Biobleaching

Recently, in an effort to eliminate chlorine altogether, "Biobleaching" has been examined. There is huge number of microorganisms take advantage of the world's largest energy storage which is to be in plant biomass. A broad spectrum of enzymes was produced by those organisms that catalyze the hydrolysis of the various components that are present in plant material. It could be possible to find an enzyme which can assist in the removal of lignin by identifying those enzymes which target specific components of wood fiber. At present, enzymes are being considered as only partial replacements for chemicals. However, there is hope that as the mechanisms of enzymatic treatment are elucidated, refinements in the process may allow enzyme assisted "Total Chlorine Free" (TCF) bleaching sequences to become an effective and economic method of pulp treatment (Bajpai, 1993).

2.2.2.3 Cotton Treatment

Xylanase used in wet-processing treatments of cotton, such as scouring and stonewashing in the textile industry and the possibilities of enzyme treatment for other fibers, especially wool, are under extensive research (Coughlan and Hazlwood, 1993).

2.2.2.4 Cereal Industry

The enzyme xylanase has long been used by the cereal industry to standardize and improve flour performance. Until now, the performance of microbial xylanases has varied from flour to flour, due to the natural content of xylanase inhibitors in the wheat (Saddler *et al.*, 2008).

2.2.2.5 Food Industry

In the food industry, xylanases are used in fruit, vegetable and plant processing, in wine making and brewing, in baking, milling, pastry and confectionery as well as in coffee processing. Their functions in these industries are very diverse. For example, in fruit and plant processing they improve the maceration process, juice clarification, the extraction yield and filtration efficiency, hence improving the process performance and product quality. Xylanases also reduce the wart viscosity in beer making, improve grape skin maceration in wine making and reduce haze in the final products. In baking, xylanases improve elasticity and strength of doughs, thereby allowing easier handling, larger loaf volume and improved bread texture. In coffee processing, xylanases reduce the viscosity of coffee extracts and improve the drying or lyophilization processes (Saddler *et al.*, 2008).

2.3 Expression System

An expression system consists minimally of a source of DNA and the molecular machinery required to transcribe the DNA into mRNA and translate the mRNA into protein using the nutrients and fuel provided. In the broadest sense, this includes every living cell capable of producing protein from DNA. However, an expression system more specifically refers to a laboratory tool, often artificial in some manner, used for assembling the product of a specific gene or genes. It is defined as combination of an expression vector, its cloned DNA and the host for the vector that provide a context to allow foreign gene function in a host cell, that is produce proteins at a high level (Hegde and Kang, 2008).

In addition to these biological tools, certain naturally observed configurations of DNA (genes, promoters, enhancers, repressors) and the associated machinery itself are referred to as an expression system, as in the simple repressor 'switch' expression system in Lambda phage. It is these natural expression systems that inspire artificial expression systems (Hebert and Molinari, 2007).

2.3.1 Yeast Expression System

In general, fungi are excellent hosts for the production of recombinant proteins. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media. As eukaryotes, they are able to perform protein modifications like glycosylation (addition of sugars), thus producing even complex foreign proteins that are identical or very similar to native products from plant or mammalian sources. The first yeast expression platform was based on the commonly known baker's yeast *Saccharomyces cerevisiae*. However the baker's yeast is only one of more than 800 different yeasts with different characteristics and capabilities. For instance some of them grow on a wide range of carbon sources and are not restricted to glucose, as it is the case with baker's yeast. Several of them are also applied to genetic engineering and to the production of foreign proteins (Zhang *et al*., 2003)

Suitable yeast strains are transformed by a vector, a so-called plasmid that contains all necessary genetic elements for recognition of a transformed strain and the genetic advice for the production of a protein. A selection marker, required to select a transformed strain from an untransformed background. This can be done if for instance such an element enables a deficient strain to grow under culturing conditions void of a certain indispensable compound like a particular amino acid that cannot be produced by the deficient strain (Tanaka *et al*., 2003).

Since the yeasts differ in their characteristics to produce a certain protein it cannot be excluded at the beginning of a development that selected yeast will not be able to produce the desired compound at all. This in turn can lead to costly time-consuming failures. It is therefore advisable to assess several yeast platforms in parallel for their capabilities to produce such a compound. Therefore, a plasmid system was developed that can be targeted in functional form to all yeast in parallel (Tanaka *et al*., 2003). It is composed in modular way of element for selection, a "universal" targeting sequence that is present in all yeasts (the rDNA) and it contains within the expression cassette a promoter that is active in all yeast.

2.3.2 Kluyveromyces lactis Expression System

The yeast *Kluyveromyces lactis* has been used over a decade for the industrial-scale production of commercially important proteins *K. lactis* cells can efficiently secrete recombinant proteins, rapidly grow to high cell density and can be easily genetically manipulated, making this organism an attractive eukaryotic host for protein expression. Besides that, since *K. lactis* is present in various milk products, it has obtain food grade status which accepted as "GRAS" (generally recognized as safe) and it also excellent fermentation characteristics (Fermiñán and Domínguez, 1998).

In a typical *K. lactis* protein expression strategy, a DNA fragment containing the equipment necessary to direct the high-level transcription of a gene of interest is first assembled in *K. lactis* cells prior to its introduction into yeast cells. This fragment typically contains (in 5′ to 3′ order) (i) a strong yeast promoter, (ii) DNA encoding a secretion leader sequence (if secretion of the protein is desired), (iii) the gene encoding the desired protein, (iv) a transcription terminator sequence and (v) a yeast-selectable marker gene (Colussi and Taron, 2005).

In *K. lactis*, the expression of heterologous genes has been achieved using various promoters isolated from native *K. lactis* genes or using promoters originating from other yeasts. However, the *K. lactis LAC4* promoter (P_{LAC4}) is often used because of its strength and inducible expression. *K. lactis* P_{LAC4} drives expression of the *LAC4* gene that encodes a native lactase (β-galactosidase) that is an essential part of the lactose-galactose regulon that allows this organism to utilize lactose as a carbon and energy source. Two upstream activating sequences (UAS I and UAS II) located in a 2.6-kb intragenic region between *LAC4* and *LAC12* regulate the transcription of *LAC4*, which can be induced 100-fold in the presence of lactose or galactose (Colussi and Taron, 2005).

2.4 Physical Factor Affecting the Expression of Recombinant Protein

Physical cultural conditions during cultivation of any microbes is very important. All parameters involved must be monitor closely to ensure the growth of cultivates microbes develop well and can produce maximum enzyme of interest. There are several factors that affecting the growth condition of microbes.

2.4.1 Temperature

The cultivation temperature does not only affect the growth rate of an organism, but it can also have a marked effect on the level of xylanase production. For example, *T. reesei* Rut C-30 grew well at 17, 28 and 37^{o}C when cultivated on lactose substrate, but xylanase production was significantly increased at higher temperature, whereas cellulase production was reduced (Ellaiah *et al*., 2002). An initial phase of cultivation of *T. reesei* Rut C-30 at 37^{o}C followed by a shift to 28^{o}C in the beginning of the enzyme production phase was advantageous for both the amount of xylanase activity obtained and the ratio of xylanase to cellulose (Ramesh and Lonsame, 1989). By applying the temperature shift during laboratory cultivation, xylanase activity could almost be doubled, whereas the xylanase/cellulase ratio was three-fold higher in comparison to cultivation at a constant temperature of 28^{o}C (Xiong, 2004).

