Cloning and Expression of Pullulanase a Gene from Locally Isolated Bacillus

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ABSTRACT

A pullulanase-producing bacteria has been identified as *Exiguobacterium sp. MAAC-1* using 16S rRNA gene sequence analysis. *Exiguobacterium sp*. MAAC-1 achieved optimum pullulanase production at 22 hours incubation at 37 ^{o}C in modified Peptone Yeast Extract (PYE) medium. The optimum temperature and pH of the crude enzyme were 60 ^{o}C and pH 9.0 respectively. Plackett-Burman design was applied in the screening process of 17 nutrients for pullulanase production and five nutrients were identified as significant and effective factors for the pullulanase production. The five significant factors are sago starch, NH_4Cl, Na_2HPO_4, KCl and $MgSO_4$. The five nutrients were selected for further optimization studies using Central Composite Design (CCD). Optimum pullulanase production was achieved using 3.86%w/v sago starch, 0.002% w/v NH_4Cl, 0.05%w/v Na_2HPO_4, 0.015%w/v KCl and 0.025 %w/v $MgSO_4$, with predicted pullulanase activity, 1.252 U/ml. The experimental pullulanase activity was achieved at 1.208 U/ml. About 9.6-fold increment of pullulanase production was achieved after medium optimization process. For the pullulanase gene isolation, 1177 bp of partial pullulanase gene was amplified and showed the highest homology of 60 % with pullulanase gene from *Exiguobacterium sp*. 255-1. The four conserved regions of amylolytic enzyme, conserved region I, II, III and IV, and a highly conserved region of pullulanase type I (motif YNWGYDP) were found in the partial pullulanase gene.

ABSTRAK

Bakteria yang menghasilkan pullulanase telah dikenalpasti sebagai *Exiguobacterium sp. MAAC-1* melalui analisis jujukan gen 16S rRNA. *Exiguobacterium sp*. MAAC-1 menghasilkan pullulanase secara optimum pada 22 jam pengeraman pada 37 ^{o}C dalam media Peptone Yeast Extract (PYE) yang diubahsuai. Suhu dan pH optimum untuk penghasilan pullulanase adalah pada suhu 60 ^{o}C and pH 9.0. Rekabentuk Plackett-Burman telah diaplikasi untuk proses penyaringan 17 nutrient untuk penghasilan pullulanase. Lima nutrient telah dikenalpasti sebagai nutrient yang yang memberi kesan efektif terhadap penghasilan pullulanase. Nutrien yang memberi kesan efektif adalah kanji sagu, NH_4Cl, Na_2HPO_4, KCl dan $MgSO_4$. Kelima-lima nutrient dipilih untuk pengoptimuman menggunakan Rekabentuk Central Composite (CCD). Pullulanase optimum dihasilkan menggunakan 3.86% (w/v) kanji sagu, 0.002% (w/v) NH_4Cl, 0.05%(w/v) Na_2HPO_4, 0.015% (w/v) KCl and 0.025 % (w/v) $MgSO_4$, dengan anggaran aktiviti pullulanase , 1.252 U/ml berbanding dengan aktiviti pululanase secara eksperimental 1.208 U/ml. Penghasilan pullulanase aktiviti secara optimum adalah 9.6 kali ganda berbanding dengan sebelum pengoptimuman medium. Separa pemencilan gene pullulanase dengan 1177bp mempunyai homology tertinggi adalah 60% bersamaan dengan *Exiguobacterium sp*. 255-1. Empat konserv region untuk enzyme amylolitik iaitu konserv region I, II, III and IV, dan konserv region tertinggi pullulanase type I (motif YNWGYDP) ditemui dalam gen pullulanase yang dipencilkan secara separa.

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CHAPTER I

INTRODUCTION

1.1 Introduction

Pullulanase (EC 3.2.1.41) is one of the members from the family of 13 glycosyl hydrolases, which is also called α-amylase family. Pullulanase has an interesting feature, where it can specifically attack branch points in pullulan, starch and dextrins. Pullulanases are classified as Type I and Type II. Type I pullulanases are able to hydrolyze α-1,6-glucosidic linkages, and Type II pullulanases are able to hydrolyze α-1,6-glucosidic linkages and also α-1,4-glucosidic linkages.

Since pullulanase was discovered by Bender and Wallenfels in 1961, the enzyme has been detected or isolated from many sources. The presense of pullulanase has been reported from mesophiles, e.g., *Bacillus sp*. strain KSM-1378 (Ara *et al*., 1995), *Bacillus sp*. strain S-1 (Lee *et al*., 1997), *Bacillus sp*. strain KSM-1876 (Hatada *et al*., 2001), etc. Besides, pullulanase also has been found from thermophiles, e.g. *Anaerobranca gottschalkii* (Bertoldo *et al*., 2004), *Bacillus thermoleovorans* US105 (Messaoud *et al*., 2002), and also from hyperthermophiles *Fervidobacterium pennavorans* Ven5 (Bertoldo *et al*., 1999), *Rhodothermus marinus* (Gomes *et al*., 2003), etc.

Due to the specific debranching ability, the use of pullulanase in starch-processing industry is mostly promoted. Pullulanase is employed in the saccharification process to enhance the efficiency of the process. Beside, the combined application of pullulanase with other amylolytic enzymes may increase

the quality of sugar syrups. The products from the starch-processing have wide application in various industries, such as beverages, confectionary, canning, ice-cream, etc. Pullulanase can also be applied in baking industry and detergent industry. Recently, there are some reports on the application of pullulanase in the synthesis of branched-CD. Those reports also stated that the branched-CDs, especially heterobranched-CDs, can contribute to the pharmaceutical field as drug-carrier due to its higher aqueous solubility and cell-targeting ability.

The wide application has encouraged studies on pullulanase from various microorganisms isolated from different location. Medium development is one of the important aspects to enhance pullulanase production. The culture condition and medium composition will greatly influence the pullulanase production and also the production cost. For the industrial purpose, high pullulanase production but involving lower cost is an important aspect to be considered in production studies. Besides, the studies using molecular biology techniques may also improve the pullulanase enzyme and the production. The molecular studies also provided better understanding on the reaction mechanism.

1.2 Objective

The objective of this research is to enhance pullulanase production by *Exiguobacterium sp.* MAAC-1 through statistical screening and optimization. Besides, part of the pullulanase gene was also isolated.