2.4.2 Agitation Rate

When cultivating of filamentous fungi for enzyme production, the agitation rate influence the fungal growth and secretion of enzymes. The shearing action on the morphology and productivity of filamentous fungi also deserves attention. Too strong agitation would be harmful for the production of xylanase. In large-scale fermentations, the stirrer speed had an even more pronounced effect on the

production of xylanase. (Kammoun, 2007). The enzyme production by *T. reesei* was seriously affected by agitation. When using lactose as the substrate in a 15-litre fermentor, the optimal agitation rate was found to be 200 rpm. Low xylanase activities were obtained at 130 rpm, most probably due to oxygen or mass transfer limitations, while at 400 rpm almost no xylanase was produced. The latter result could be explained by a low production rate caused by the increased shear stress (Xiong, 2004).

2.4.3 pH

pH is an important parameter in the production of enzymes by *Trichoderma reesei*. Earlier reports indicated that a rather high pH (7.0) is essential for good production of xylanases by *T. reesei* Rut C-30 on cellulose and xylan based growth media, although growth (broth viscosity) was evidently better at pH 4.0 than at pH 7.0 (Kobayashi *et al*., 1998). Meanwhile, good production of cellulases was found at low pH (4.0). A high pH (7.0) was essential for high xylanase production by *Trichoderma longibrachiatum* in cellulose medium. During the course of the fermentation, the nitrogen source can significantly influence the pH of the medium. The pH of *T. reesei* culture broth decreased during the cultivation when ammonium salts were used as the nitrogen source, whereas the pH increased when urea was the nitrogen sources (Xiong, 2004).

2.5 Optimization of Enzyme Production by Using Response Surface Methodology (RSM)

The conventional study was performed by manipulating one parameter while keeping the others at constant level do not reflect the interaction effects among these variables employed. This kind of study does not depict the net effect of the various factors on the enzyme activity. In order to overcome this major problem, optimization study is done using Response Surface Methodology (RSM) which is a

mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes (He and Tan, 2006).

Response Surface Methodology (RSM) has been extensively applied to optimize culture medium and other process parameters for the production of lipase (He and Tan, 2006; Liu *et al*., 2006), tannase (Battestin and Macedo, 2007), α-amylase (Rao and Satyanarayana, 2007), β-cyclodextringlucanotransferase (Ibrahim *et al*. 2005), dextran dextrinase (Naessens *et al*., 2004) and chitinase (Nawani and Kapadnis, 2005).

Chen *et al*. (1992) studied the optimization of β- galactosidase production using a statistical and mathematical approach. These authors used a central composite design to optimize the medium composition for lactase production by *Kluyveromyces marxianus* and found the optimal medium composition as 80.6 g l^{-1} lactose, 107.7 g l^{-1} corn steep liquor, 4.1 g l^{-1} glucose and 9.6 g l^{-1} glycerol. Becerra and Siso, (1996) used a full-factorial design to optimize β-galactosidase production by *Kluyveromyces lactis* in solid-state fermentations on corn grits or wheat bran moistened with deproteinized milk whey.

CHAPTER 3

MATERIALS AND METHODS

3.1.1 Strategies for Optimization of Temperature and Agitation Rate on Expression of Recombinant Xylanase

This chapter will elaborate on the materials and methods that have been applied in this experiment. To ensure the flow of experiment goes smooth, it has been divided into 4 stages. The first stage of the experiment was to obtain the standard curve of xylose concentration versus optical density at 575nm (OD_{575}). The second stage was performed to observe the most suitable pH, incubation time and temperature for the enzyme assay purpose. The 3rd stage of the experiment was carried out to obtain the best range of temperature and agitation rate. One factor at a time method (OFAT) was applied at this stage. For the 4th stage of this experiment, Response Surface Methodology (RSM) was used to further optimize the data gained from stage 3. Interaction of all parameters was analyzed at this stage. Figure 3.1 shows the flow diagram that simplified the strategies of this research.

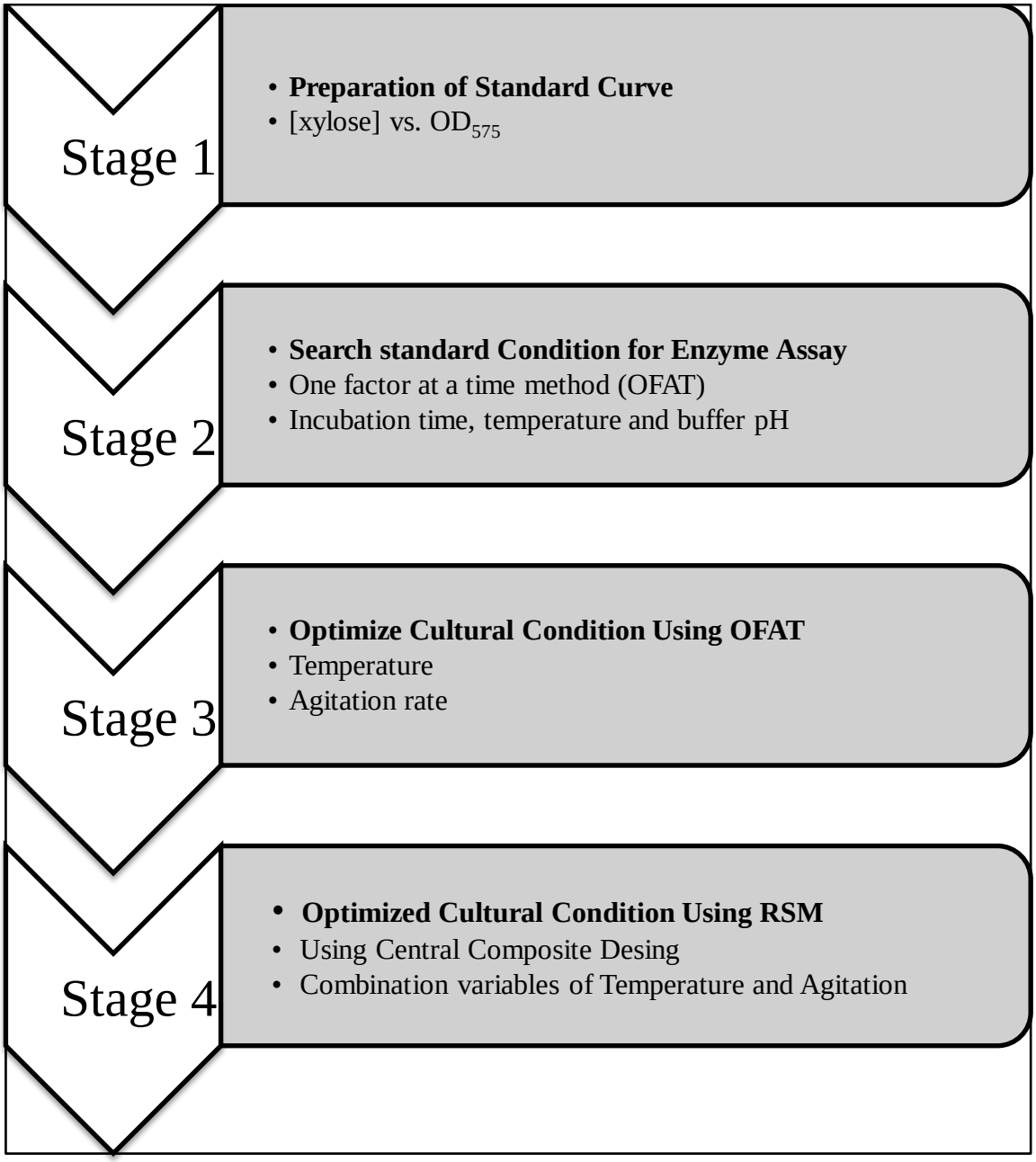

Figure 3.1: Research design for optimization of expression of recombinant xylanase

3.2 Chemicals and Biological Substances Involved

All chemical used in this research was provided by Laboratory of Faculty of chemical Engineering and Natural Resources (FKKSA), UMP. Most chemical was bought from Merck, Sigma and Fluka. Recombinant *K. lactis* was brought from University Teknologi Malaysia (UTM).

3.3 Culture Medium

The preparation of culture medium is depend on vector promoter. The promoter in *K. lactis* used in this research is LAC4. To prepare the culture medium, glucose stock culture must be prepared first and followed by Yeast Peptone Dextrose (YPD). The 20%w/v glucose was used in preparation of culture medium. 20 g glucose was dissolved into 100 ml dH_2O. Ten grams of Yeast Extract and 20 g Bacto^{TM} Peptone was dissolved in 900 ml dH_2O. The solution was mixed together and autoclave for 20 min at 121^{o}C and 15 psi. The solution was let cool to room temperature after 20 min. The YPD culture medium was stored in refrigerator.

3.4 Cultivation Method

Sterilized 500ml conical flask with cap was filled up with 50 ml sterilized YPD medium. By using pre-heated inoculums loop, inoculate some *K. lactis* from frozen stock culture which mixed with glycerin and immerse the loop into the culture medium in the flask. This step was repeated for 2 to 3 times. Pipette some sample from the flask into a cuvette and the OD_{575} was read using spectrophotometer. The flask that containing microbe was incubated using incubator shaker.

3.5 Preparation of Standard Curve

Xylose stock solution was prepared by disolved 0.1g xylose into 100 ml distilled water. The concentration of the stock solution was 1 g/L. the siries of different concentration of xylose solution from 0.1 g/L until 1.0 g/L was prepared in test tube by adding distiled water into stock solution. The volume of each test tube was adjusted to 1.0 ml. the one tenth dilution was performed by adding 0.1 ml solution into 0.9 ml distiled water. Diluted solution with volume of 0.75 ml of the was separate and mixed with 0.25 ml of oat spelt xylan (OSX) solution that acts as substrate. The solution then was added with 1ml citrate buffer at pH 6. The mixture was incubated in water bath for 20 minutes at 50^{o}C. The incubation was important to allow reaction between xylose and the OSX. Then, the solution removed from water bath and 0.75 ml DNS and 0.5 ml NaOH was added into the solution to terminate the reaction.the solution was boiled for 15 minutes to allow DNS and NaOH works. Then it was cool down under flowing water. The optical density was read using spectrometer with wavelenght 575 nm. Standard curve was ploted by xylose concentrated versus OD_{575}. Table 3.1 shows concentration of xylose involed in preparation of standard curve.

Table 3.1: Concentration of xylose involved in preparation of standard curve

Xylose Concentration mg/ml	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
dH_2O	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
Volume, ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
OD_{575}										

3.6 Determination of Enzyme Standard Reaction Condition

Determination of enzyme stability in term of incubation temperature, duration and pH buffer is important as the data was needed in enzyme assay. Determination of enzyme standard reaction condition only take place during incubation step in enzyme assay. During incubation step, the enzyme react with substrate which the xylanase degrade cellulose in oat spelt xylan (OSX) into simpler sugar. Manipulation of incubation parameters during the reaction is neccessary to determine the optimum conditions for enzyme reaction.

In determinantion of optimum incubation time, timing for incubation was set at 5, 10, 15, 20, 25 and 30 minutes. Temperature and pH buffer were set constant at 60 ^{o}C and pH 5 respectively. After the first set of assay finished and the optimum incubation time can be determined by plotting a graph incubation time vs. OD_{575}. Maximum OD is selected for the optimum incubation time. Second step was to determine the incubation temperature. The incubation temperature were set at 50^{o}C, 60^{o}C and 70^{o}C. The optimum incubation time from the first step was applied and buffer pH 5 was set constant. When incubation process and enzyme assay were finished, the optimum temperature was determined by plotted graph temperature versus OD_{575}. The optimum temperature and incubation time obtained was set to be constant for 3rd set incubation. The pH of citrate buffer used was set at 3,4,5,6 and 7. After the assay for 3rd set finish the optimum pH was determined using pH vs. OD_{575} curve. After all the incubation parameter has obtain, all the parameter can be applied as standard condition for xylanase assay. Table 3.2 simplified the data of determination of recombinant xylanase incubation time, temperature and pH buffer.

Table 3.2: Determination of incubation time, temperature and pH buffer of recombinant xylanase reaction

Set	Parameter to Determine	Manipulated Value						Constant Value
1	Incubation time, min	5	10	15	20	25	30	60^{o}C, pH 5
2	Temperature,^{o}C	40	50	60	70			pH 5, optimized time
3	Buffer pH	3	4	5	6	7		optimized time & temperature

3.7 Selection the Best Range of Parameters Using Conventional Method

K. lactis yeast was cultured at different temperature in incubator shaker with 200 rpm. The temperature of the incubator shaker was set at 25, 30, 35 and 40 ^{o}C. After the second day or each of the culture have reach OD_{575} 1.50 and above, the xylanase was extracted and proceed with xylanase assay. After the best range of culture temperature was obtained, the value was used in the next step which was to determine best range of agitation rate.

The next step is to determine the best range of agitation rate for recombinant *K. lactis* to grow. To obtain the best range of agitation rate, the rate must be varied while the temperature must be set constant. Four different agitation rates were set at 150, 200, 250 and 300 rpm. The temperature for each set of culture was set constant at the best temperature obtained in the first step. Table 3.3 shows the simplified data in screening the best cultural condition using conventional method.

Table 3.3: Cultural condition in screening best cultural condition using conventional method.

Set	Parameter	Manipulated Value				Constant Value
1	Temperature,^{o}C	25	30	35	40	250 rpm
2	Agitation rate, rpm	150	200	250	300	35^{o}C

3.8 Optimization of Temperature and Agitation Rate on Expression of Recombinant Xylanase Using Response Surface Methodology (RSM)

Based on the previous result of one at a time method, the best and smaller range of temperature and agitation rate were used for further study using Response Surface Methodology (RSM). For optimization of cultural conditions, the design was made up of a full 2^2 factorial with a total of 13 experiments using Central Composite Design (CCD). Among of the 13 experiments, there are eight star points and 5 replicates at the centre points. The value of alpha was set at 1.41421. The value of alpha determines the location of the star points in a central composite design. Table 3.4 shows the parameters and level involved in optimization of cultural conditions.