1.3 Scope

The scope of this research includes:

(a) screening of pullulanase-producer
(b) identifying the bacteria at phylogenetic level
(c) screening of best nutrients for pullulanase production using experimental design
(d) enhancing pullulanase production using experimental design
(e) isolating part of pullulanase gene

CHAPTER II

LITERATURE REVIEW

2.1 Starch

Starch ranks among the most abundant carbohydrate polymers on Earth. Starch is found in many plants as a food reserve polysaccharide, and it represents carbon as well as energy source for animals, higher plants and various microorganisms (Janeček, 1997). Starch is a biopolymer consisting of α-D-glucose residues connected to form large macromolecules (Nigam and Singh, 1995; Kujawski *et al*., 2002). Starch is composed of two polymers: amylose and amylopectin. Depending on the plant sources, the average amylose and amylopectin content in starches vary between 15-25 % and 75-85 %, respectively (Gomes *et al*., 2003; Koch *et al.*, 1997). Amylose is an essentially linear polymer consisting of up to 6000 glucose residues linked through α-1,4 glycosidic bonds. Amylose has a double helical structure with the helix contains six D-glucose residues per turn. Amylopectin is a highly branched polymer that consist of short α-1,4 linked linear chains of 10-60 glucose residues and α-1,6 linked side chains with 15-45 glucose residues. The complete amylopectin polymer contains around 2000000 glucose residues. Amylopectin contains 4-6 % of branching point averagely depending on the plant origin (Nigam and Singh, 1995).

2.2 Starch-processing Enzymes

The application of enzymes in many processes has been known since the ancient Greece century. They used enzymes from microorganisms in baking, brewing, alcohol production, cheese making and so on.

Nowadays, the starch processing industry is getting important due to the high demand on products from starch processing. The starch industry is one of the major users of enzymes for hydrolysis of starch to useful products. Most agriculture biomass containing starch, such as tapioca, sago, wheat is potential substrate for the production of gaseous or liquid fuels, feed proteins, and chemicals by microbial processes (Nigam and Singh, 1995).

Industrial hydrolysis of starch to low-molecular-weight dextrin and glucose is done chemically or enzymatically. In the past decades, there is a shift from the acid hydrolysis of starch to the use of starch-processing enzymes. Traditional chemical hydrolysis has been prevailed by enzymatic treatment due to enzymatic hydrolysis having several advantages (Nigam and Singh, 1995). First, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in fewer side reactions and causing less unwanted changes like “browning”. Enzymes applied in starch-processing industry are usually used to hydrolyze α- 1,4- glycosidic bonds or amylopectin branches.

Generally, there are four groups of starch-processing enzymes, which are endoamylases, exoamylases, debranching enzymes and transferases (van der Maarel *et al*., 2002). Figure 2.1 depicts the action patterns of starch-hydrolyzing enzymes.

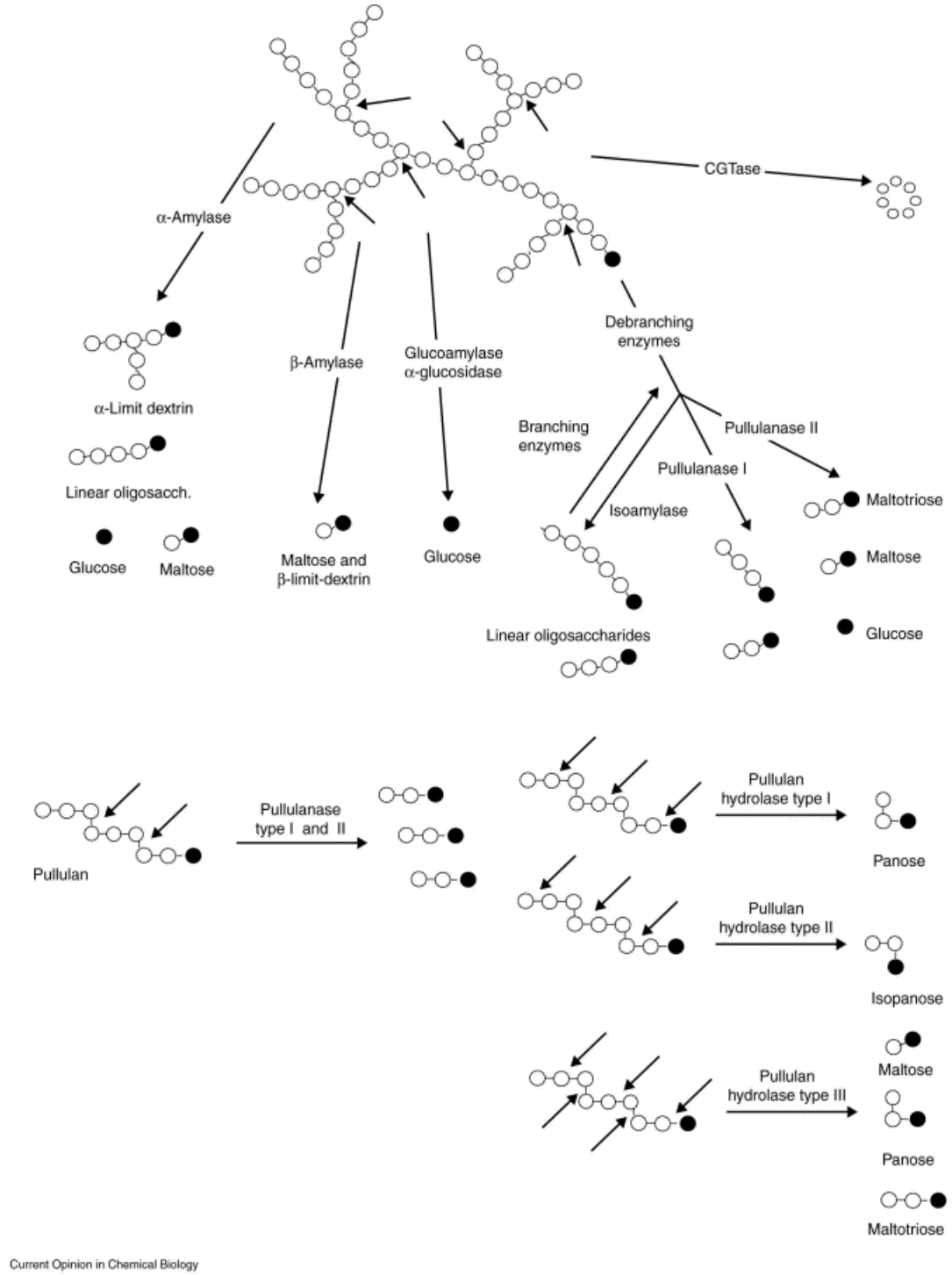


Figure 2.1: The action patterns of starch-hydrolyzing enzymes (Bertoldo and Antaranikian, 2002)

Enzymes of endoamylases, such as α-amylase (EC 3.2.1.1), are able to hydrolyze internal α-1,4- glycosidic bonds in amylase and amylopectin chain producing oligosaccharides with varying length with an α-configuration and α-limit dextrins.