Table 3.4: Initial data used in RSM

Factors	**Symbol**	**Units**	**Low start Point**	**Low Level**	**Centre Point**	**High Level**	**High Start Point**
			- α	**-1**	**0**	**+1**	**+ α**
Temperature	X1	^oC	30.7	32	35	38	39.2
Agitation Rate	X2	rpm	208	220	250	280	292

3.9 Enzyme Assay

Enzyme assay is important to determine the concentration of the xylose which is converted from xylan catalyzed by xylanase enzyme in a sample. The method used was as described by Bailey *et al*.(1989). After two days or the culture have achieved OD_{575} = 1.5 and above, the enzyme can be extract for assay purpose. Before proceed with assay, some pretreatment of enzyme take place to remove cell and culture media. Sample with amount of 1.0 ml was taken from each shake flask containing recombinant and non-recombinant *K.lactis* which represent control sample. Sterile distiled water with amount of 9.0 ml was added to the sample for one tenth dilution. The diluted sample with amount of 10 ml was centrifuged at 6000 rpm

and 25^{o}C for 5 minutes. After centrifuged, the supernatant was discarded. The pallet then washed by sterile distiled water and centrifuged again to remove the remaining YPD media. The pellet was added with sterile distiled water to 10 ml.

The sample then ready to be proceed with enzyme assay. The sample with amount of 0.1 ml was added together with 0.45 ml 1% OSX and 0.2 ml buffer. The mixture was incubated in water bath using standard assay condition which are 20 minutes, 60^{o}C and pH 5 (Appendix A4). The purpose of incubation is to allow the reaction between enzyme and substrate take place. The 0.75 ml DNS and 0.05 ml of 1 M NaOH which acts as indicator was added. Then the mixture was boil in 100 ^{o}C water for 15 minutes. After that, the mixture was allowed to cool under flowing tap water. The absorbance of the mixture was measured using spectrophotometer using 575 nm wavelenght. One xylanase unit is defined as the amount of enzyme that produced reducing carbohydrates having a reducing power corresponding to one mmol xylose from birch xylan in one minutes under assay conditions (Bailey *et al*., 1989). The activity was calculated using the Equation 3.1.

$$\text{Xylanase activity} = \frac{X \times D}{MW \times T \times V} \quad \textbf{(Equation 3.1)}$$

.

X - Xylanase concentration from standard curve

D - Dilution factor

MW - molecular weight of xylanase (150.14 mg/mmol)

T - Incubation time

V - volume enzyme

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Screening of the Best Range of Temperature and Agitation Rate on Expression of Recombinant Xylanase Using Conventional Method

Studies on the parameters of cultural condition that affect the expression level of recombinant xylanase were carried out by using the conventional method. The method used was one factor at a time which manipulating one parameter while keeping the other at a certain level. This study was done to determine the best range for all parameters for further optimization process. Basically, the low and high levels of every parameter were obtained before and after peak values respectively in the appropriate study. The parameters involved in this study were temperature and agitation rate.

4.1.1 The Effect of Temperature Using Conventional Method on Expression of Recombinant Xylanase.

The effect of temperature was studied to determine the best range of temperature on expression of recombinant xylanase. The temperature was manipulated at 25, 30, 35 and 40^{o}C while agitation rate was kept constant at 250 rpm. The data obtained from the experiment is shown in Figure 4.1.

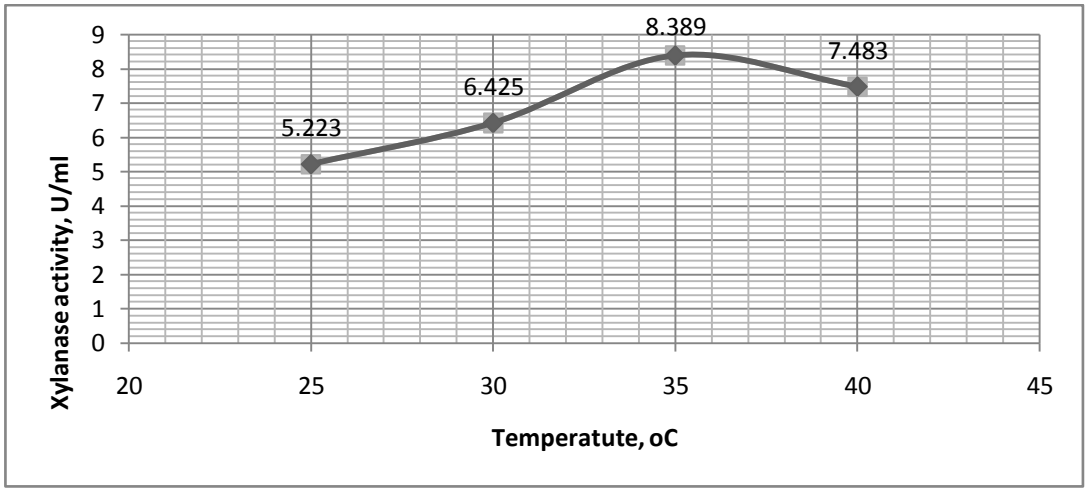

Figure 4.1: The effect of temperature on recombinant xylanase activity

From the graph, the activity was inclined until temperature 35^{o}C and started to decline after temperature 35^{o}C. The result shows the best range of temperature between 33^{o}C until 38^{o}C. This fact supported by previous research. The temperature of 33^{o}C until 37^{o}C has been used for galactosidase production by many researchers (Ku and Hang, 1992; Hewitt and Wassink, 1984; Artolozaga *et al.*,1998). Temperature at 35^{o}C was the best temperature and recombinant xylanase activity achieved by this temperature was 8.389 U/ml. Matheus and Rivas, (2003) have reported that 34.3^{o}C as an optimal temperature for B-D-galactosidase production by *K. lactis.*

The best range of temperature obtained will be used for the next step in order to study the effect of agitation rate on the expression of recombinant xylanase while the temperature was kept constant.

4.1.2 The Effect of Agitation Rate Using Conventional Method

The effect of agitation rate study was done to determine the best range of agitation rate on expression of recombinant xylanase using a constant temperature at 35^{o}C which obtained from the previous study. The agitation rate was set at 150, 200, 250 and 300 rpm. The result from the experiment shown in Figure 4.2.

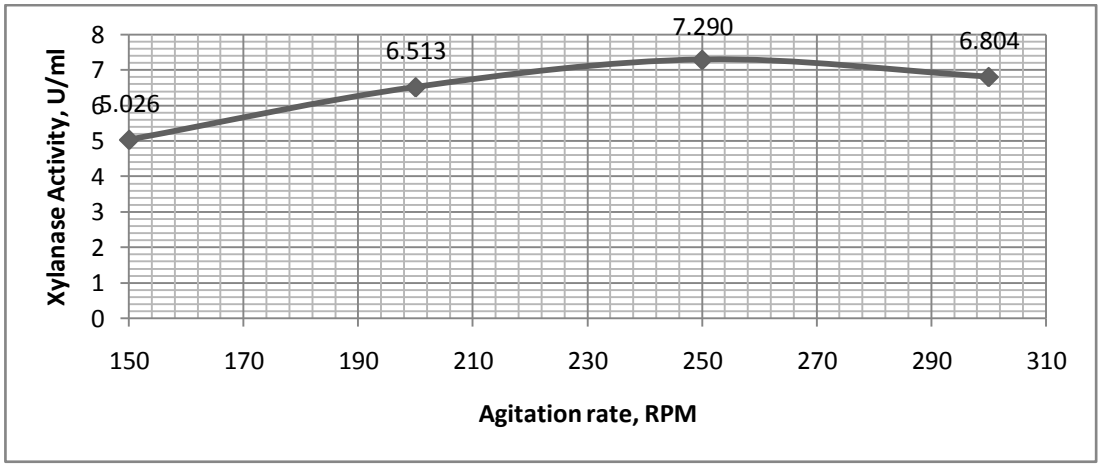

Figure 4.2 : The effect of cultivation agitation rate on recombinant xylanase activity

From Figure 4.2, the maximum activity has achieved at 250 rpm with 7.290 U/ml of recombinant xylanase activity. The recombinant xylanase activity was increased proportionally with agitation rate increment. Kammoun (2008) reported that the α-amylase output increased with increasing the agitation from 150 to 250 rpm. The agitation rate at 250 rpm found to be the best agitation rate and it was selected to be used in further studies using RSM.

As conclusion, the main results from previous study are 35^{o}C and 250 rpm for temperature and agitation rate respectively. Those values will be used in the Response Surface Analysis (RSM) as centered values. The smaller parameter range to be further study in Response Surface Methodology (RSM) for temperature is between 33^{o}C to 38^{o}C and range for agitation rate is between 230 rpm to 280 rpm.

4.2 Determination of the Optimum Temperature and Agitation Rate on Expression of Recombinant Xylanase Using Response Surface Methodology

Optimization of temperature and agitation rate on the expression of recombinant xylanase was carried out using response surface methodology (RSM).

The cultural conditions involved are temperature and agitation rate. By using the Central Composite Design (CCD), the experiment with different combination of temperature and agitation were performed. The combination of data was arranged using Design Expert Software was listed in Table 4.1. The xylose concentration which indicated recombinant xylanase was analyzed by enzyme assay.

The result was analyzed using analysis of variance (ANOVA) as appropriate to the experiment design used. The regression equation (Equation 4.1) below was obtained from the analysis of variance and all term regardless of their significance are included in the following equation.

$$1/Y = +0.11 + 0.012\ X_1 - 3.836\times10^{-3}\ X_2 - 3.383\times10^{-4}\ X_1.X_2 + 0.058\ X_1^2 + 0.015\ X_2^2 \quad \textbf{(Equation 4.1)}$$

Y is the predicted response, X_1 is coded value for temperature and X_2 is coded value for agitation rate. A base of inverse transformation was performed with quadratic as original design model which consist of 1 offset, 2 linear, 2 quadratic and 1 interaction time. The predicted levels of recombinant xylanase activity from recombinant *Trichoderma reesei* in *K. lactis* at each experimental point using Equation 4.1 are given in Table 4.1 along with the experimental result. The highest production of recombinant xylanase which produced 9.358 U/ml of enzyme activity was read on Standard no. 12. The parameters of Standard no. 12 are 35^{o}C temperature 250 rpm. The lowest xylanase activity was recorded at Standard no. 6 with 4.154 U/ml enzyme activities. The cultural parameters for Standard no. 6 were 39.2^{o}C and 250 rpm. Coded activity was the inverse of actual enzyme activity. The inverse mode for transformation was selected as recommended by software to ensure standard transformation falls closest to the best lambda value and is within the confidence interval.

Table 4.1: Central composite design matrix, the predicted and experimental value obtained for the expression of recombinant xylanase.

Standard	Temperature,°C		Agitation, rpm		Xylanase Activity, U/ml			
	Coded	Actual	Coded	Actual	Actual Value	Actual Coded	Predicted Value	Predicted Coded
1	-1	32	-1	220	5.803	0.17	5.687	0.17
2	1	38	-1	220	4.930	0.20	5.147	0.20
3	-1	32	1	280	6.158	0.16	5.254	0.17
4	1	38	1	280	5.221	0.19	5.522	0.19
5	-α	30.8	0.0	250.0	4.703	0.21	4.367	0.21
6	α	39.2	0.0	250.0	4.154	0.24	4.175	0.24
7	0.0	35.0	-α	207.6	6.966	0.14	6.554	0.15
8	0.0	35.0	α	292.4	7.306	0.14	6.514	0.13
9	0	35	0	250	9.100	0.11	9.126	0.11
10	0	35	0	250	9.003	0.11	9.126	0.11
12	0	35	0	250	9.035	0.11	9.126	0.11
13	0	35	0	250	8.938	0.11	9.126	0.11

The coefficient values of Equation 4.1 calculated using Design Expert Software and *P*-value of every term and the interaction are listed in Table 4.2. Based on Table 4.2, the linear term of temperature (X_1), squared terms of temperature (X_1^2), agitation rate (X_2) and squared terms of temperature (X_2^2) are significant model terms that influence the expression of recombinant xylanase due to the *P*-value less than 0.05. The interaction term of X_1X_2 seem to be insignificant. The Table 4.2 shows regression coefficients and *P*-value calculated from the model.

Table 4.2: Regression coefficients and *P*-value calculated from the model

Factor	**Coefficient**	**p-value[a] Prob > F**
Intercept	0.1096	
X_1-Temperature	0.0124	< 0.0001
X_2-Agitation	-0.0038	0.0175
X_1X_2	-0.0003	0.8526
X_1^2	0.0582	< 0.0001
X_2^2	0.0150	< 0.0001

[a] Values of *P*-value less than 0.0500 indicate model terms are significant.

Table 4.3 shows the ANOVA and regression analysis for the production of recombinant xylanase. Determination of coefficient (R^2) and correlation coefficient (R) indicates the precision of a model. Determination coefficient (R^2) implies that the independent variables tested were attributed by the sample variation of 99.66% for xylanase production. The R^2 value also indicates that only 6.49% of the total variation was not explained by the model. Normally, a regression model having an R^2 value higher than 0.9 is considered to have a very high correlation (Haaland, 1989). A better correlation between the experimental and predicted values indicated by the value of R (correlation coefficient) which closer to 1. From R^2 value, the value of R (0.9983) for Equation 4.1 indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. According to the software evaluation, the adjusted R^2 (coefficient of determination) was calculated to be 99.42%. The value indicates a good agreement existed between the experimental and predicted values of xylanase activity.

When significance of the regression model was tested, it was found that *P*-values obtained were small, <0.0001 (Table 4.3) compared to a desired significance level which is 0.05. It is indicates that the regression model was accurate in predicting the pattern of significance to the production of recombinant xylanase.

Table 4.3: ANOVA for response surface quadratic model for the production of xylanase from recombinant *K. lactis*

Source	Sum of Squares	Degree of freedom	Mean	F Value	p-value Prob > F	R^2
Model	0.02534	5	0.005068	411.5556	< 0.0001	0.9966
Residual	8.62E-05	7	1.23E-05			
Lack of Fit	6.97E-05	3	2.32E-05	5.612122	0.0645	
Pure Error	1.65E-05	4	4.14E-06			
Correlation Total	0.025426	12				

To further investigate the effect of the both temperature and agitation rate on recombinant xylanase production, a three dimension (3D) plot was performed by the software. Figure 4.3 is the response surface curves for the both parameters involved. The 3D plot representing the recombinant xylanase activity was a function of both temperature and agitation rate of culture conditions. The 3D plot is very useful as it can show the interaction of all parameters involved. Besides that, the graduated color of the contour can ease the researcher to locate the area of maximum xylanase production lies. The red color region in the curve indicate the area which can produce high activity.