Exoenzymes only attack external glycosidic linkages. β-amylase (EC 3.2.1.2), amyloglucosidase or glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) are the member of this group. β-amylase exclusively hydrolyze α-1,4-glycosidic linkages producing β-limit dextrin, while glucoamylase and α-glucosidase hydrolyze α-1,4- and α-1,6-glycosidic linkages producing glucose and maltose.α-glucosidase is better in hydrolyzing linkages in short maltooligosaccharides and liberates glucose with an α-configuration while glucoamylase hydrolyzes long-chain polysaccharides better. β-amylase and glucoamylase are able to convert the anomeric configuration of the liberated maltose from α to β (van der Maarel *et al*., 2002). Besides, there are many other enzymes in exoamylases group. For example, cyclodextrin glycosyltransferase (EC 2.4.1.19) has transglycosylation activity and maltogenic α-amylase (or glucan α-1,4-glucanhydrolase, EC 3.2.1.133) which is a kind of amylase from *Bacillus stearothermophilus* producing maltose, and so on.

Debranching enzymes are the third group of starch-degrading enzymes, which are able to attack branching point or α-1,6- glycosidic linkages. Isoamylase (EC 3.2.1.68) and pullulanase type I (3.2.1.41) are debranching enzymes that specially hydrolyze α-1,6- glycosidic linkages producing long linear polysaccharides. The difference between pullulanases and isoamylase is the ability to hydrolyze pullulan. Pullulanases are able to hydrolyze the α-1,6- glycosidic linkages in pullulan and amylopectin, while isoamylase can only hydrolyze α-1,6 linkages in amylopectin. Pullulanase type II is able to hydrolyze both α-1,4 and α-1,6 glucosidic linkages. Pullulanase type II is also referred as amylopullulanase or amylase-pullulanase.

The last group of starch-degrading enzymes is transferases, which cleave an α-1,4-glycosidic linkage of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond (van der Maarel *et al*., 2002). The member of this group are amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) that form a new α-1,4-glycosidic linkage, and branching enzyme (EC 2.4.1.18) that form α-1,6-glycosidic linkage. The difference between amylomaltase and cyclodextrin glycosyltransferase is the end product from the transglycosylation reaction, where amylomaltase produces a linear product while cyclodextrin glycosyltransferase produce cyclic product with 6, 7, or 8 glucose residues.

2.3 The α-amylase Family

All enzymes known to date are grouped or classified based on the recommendations of the International Union of Biochemistry and Molecular Biology (IUBMB). This enzyme classification is based on two characteristics, the reaction catalyzed and the substrate specificity (Kuriki and Imanaka, 1999). Enzymes hydrolysing the *O*-glycosyl linkage of starches or sugars are so-called glycoside hydrolases. There are several classification systems for glycoside hydrolases, including the IUBMB classification that is based on substrate and product specificity, mode of attack (*exo* or *endo*) and stereochemical mechanism (Henrissat and Davies, 1997). The IUBMB classification of glycoside hydrolase is expressed in the EC number for a given enzyme. *O*-glycoside hydrolases are given the code EC 3.2.1.x, where x represents the substrate specificity, molecular mechanism or the type of linkage (Henrissat and Davies, 1997). Some controversies occurred when those classifications fail to reflect the 3D structural features of these enzymes. Moreover, the classifications also fail to account for the evolutionary and mechanistic resemblance of the enzymes (Henrissat and Davies, 1997).

Therefore, a new classification of glycoside hydrolases based on amino acid sequence similarities was proposed in 1991. This classification has been frequently updated and is currently most accessed on the web (http:www.expasy.ch/cgi-

bin/list?glycosid.txt). Based on the presence of the common features in amino acid sequences, glycoside hydrolases and related enzymes have been classified together with *O*-glycosyl hydrolases into more than 60 sequence-based families (Janeček, 1997; Henrissat and Davies, 1997). β-amylase and glucoamylase form their own families, family 14 and 15, respectively. The rest of glycoside hydrolases are classified into the family 13 glycosyl hydrolases or α-amylase family (Janeček, 1997). Remarkably, a few transferases (EC 2.4.1.x), such as cyclodextrin glycosyltransferase, amylomaltase, etc. are classified together with glycoside hydrolases due to their glycosyl hydrolase/transferase activity.

The α-amylase family enzymes are multidomain enzymes, however, they share a common catalytic domain in the form of a parallel $(\alpha/\beta)_8$ barrel, which is a barrel of eight parallel β-strands surrounded by eight helices, the so-called domain A. The domain A also is the most conserved domain in all α-amylase family enzymes. Besides, the α-amylase family share a common characteristic that all the enzymes employ the α-retaining mechanism but they vary widely in their substrate and product specificities.

The α-amylase family enzymes should have the following criteria (Kuriki and Imanaka, 1999; van der Maarel *et al*, 2002):

i) They act on α-glycosidic linkages
ii) They hydrolyze α-glycosidic linkages to produce α-anomeric mono- or oligosaccharides by hydrolysis, or form α-1,4 or α-1,6 glycosidic linkages by transglycosylation, or a combination of both activities.
iii) They have four highly conserved regions in their primary sequence, which contain all the catalytic, and most of the important substrate-binding sites.
iv) They posses a $(\beta/\alpha)_8$ or TIM barrel structure containing Asp, Glu and Asp residues as catalytic sites corresponding to Asp-206,Glu-230, and Asp-297 of Taka-amylase A.

The enzymes of the α-amylase family that match the criteria above are listed in Table 2.1.

Table 2.1: The members of the α-amylase family (van der Maarel *et al*., 2002)

Enzyme	EC number	Main substrate
Amylosucrase	2.4.1.4	Sucrose
Sucrose phosphorylase	2.4.1.7	Sucrose
Glucan branching enzyme	2.4.1.18	Starch, glycogen
Cyclodextrin glycosyltransferase	2.4.1.19	Starch
Amylomaltase	2.4.1.25	Starch, glycogen
Maltopentaose-forming amylase	3.2.1.-	Starch
α-Amylase	3.2.1.1	Starch
Oligo-1,6-glucosidase	3.2.1.10	Amylopectin
α-Glucosidase	3.2.1.20	Starch
Pullulanase	3.2.1.41	Pullulan
Amylopullulanase	3.2.1.41/3.2.1.1	Pullulan
cyclomaltodextrinase	3.2.1.54	cyclodextrin
Isoamylase	3.2.1.68	Amylopectin
Isopullulanase	3.2.1.57	Pullulan
Maltotetraose-forming amylase	3.2.1.60	Starch
Glucodextranase	3.2.1.70	Starch
Trehalose-6-phosphate hydrolase	3.2.1.93	Trehalose
Maltohexaose-forming amylase	3.2.1.98	Starch
Maltogenic amylase	3.2.1.133	Starch
Neopullulanase	3.2.1.135	Pullulan
Malto-oligosyl trehalase hydrolase	3.2.1.141	Trehalose
Malto-oligosyl trehalase synthase	5.4.99.15	Maltose

2.3.1 Catalytic Mechanism

According to MacGregor *et al*. (2001), all the glycoside hydrolases have a similar catalytic mechanism throughout the family, and the catalytic amino acid residues are conserved in all the enzymes. Briefly, there are two major catalysis mechanisms that two amino acid residues participate in a single-displacement or double-displacement reaction resulting in inversing or retaining the configuration, respectively (Stam *et al*., 2005; Henrissat, 1991). Generally, all enzymes of α-amylase family employ the α-retaining double-displacement catalytic mechanism.