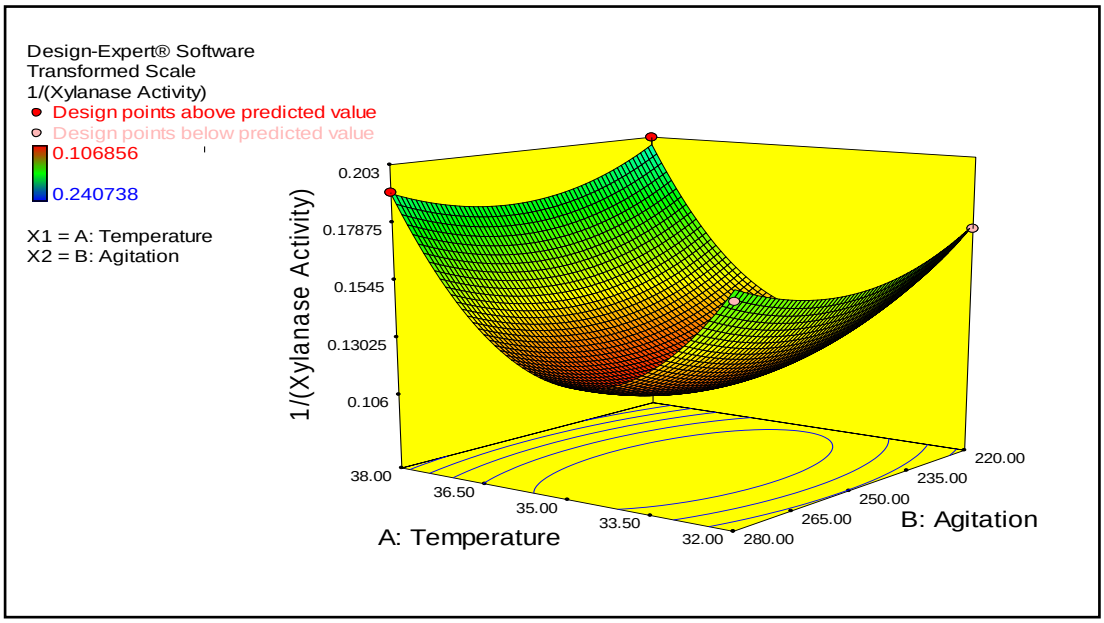

Figure 4.3: Response surface plot of recombinant xylanase production: temperature vs. agitation rate

The culture temperature gave significant effect to the expression of recombinant xylanase. Optimum value of temperature lays in red area in Figure 4.3 which is between 33.5^{o}C until 36.5^{o}C. Out of that range, the activity of xylanase is relatively low. The maximum activity achieved at temperature 34.6^{o}C. Activity recorded at this temperature is 0.109 for coded value and 9.1743 U/ml when converted to actual value. The literature review reported that most amylase production studies have been done with mesophilic fungi having a temperature range of 25 to 37 C (Gupta *et al.*, 2003). This fact also supported by previous research which the temperature of 33^{o}C until 37^{o}C has been used for galactosidase production by many researchers (Ku and Hang, 1992; Hewitt and Wassink, 1984; Artolozaga *et al.*,1998).

From Table 4.2, the both temperature and agitation effect are significant. However, by refering to Figure 4.3, agitation rate give less effect on expression of xylanase activity compare to temperature. It is can be verified by refering to the curve along the agitation axis in Figure 4.3 does not bend too much and almost flatten compare to the curve along the temperature axis.

Eventough the effect of agitation is small, there still have some spot in the curve where the activity can be maximum. From Figure 4.4, the lowest coded activity can be obtained. With temperature of 34.74^{o}C and agitation rate at 258 rpm, the highest activity can be achieved at 0.109091 of coded activity or 9.17 U/ml of actual activity value. Lower or higher agitation rate caused the activity decrease due to the inverse transformation applied to the curve in Figure 4.3.

The agitation rate obtained was suuported by Panesar (2008) which stated that progressive increase in enzyme activity with increase in agitation rate up to 250 rpm was observed. However, no improvement in the enzyme activity was observed at higher agitation rate. The increase in enzyme activity with agitation mode may be attributed to the uniform distribution of the yeast culture in the medium resulting in better nutrient availabilty and oxygen transfer rate. The earlier studies also supported the agitation mode for enzyme production (Pedrique and castillo, 1982; Champluvier *et al*., 1988 Chen *et al*., 1992).

4.2.1 Optimization of Temperature and Agitation Rate on Expression of Recombinant Xylanase

The optimum activity of recombinant xylanase can be predicted using response surface plots that were employed by the software using the Equation 4.1. From the plots in Figure 4.3, the effect of the cultural condition on expression level of the recombinant xylanase can be determined. By choosing the appropriate target at the numerical criteria in the software interface, highest desirability of a pair of temperature and agitation rate was able to determine.

Result from the response surface analysis shown the optimum levels of the predicted variables for maximum activity of xylanase were 34.6^{o}C and 254 rpm for temperature and agitation respectively. The highest activity that can be achieved by the optimized parameters was predicted as 9.1986 U/ml. In order to verify the predicted of cultural condition, an experiment of rechecking was performed according to the proposed parameter. The maximum value of the xylanase activity achieved after optimization of cultural condition is 9.221 U/ml.

Base on Table 4.4, the expression of recombinant xylanase was successfully optimized and the activity was improved about 1.78-fold after the optimization was done. The predicted and experimental enzyme activity that was optimized by cultural conditions is detailed in Table 4.4.

Table 4.4: Summary of the optimized cultural conditions for recombinant xylanase production in *K. lactis.*

Cultural Conditions	**Before Optimization**		**After Optimization**		
	Parameter Values	**Xylanase Activity (U/ml)**	**Parameter Values**	**Xylanase Activity (U/ml)**	
				Predicted	**Experimental**
Temperature	38^{o}C	5.221	34.6°C	9.198	9.221
Agitation rate	280 rpm		254 rpm		

From the Table 4.4, the value of the temperature and agitation rate was reduced after the optimization compared to before optimization. This shows the overall improvement in term of enzyme production and cultural condition. Optimization of temperature, agitation rate and pH medium ran on galactosidase production by Panesar (2008) also shows a progressive result which the production increase by 8.3-fold.

CHAPTER 5

CONCLUSION AND RECOMENDATION

5.1 Conclusion

The research on the optimization of temperature and agitation rate on expression of recombinant xylanase in *K. lactis* was successfully carried out. By using conventional method, one factor at a time (OFAT), the best range of both temperature and agitation rate was able to be determined. The optimum range of temperature was within 32^{o}C to 38^{o}C and agitation rate range was within 220 rpm to 280 rpm. The both values are valuable for further optimization using Response Surface Methodology (RSM).

The objective of this research which is to determine the optimum temperature and agitation on expression of xylanase in *K. lactis* was achieved. The optimized temperature and agitation rate are 34.68^{o}C and 254 rpm respectively. By using the optimized conditions, the activity of recombinant xylanase was 9.281 U/ml. The activity was increased 43.75% or 1.78-fold than initial xylanase activity.

In term of reduction of energy consumption, 8.7% energy for heating can be reduced due to reduction of temperature from 38^{o}C to 34.6^{o}C. The reduction of agitation rate from 280 rpm to 254 rpm leads to 9.3% reduction of energy consumption for agitation power. The increment of xylanase activity and reduction of energy consumption can reduce the operating cost. Therefore, the expression of recombinant xylanase was successfully optimized and improved in term of

production and energy consumption by using Response Surface Methodology (RSM).

5.2 Recommendation

To obtain more significant increment in optimization of recombinant xylanase activity, additional of more parameter such as pH buffer, type of medium culture or concentration of substrate may increase the xylanase production. Optimization of many parameters will give the best cultural condition for recombinant xylanase expression. The production can be multiple folds than initial production.

For further study of optimization on temperature effect, using temperature shift due to growth phase changes in culture growth might give significant effect. It is because each growth phase needs different culture temperature because biochemical reaction which depends to temperature in each phase is different.

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APPENDIX A

MATERIALS AND METHODS

Appendix A1

Buffer Table

Buffer Table A-1: Ratios of acid and base to provide adequate pH

0.1 M citric acid	**0.1 M sodium citrate**	**pH**
46.5	3.5	3
43.7	6.3	3.2
40	10	3.4
37	13	3.6
35	15	3.8
33	17	4
31.5	18.5	4.2
28	22	4.4
25.5	24.5	4.6
23	27	4.8
20.5	29.5	5
18	32	5.2
16	34	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	41.5	6
7.2	42.8	6.2

(Gomori, 1955) stock solutions: **A**: 0.1 M citric acid; **B**: 0.1 M sodium citrate. Use *x* ml A + *y* ml B and dilute to 100 ml with 50 ml DI.

Appendix A2

DNS preparation:

Chemical Involved:

10g Sodium Hydroxide

182g Pottasium sodium tartarate

10g Dinitrosalicylic acid

2g Phenol

0.5g Sodium sulfite

Solving al the chemicals in 600 ml of distilled water. Stir the solution until all materials dissolved.The volume of the solution is adjust to 1L.The solution should be stored in dark Schott bottle

Appendix A3

Standard Curve of xylanase activity

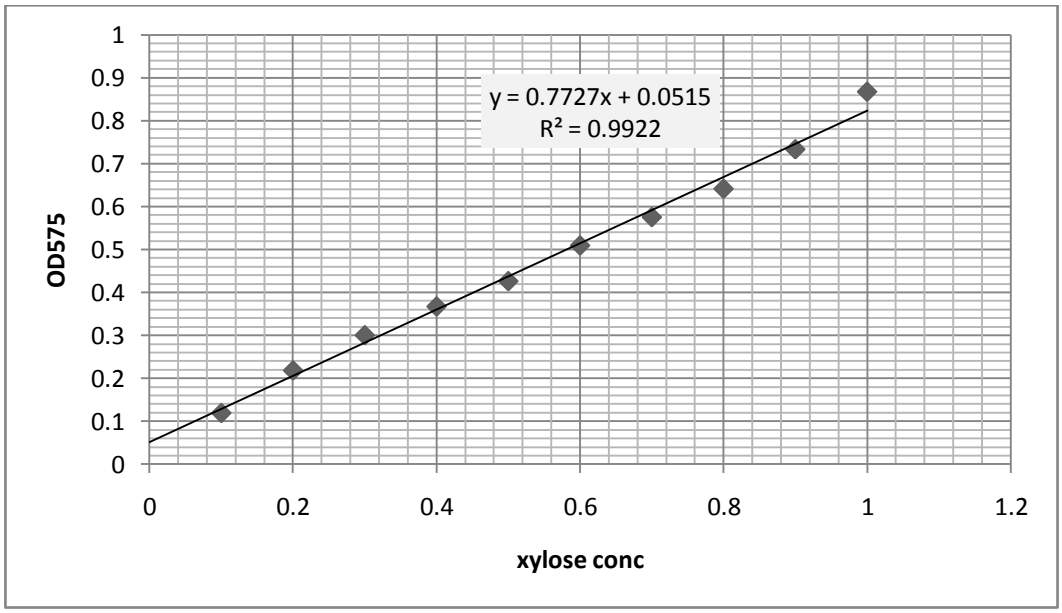


Figure A-3 : Standard curve of xylanase activity

Appendix A4

Standard Recombinant Xylanase Reaction Condition

Standard Enzyme Reaction Condition was done using one factor at a time (OFAT). The standard recombinant xylanase reaction condition for temperature, incubation time and pH buffer are 60°C, 20 minutes and pH 5. The following graphs is the result of using OFAT in determine standard recombinant xylanase reaction condition.

OFAT of Incubation Temperature of Xylanase Reaction Period

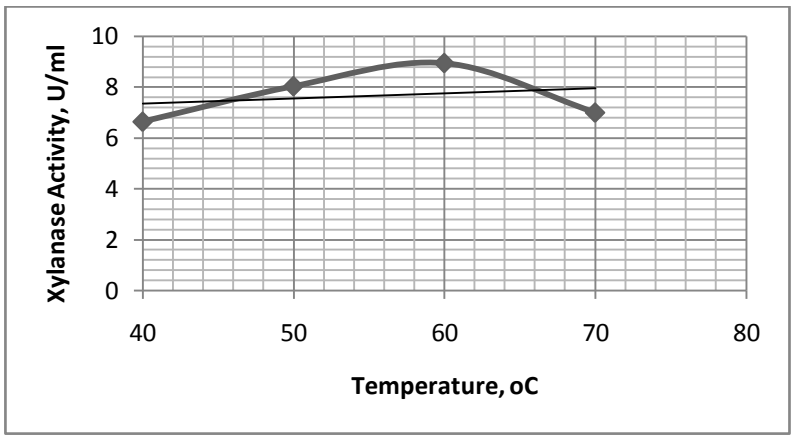


Figure A-4: Plots of incubation temperature vs. xylanase activity.
The best temperatre is 60°C

OFAT of Incubation Duration of Xylanase Reaction Period

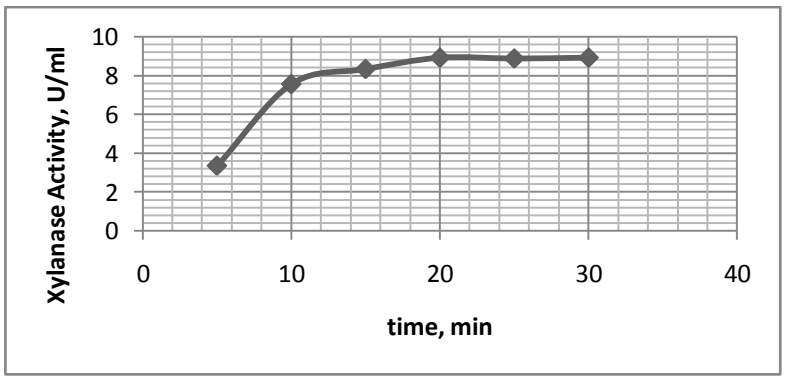


Figure A-5 : Plots of incubation time vs. Xylanase Activity.
The best incubation time is 20 minutes