The anomeric configuration is retained when the enzymes act on α-linkages in glucosides and yields α-linked products via the double-displacement mechanism (Figure 2.2).

The first displacement start after the substrate has bound in the active site. MacGregor *et al*. (2001) proposed that the active site of α-amylase family is made-up of a number of subsites, where each subsite can interact with one glucose residue of the substrate (Figure 2.3). Cleavage occurs between subsites -1 and +1 as indicated by the arrow and the reducing end group is bound at subsite +3. In the first displacement (Janeček, 1997; van der Maarel *et al*., 2002), the conserved glutamic acid (Glu230; Taka-amylase A numbering will be used throughout this thesis), which is in the acid form, protonates the oxygen of the glucosidic linkage located between two glucose molecules at the subsites -1 and +1. Meanwhile, the nucleophilic aspartate (Asp206), a general base catalyst, and attacks the C1 of glucose at subsite -1 and transient formation of an oxocarbenium ion-like transition state. Then, a nucleophilic acid group of the enzyme attacks at the sugar anomeric centre to give a β-glycosyl enzyme intermediate while the aglycone of the substrate leaves the active site.

In the second displacement, hydrolysis process is involved. During the second displacement the protonated glucose molecule at subsite +1 leaves the active site. Then, a water molecule or a new glucose molecule moves into the active site, and attacks the covalent bond between the glucose molecule at subsite -1 and the aspartate (Asp206) (MacGregor *et al*., 2001). Again, an oxocarbonium ion-like transition state is formed. After that, the base catalyst accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subsite -1 and the aspartate forming a new hydroxyl group at the C1 posotion of the glucose at subsite -1 (hydrolysis) or a new glycosidic linkage between the glucose at subsite -1 and +1 (transglycosylation) (van der Maarel *et al*., 2002).

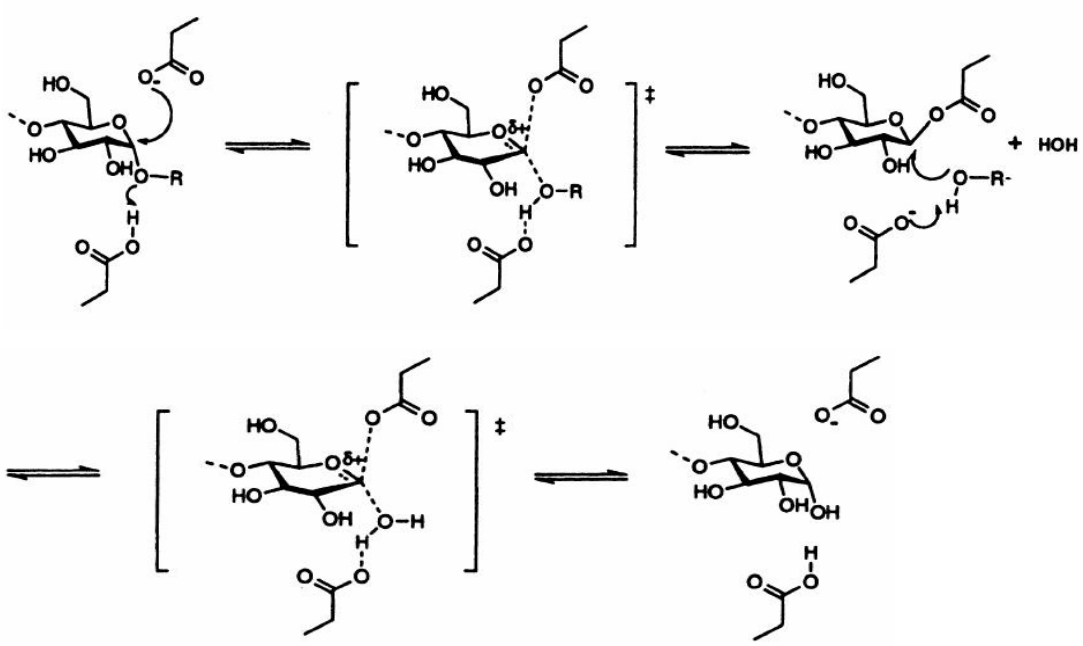


Figure 2.2: The steps of catalytic double-displacement mechanism of α-amylase family (MacGregor *et al*., 2001)

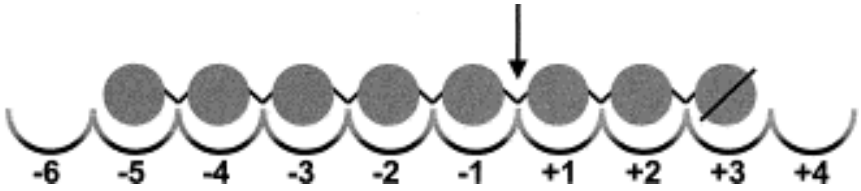


Figure 2.3: Schematic of subsite arrangement with oligosaccharide occupying subsites -5 through +3 (MacGregor *et al*., 2001)

2.3.2 Conserved Regions

The α-amylase family have a very interesting feature. The enzymes exhibit generally very low degree of sequence similarity, they contain several well-defined and highly conserved regions in their amino acid sequences. Kuriki and Imanaka (1999) has pointed out that the α-amylase family enzymes have four highly conserved regions in their primary sequence. Table 2.2 shows the four conserved regions and the corresponding β-sheets found in the amino acid sequence of enzymes from α-amylase family. The four conserved regions are easily identifiable

in all enzyme specificities in α-amylase family. Thus, the four conserved regions are believed to form the catalytic and substrate-binding sites, as well as some of the amino acid of the conserved regions are essential for the stability of the conserved TIM barrel topology (van der Maarel *et al*., 2002; Kuriki and Imanaka, 1999).

Although different enzymes of α-amylase family have different linkage-attack specificities but they act on the same α-linked glucose residue that are located at subsite -1, strong similarities should be found in the amino acids that make up the subsite. The five amino acids are: the proton donor Glu230, the catalytic nucleophile Asp206, the second conserved aspartic acid Asp297, and two histidine residues His122 and His296, were confirmed as being important at subsite -1 (MacGregor *et al*., 2001). The three amino acid residues (Asp206, Glu230 and Asp297) were identified to play an important role in the catalysis mechanism of α-amylase family enzymes, and are found at the centre of the active site (Kuriki and Imanaka, 1999; Janeček, 1997; MacGregor *et al*., 2001). Two conserved hisditine residues, which are His122 and His296, are involved in the substrate recognition in the active site of the α-amylase family enzymes. Besides, the segment around the Asp175 is involved in the binding of a calcium ion (Janeček, 1997).