OFAT of Incubation Temperature of Xylanase Reaction Period

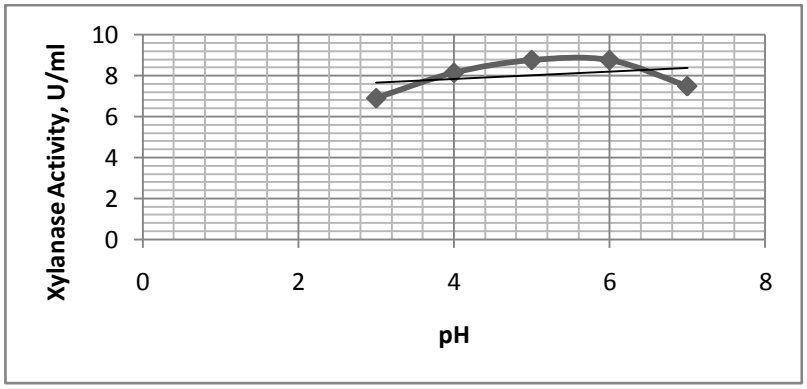


Figure A-6: Plots of incubation temperature vs. xylanase activity. The best pH buffer for incubation is pH 5

APPENDIX B

RESULT AND DISCUSSION

Appendix B1

Experiment Data

Table B-2 : OFAT on Temperature

Temperature	OD Black	OD xylanase	Enzyme activity
25	0.02	0.26	5.223
30	0.06	0.284	6.425
35	0.03	0.315	8.389
40	0.014	0.296	7.483

Table B-2: OFAT on agitation rate

Agitation rate	OD Black	OD xylanase	Enzyme activity
150	0.05	0.207	5.170519
200	0.06	0.253	6.319523
250	0.09	0.277	7.290
300	0.02	0.262	6.544328

Table B-3 : Experiment data based on RSM suggested parameter

Standard	Temperature	Agitation rate	OD blank	OD xylanase	Xylanase activity
1	32	220	0.016	0.236	5.640899
2	38	220	0.009	0.218	5.091356
3	32	280	0.004	0.197	5.285312
4	38	280	0.013	0.22	5.414616
5	30.75736	250	0.024	0.226	4.541812
6	39.24264	250	0.004	0.192	4.218552
7	35	207.5736	0.013	0.269	6.610681
8	35	292.4264	0.021	0.295	6.739985
9	35	250	0.015	0.348	9.099788
10	35	250	0.015	0.245	9.00281
11	35	250	0.015	0.352	9.229092
12	35	250	0.015	0.356	9.358396
13	35	250	0.015	0.343	8.938158

Appendix B2

RSM Analyzing

Table B-4 : ANOVA data

Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	0.025	5	5.07E-03	411.56	< 0.0001	significant
A-Temperature	1.24E-03	1	1.24E-03	100.4	< 0.0001	
B-Agitation	1.18E-04	1	1.18E-04	9.56	0.0175	
AB	4.58E-07	1	4.58E-07	0.037	0.8526	
A^2	0.024	1	0.024	1916.2	< 0.0001	
B^2	1.57E-03	1	1.57E-03	127.29	< 0.0001	
Residual	8.62E-05	7	1.23E-05			
Lack of Fit	6.97E-05	3	2.32E-05	5.61	0.0645	not significant
Pure Error	1.66E-05	4	4.14E-06			
Cor Total	0.025	12				

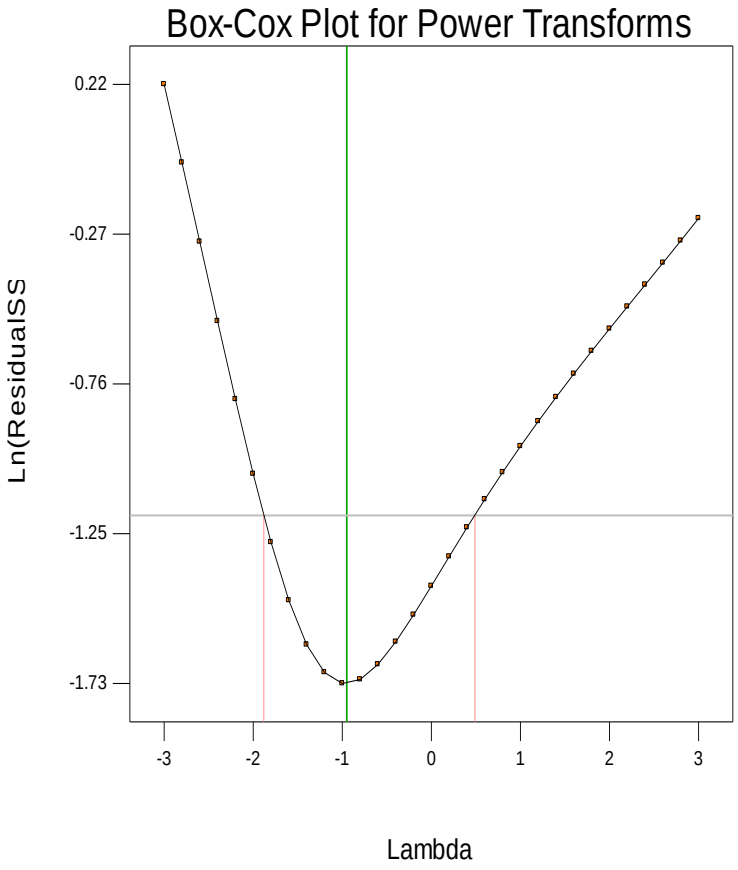
Appendix B3

Box Cox Analysis

Design-Expert® Software
1/(OD)

Lambda
Current = -1
Best = -0.95
Low C.I. = -1.88
High C.I. = 0.49

Recommend transform:
Inverse
(Lambda = -1)



Appendix B4

RSM Analyzing Report

Table B-4: Diagnostic Report

					Internally	Externally	Influence on		
Actual	Predicted			Standard	Studentized	Studentized	Fitted Value	Cook's	Run
Value	Value	Residual	Leverage	Order	Residual	Residual	DFFITS	Distance	Order
0.17	0.17	-1.59E-03	0.625	1	-0.74	-0.714	-0.921	0.152	3
0.2	0.2	3.38E-03	0.625	2	1.574	1.814	* 2.34	0.688	6
0.16	0.17	-4.55E-03	0.625	3	-2.116	-3.262	* -4.21	* 1.24	1
0.19	0.19	4.27E-04	0.625	4	0.199	0.185	0.238	0.011	13
0.21	0.21	4.10E-03	0.625	5	1.907	2.548	* 3.29	* 1.01	10
0.24	0.24	-2.94E-03	0.625	6	-1.366	-1.477	-1.906	0.518	5
0.14	0.15	-1.51E-03	0.625	7	-0.702	-0.674	-0.87	0.137	11
0.14	0.13	2.67E-03	0.625	8	1.243	1.304	1.684	0.429	7
0.11	0.11	2.81E-04	0.2	9	0.09	0.083	0.041	0	2
0.11	0.11	1.47E-03	0.2	10	0.467	0.439	0.219	0.009	12
0.11	0.11	-1.26E-03	0.2	11	-0.401	-0.376	-0.188	0.007	9
0.11	0.11	-2.76E-03	0.2	12	-0.878	-0.862	-0.431	0.032	4
0.11	0.11	2.27E-03	0.2	13	0.723	0.696	0.348	0.022	8