For all enzymes in α-amylase family, the specificity of the enzymes varies due to the nature of substrate binding at subsites +1 and +2. Therefore, the amino acid

Table 2.2: The four conserved regions and the corresponding β-sheets found in the amino acid sequence of enzymes from α-amylase family. Invariable three catalytic sites are indicated by bolded capital letter (Kuriki & Imanaka, 1999; Janecek, 1997)

Enzyme	Origin	Region 1 β3	Region 2 β4	Region 3 β5	Region 4 β7
α-Amylase	*Aspergillus oryzae*	117 DVVANH	202 GLRI**D**TVKH	230 **E**VLD	292 FVENH**D**
CGTase	*Bacillus macerans*	135 DFAPNH	225 GIRF**D**AVKH	258 **E**WFL	324 FIDNH**D**
Pullulanase	*Klebsiella aerogenes*	600 DVVYNH	671 GFRF**D**LMGY	704 **E**GWD	827 YVSKH**D**
Isoamylase	*Pseudomonas amyloderamosa*	292 DVVYNH	371 GFRF**D**LASV	435 **E**PWA	505 FIDVH**D**
Branching enzyme	*Escherichia coli*	335 DWVPGH	401 ALRV**D**AVAS	458 **E**EST	521 LPLSH**D**
Neopullulanase	*Bacillus stearothermophilus*	242 DAVFNH	324 GWRL**D**VANE	357 **E**IWH	419 LLGSH**D**
Amylopullulanase	*Clostridium thermohydrosulfulcum*	488 DGVFNH	594 GWRL**D**VANE	627 **E**NWN	699 LLGSH**D**
α-Glucosidase	*Saccharomyces carlsbergenesis*	106 DLVINH	210 GFRI**D**TAGL	276 **E**VAH	344 YIENH**D**
Clyclodextrinase	*Thermoanaerobacter ethanolicus*	238 DAVFNH	321 GWRL**D**VANE	354 **E**VWH	416 LIGSH**D**
Oligo-1,6-glucosidase	*Bacillus cereus*	98 DLVVNH	195 GFRM**D**VINF	255 **E**MPG	324 YWNNH**D**
Dextran glucosidase	*Streptococcus mutans*	98 DLVVNH	190 GFRM**D**VIDM	236 **E**TWG	308 FWNNH**D**
Amylomaltase	*Streptococcus pneumoniae*	224 DMWAND	291 IVRI**D**HFRG	332 **E**ELG	391 YTGTH**D**
Glucan debranching enzyme	Rice	502 DVVYNH	572 GFRF**D**LMGH	613 **E**GWD	740 YVSAH**D**

residues that make up subsites +1 and +2 of an enzyme may be varied depending on the specificity of the enzyme (MacGregor *et al*., 2001). The amino acids residues in conserved regions II and III make up the subsite +1 and subsite +2. Particularly, the amino acid residues of 209, 210, 231, 232 and possibly 233 and 234, may play an essential role in the specificity of bond-type of enzymes. However, some of the roles of these amino acid residues are still not clearly confirmed yet.

2.4 Pullulanases

Pullulanase (pullulan α-glucano-hydrolase; EC 3.2.1.41) is an extracellular carbohydrase, was first found by Bender and Wallenfels (1961) from mesophilic organisms, *Klebsiella pneumoniae* (formerly known as *Aerobacter aerogenes* or *Klebsiella aerogenes*). Pullulanases are defined as debranching enzymes that are able to hydrolytically cleave the α-1, 6 glucosidic bonds of the linear α-glucan pullulan producing maltotriose as the final product (Lévêque *et al*., 2000; Kriegshäuser and Liebl, 2000; Bertoldo *et al*., 2004). Depending on their inability or ability to hydrolyze α-1,4 glucosidic linkages in other polysaccharides, pullulanases are divided into two categories based on substrate specificity: type I and type II pullulanase respectively. Type I pullulanase specifically hydrolyze the α-1,6 linkages in branched oligosaccharides, such as pullulan, starch, amylopectin and glycogen, forming linear α-1,4-linked oligomers. Type II pullulanase or amylopullulanases are able to hydrolyze α-1,6 glycosidic linkages in pullulan and branched substrates in addition to the α-1,4 glycosidic linkages in polysaccharides (Bertoldo *et al*., 2004).

Pullulan is a biodegradable water-soluble polysaccharide, which is produced by the yeast-like fungus *Aureobasidium pullulans* (formerly known as *Pullularia pullulans* or *Dematium pullulans*) (Leathers, 2005; Marshall, 1973). Pullulan is a linear polymer of maltotriose (shown in Figure 2.4), units of three a-1,4-linked glucose molecules, which are polymerized in a linear fashion via a-1,6-linkages. Pullulan serves as a model substrate for pullulanase. Pullulanases particularly

hydrolyze the α-1,6 linkages in pullulan and convert the polymer almost quantitavely to maltotriose.

According to Drummond *et al*. (1969), pullulanases are able to attack pullulan by either one of the two action patterns shown in Figure 2.5. Both action patterns are exo and endo enzyme actions. The hydrolysis of exo action is restricted to the α-1,6 linkage nearest to the terminal of non-reducing end (or reducing end), with the stepwise release of maltotriose as the only low-molecular-weight product of the reaction. The endo action, however, in which initial hydrolysis can occur at internal as well as external α-1,6 linkages with the intermediate production of hexa-, nona- and larger oligosaccharides, in addition to maltotriose.

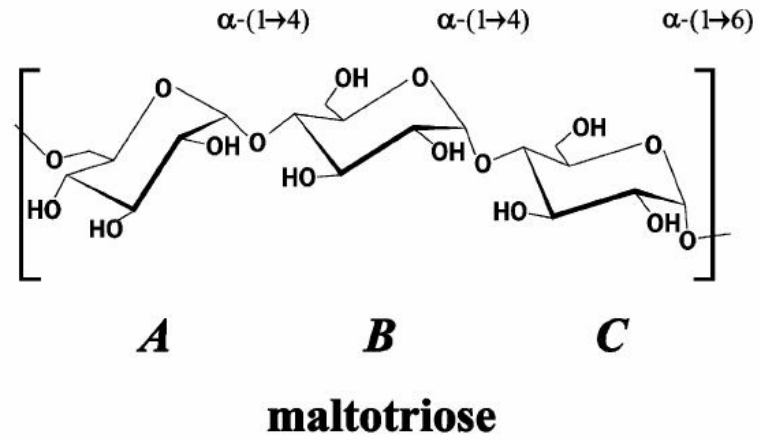


Figure 2.4: Repeated units of pullulan (Delben *et al*., 2006)

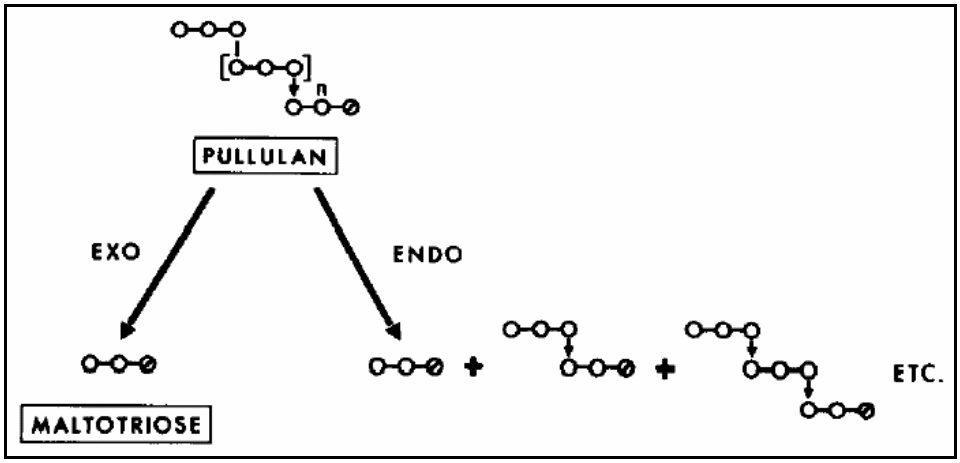


Figure 2.5: The action patterns of pullulanase

In addition, pullulanase have an interesting feature in the sequence similarity. The sequence information reveals that the enzyme contains a highly conserved region consisting seven amino acids, YNWGYDP, found in all type I pullulanase (Bertoldo *et al*., 2004). The type II pullulanase sequences do not contain this conserved region (Bertoldo *et al*., 1999). Yamashita *et al.* (1997) proposed that the YNWGYDP motif may be involved in the degradation of α-1,6 glycosidic linkages or substrate binding.

2.4.1 Properties and Sources

Pullulanase is also a starch debranching enzyme, which can cleave the α-1,6 glycosidic linkage. Moreover, pullulanase type II are also able to hydrolyze α-1,4 glycosidic linkages despite of α-1,6 glycosodic linkages.

In recent years, many pullulanases have been identified and studied, since the interest in pullulanases is motivated by industrial applications (Erra-Pujada *et al*., 2001., Bertoldo *et al*., 1999). To date, there are many other mesophiles that have been identified as pullulanase-producer, such as *Bacillus sp*. strain KSM-1876 (Hatada *et al*., 2001), *Bacillus sp*. strain KSM-1378 (Ara *et al*., 1995), *Bacillus sp*.

strain S-1 (Lee *et al*., 1997), *Bacillus sp*. US149 (Roy *et al*., 2003), and *Micrococcus halobius* OR-1 (Devi & Yogeeswaran, 1999). Pullulanase has also been detected in the thermophiles *Bacillus thermoleovorans* US105 (Messaoud *et al*., 2002), *Anaerobranca gottschalkii* (Bertoldo *et al*., 2004), *Bacillus sp*. DSM 405 (Brunswick *et al*., 1999), *Clostridium thermosulfurogenes* SV9 (Swamy and Seenayya, 1996), and in the hyperthermophile *Fervidobacterium pennavorans* Ven5 (Bertoldo *et al*., 1999), *Rhodothermus marinus* (Gomes *et al*., 2003), *Thermococcus hydrothermalis* (Gantelet & Duchiron, 1998). The source of microorganisms and properties of pullulanases are summarized in Table 2.3.

The molecular weight of pullulanase varies widely from different sources. Generally, the molecular weight of pullulanase type I is 70-80 kDa, and 100-210 kDa for pullulanase type II (Kim *et al*., 2000). Few studies have found that Ca^{2+} ion have no effect on the stabilization and activity of pullulanase enzyme (Stefanova *et al*., 1999; Lee *et al*., 1997, Ara *et al*., 1995). However, it was found that in the presence of increasing amounts of Ca^{2+} ions, similar significant increases are observed in the enzyme thermoactivity and thermostability (Erra-Pujada *et al*., 2001; Gantelet & Duchiron, 1998; Antranikian *et al.*, 1987). According to Saha *et al*. (1988), pullulanase enzyme may not require Ca^{2+} ions for its activity, but Ca^{2+} ions may play an important role in thermal stability and may maintain the conformation of the enzyme. According to Lévêque *et al*. (2000), there was no conclusion that can be drawn out about the mechanism by which Ca^{2+} ions stabilize and activate the pullulanase enzyme.

Table 2.3: Source and properties of pullulanases.

Source	Molecular weight	Opt. pH	Opt. Temp.	Type[1]	Reference
Bacillus sp. US149	200	5.0	60°C	II	Roy *et al*., 2003
Rhodothermus marinus	nd[2]	6.5	65 °C	nd	Gomes *et al*., 2003
Anaerobranca gottschalkii	96	8.0	70 °C	I	Bertoldo *et al*., 2004
Fervidobacterium pennavorans Ven 5	77	6.0	85 °C	I	Koch *et al*., 1997
Thermatoga maritima	89	5.9	90 °C	I	Kriegshäuser & Liebl, 2000
Clostridium thermosulfurogenes SV2	nd	6.0	75 °C	nd	Reddy *et al*., 1999(a,b)
Bacillus acidopullulyticus	97	5.5	60 °C	nd	Stefanova *et al*., 1999
Bacillus sp. DSM 405	126	6.0	70 °C	II	Brunswick *et al*., 1999
Thermococcus hydrothermalis	nd	5.5	95 °C	II	Gantelet & Duchiron, 1998
Bacillus sp. S-1	140	9.0	60 °C	I	Lee *et al*., 1997
Bacillus sp. KSM-137	210	9.5	50 °C	II	Ara *et al*., 1995
Thermus IM6501	80	6.0	70 °C	I	Kim *et al*., 2000
Micrococcus halobius OR-1	nd	8.0	60 °C	I	Devi & Yogeeswaran, 1999
Desulfucoccus mucosus	74	5.0	85 °C	II	Duffner *et al*., 2000
*Bacillus thermoleovorans U*S105	nd	5.6	75 °C	I	Messaoud *et al*., 2002
Clostridium thermohydrosulfuricum	136.5	5.5	90 °C	I	Saha *et al*., 1988

[1] Pullulanase type- I: type I pullulanase; II: type II pullulanase
[2] nd: not determined

The divalent ions or some reagents may affect the activity of pullulanase. Different sources of pullulanase may have different degrees of divalent ions effect. *Clostridium thermohydrosulfuricum* pullulanase is inhibited by cyclodextrins, EDTA and *N*-bromosuccinimide, but not by acarbose and *p*-chloromercuribenzoate (Saha *et al*., 1988). Kim *et al* (2000) showed that the activity of pullulanase from *Thermus* IM6501 was strongly inhibited by Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and Ag^{2+}, while enhanced slightly by Ca^{2+}, Ba^{2+}, Li^{2+} and Mg^{2+}. EDTA also inhibited the

pullulanase activity by 70%. On the other hand, Zn^{2+}, Cu^{2+}, Fe^{2+}, *N*-bromosuccinimide and EDTA were able to inhibit the pullulanase from *Desulfurococcus mucosus*, while Ca^{2+}, Mn^{2+} and cyclodextrins had no inhibition effect on the enzyme activity (Duffner *et al*., 2000).

According to Ara *et al*. (1995), the pullulanase from *Bacillus sp*. KSM-1378 was inhibited by diethyl pyrocarbonate, phenylmethanesulphonyl fluoride, *N*-bromosuccinimide, α-CD and β-CD, meanwhile *N*-ethylmaleimide, 4-chloromercuribenzoate and monoiodoacetate had no effect on the enzyme activity. For the effect of divalent ions, *Bacillus sp*. KSM-1378 pullulanase was strongly inhibited by Hg^{2+}, Cd^{2+}, Pb^{2+} and Mn^{2+} ions, and Co^{2+} ions slightly stimulated the pullulanase activity.

The optimum pH of most pullulanase occurs between pH 5.0 and 7.0, and optimum temperature between 45 to 60 ^{o}C (Saha *et al*., 1988; Ara *et al*., 1995; Kelly *et al*., 1983). But the optimum pH for alkaliphilic enzyme is between pH 8.0 to 10.0 (Bertoldo *et al*., 1999).The optimum temperature of archaeal pullulanases are typically between 80 to 100 ^{o}C (Lévêque *et al*., 2000), and some thermophiles and hyperthermophiles pullulanase are generally above 70 ^{o}C.

2.4.2 Cloning of Pullulanase Gene

The world consumption of sugar syrups is continuosly increasing due to its extensive use in beverages and processed foods. Acid hydrolysis of starch has had widespread used in the past and has now been replaced by enzymatic process. Amylolytic enzymes are widely used in industry to convert starch into a variety of sugar syrups. Most amylolytic enzymes are specific for α-1,4 glucosidic linkages yet the α-1,6 glucosidic linkages must also be cleaved for complete hydrolysis of amylopectin to glucose (Devi & Yogeeswaran, 1999).

Some of the most impressive recent research in the development of new enzymes is focused on debranching enzymes. The pullulanase gene has been studied since 1980s until now. Most of the pullulanase gene studied is from archaeal and *Bacillus* species. There are also pullulanase genes isolated from *Anaerobranca gottshalkii*, *Desulfurococcus mucosus*, *Klebsiella aerogenes* and so on. Some of the studies of pullulanase gene are summarized in Table 2.4. The pullulanase gene is usually cloned in *Escherichia coli* or *Bacillus subtilis*.

Table 2.4: Studies of pullulanase gene

Organism	Type	Length (a.a)	References
Anaerobranca gottshalkii	I	865	Bertoldo *et al*., 2004
Bacillus thermoleovorans US105	I	718	Messaoud *et al*., 2002
Bacillus stearothermophilus TS-23	II	2018	Chen *et al*., 2001
Bacillus sp. KSM-1876	II	1142	Hatada *et al*., 2001
Thermus IM6501	I	718	Kim *et al*., 2000
Desulfurococcus mucosus	II	686	Duffner *et al*., 2000
Fervidobacterium pennavorans Ven5	I	849	Bertoldo *et al*., 1999
Thermacoccus hydrothermalis	II	1339	Erra-Pujada *et al*., 1999
Thermotoga maritima	I	840	Bibel *et al*., 1998
Caldicellulosiruptor saccharolyticus	I	826	Albertson *et al*., 1997
Bacillus sp. KSM-1378	II	1938	Hatada *et al*., 1996
Bacillus sp. XAL601	II	2032	Lee *et al*., 1994
Thermoanaerobacter ethanolicus 39E	II	1481	Mathupala *et al*., 1993
Klebsiella aerogenes	I	1096	Takizawa & Murooka, 1985

Pullulanase production from wild microorganism usually faces many difficulties, such as low yields of enzymes, low enzyme activity and tedious downstream purification procedure especially for the intracellular enzymes. Genetic engineering techniques can overcome all the difficulties above. Firstly, large quantities of specific gene can be isolated in pure form by molecular cloning and the target DNA or enzyme can be produced in large amounts under the control of the

expression vector (Madigan *et al*., 2000). Besides, the overexpression following the cloning step can significantly increase the enzyme yield by subcloning the target gene into a suitable expression vector. Through all the techniques above, the production cost for pullulanase enzyme can be significantly reduced and the improved enzyme properties may also meet the requirements for industrial use.

2.5 Industrial Applications

2.5.1 Starch Processing Industry

Some pullulanases are used in industries to complete the hydrolysis of starch initiated by α-amylases. Amylases hydrolyze α-1,4 glycosidic linkages in starch to produce a mixture of glucose, maltooligosaccharides and α-limit dextrins. All the remaining α-1,6 glycosidic branches in the products are hydrolyzed by pullulanase. Therefore, dextrin does not remain in the hydrolysate when starch is treated with amylase and pullulanase simultaneously, and consequently increase the efficiency of a saccharification reaction. This method has an advantage of generating higher yields of a desired end product from starch (Kim *et al*., 2000). Beside, the combined application of pullulanase with other amylolytic enzymes may increase the quality of sugar syrups.

2.5.2 Baking Industry

The baking industry is a large consumer of starch and starch-modifying enzymes. Staling effect is the major problem in the baking industry. The staling effect includes increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor, leads to the deterioration of quality (van der Maarel *et al*., 2002). Although this problem can be overcome using chemical treatment, enzymatic treatment is more preferred due to the consumers nowadays demand for products without chemicals and higher acceptance by the

consumers for enzymes, which are produced from natural ingredients, are found. Some amylolytic enzymes act as anti-staling agent to solve the staling problem. Pullulanase can specifically remove the compound responsible for the gummyness associated with α-amylase treated bakery products. Pullulanase is able to rapidly hydrolyze the branched maltodextrins of DP20-100 produced by the α-amylase (van der Maarel *et al*., 2002). Pullulanase play an important role in the enzymatic antistaling treatment.

2.5.3 Branched Cyclodextrins (CDs) Production

There is a very interesting and high economical valued application of pullulanase enzyme in branched cyclodextrins (CDs) production. CDs and branched CDs, such as maltosyl-CDs and glucosyl-CDs are homogeneous cyclic oligosaccharides, which are composed of only glucose units (Kitahata *et al*., 2000). These saccharides have a hydrophobic region and a hydrophilic region in their structures, and have the ability to form inclusion complexes with various kinds of compounds (Hamayasu *et al*., 1999). Thus, CDs and branched CDs have been widely used or stabilizing labile materials, masking odours, and solubilizing insoluble or poorly soluble drugs (Tanimoto *et al*., 2005; Okada *et al*., 1988).

2.6 *Exiguobacterium*

To date, there are only nine species of *Exiguobacterium* found. Collins *et al.* (1984) first proposed the genus *Exiguobacterium* with the type species *Exiguobacterium aurantiacum* in (López-Cortés *et al*., 2006; Kim *et al*., 2005). *Exiguobacterium aurantiacum* is a slightly alkaliphilic species isolated from potato wastewater effluent. Since then, eight new species have been added to the genus *Exiguobacterium*. The second species of this genus is *Exiguobacterium acetylicum*, which was designed in 1994 by reclassification of *Brevibacterium acetylicum* isolated from creamery waste. For the past three years, the other seven species of

Exiguobacterium were found. Frühling *et al.*(2002) have reported *Exiguobacterium undae* and *Exiguobacterium antarcticum* isolated from a microbial sediment mat of Lake Fryxell, Antarctica. In 2004, *Exiguobacterium oxidotolerans* was recovered from a drain of a fish processing plant in Japan by Yumoto *et al*.. In addition, *Exiguobacterium marinum* and *Exiguobacterium aestuarii* were isolated from a tidal flat at Yellow Sea in Korea by Kim *et al*, and *Exiguobacterium mexicanum* and *Exiguobacterium artemiae* was isolated from brine shrimp by López-Cortés *et al*., in 2005.

According to Bergey's Manual of Determinative Bacteriology (Bergey *et al*., 1994), the *Exiguobacterium aurantiacum* is an alkaliphilic bacterium that has a peptidoglycan of type lysine-glycine and contained mainly MK-7 menaquinones. The important features of genus *Exiguobacterium* are: cells in rod shape in young cultures and become almost coccoid in old cultures, with size 1.1-1.2 x 1.4-3.2 μm, stained gram positive, non-acid-fast, nonsporing, motile by peritrichous flagella, facultatively anaerobic, forming flat and pale orange colonies on nutrient agar and the pigment does not diffuse. Besides, the *Exiguobacterium* cells are alkalophilic that can grow at pH 6.5-11.5. *Exiguobacterium* cells show chemoorganotrophic, metabolism fermentative, catalase positive, oxidase negative, reduce nitrate, giving acid from glucose, sucrose, galactose and some other sugars, and the main products are lactate, acetate and formate. In addition, the *Exiguobacterium* cells also can hydrolyse starch, casein and gelatin. The optimum growth temperature is 37 ^{o}C.

2.7 Fermentation Medium Development

The application of experimental design techniques early in process development can improve process yield, reduce variability and closer conformance to nominal or target requirements, reduce development time and reduce overall costs (Montgomery, 1985). In biotechnology-based industrial processes, the formulation of cultivation media is a critical importance because their composition affects product concentration, yield and volumetric productivity. It is also important to

reduce the costs of the medium as this may affect the overall process economics and to determine the practicability of the process (de Souza *et al*., 2006; Abdel-Fattah *et al*., 2005). Ghanem *et al*. (2000) also reported that reducing the costs of the enzyme production by optimization of the fermentation medium is the basic research objective for industrial application.

The conventional one-variable-at-a-time (OVAT) method or one-dimensional search is still applied in the medium optimization study because the method is easy and simple. However, the OVAT method has some major flaws, such as interactions between components are ignored, the optimum can be missed completely, and involves a relatively large number of experiments (Kennedy and Krouse, 1999). The method is laborious and time consuming, especially for a large number of variables. Lately, the statistical experimental design is better acknowledged than the OVAT method and the use of experimental design has become more common since 1980. Experimental design is an efficient tool that offers several advantages over conventional method being rapid and reliable, short lists significant nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time, glassware, chemicals and manpower (Srinivas *et al*., 1994; Chauhan *et al*., 2005). There is a number of statistical designs available, such as full factorial, fractional factorial or Plackett-Burman, response surface methodology and so on. Kennedy and Krouse (1999) have summarized several strategies used to improve fermentation medium, including the experimental design and conventional method (Table 2.5).

Table 2.5: Experimental design and conventional strategies used in fermentation medium improvement (Kennedy and Krouse, 1999)

Strategy	Concept	Advantages	Disadvantages	*Math.
OVAT (conventional method)	Change the concentration of one component, keeping everything else constant	• easy & simple • easy to grasp results visually on a graph	• interactions ignored • optimum can be missed • large number of experiments	Nil
Full factorial	Everything possible combination tested	• Best possible coverage of experiments	• Large number of trials	Low
Partial factorial	A subset of full factorial design	• Compromise solution, with a smaller number of runs	• only potential average • some high order interactions unobserved	Moderate
Plackett-Burman	Design for seeing effects of n-1 variables in n runs	• minimum number of runs required • good screening tool • only two levels	• Interaction unobserved	Low
Central composite	Partial factorial used to estimate curvature of effects	• Estimate curvature of effects not just direction	• Moderate number of runs	Moderate
Box-Behnken	Minimalist central composite	• estimate curvature • low number of runs	• Less coverage than central composite	Moderate

* Math: mathematical expertise needed.

2.7.1 Plactkett-Burman Design

Traditionally, the screening of medium components is worked out using OVAT method, where screening is done at an arbitrarily selected level of each category at a time while keeping the other ingredients constant, again at an arbitrarily selected level (Srinivas *et al*., 1994). However, the traditional method is

time consuming and tedious and not reliable, as stated in section 2.8. Hence, experimental design is preferred. For example, full factorial design with variables varied between two levels, are capable of detecting all interactions among variables. However, the design is still laborious due to it requiring 2^n experiments, where 'n' is the number of variables examined (Milagres and Lacis, 1991). The size of a full factorial experiment increases exponentially with the number of factors and this leads to unmanageable number of experiments.

Alternatively, fractional factorial, which is in the class of factorial design, provides a solution to tackle the large number of sources of a category at a time. The fractional factorial design, a fraction of full factorial design, has lesser ability to discriminate among all main effects and interactions of variables, but they have the advantage of great efficiency. The most common fraction used is 2^{k-p} design in which the fraction is $1/2^p$. Usually, 2^k and 2^{k-p} are used to identify or screen for important factors (Gohel *et al*., 2006). Consequently, the method also involves quite a large number of experiments, and results obtained are not the pure effects of the sources as confounded with the effects of interactions among the sources of different categories used in the experiment (Srinivas *et al*., 1994).

Plackett-Burman experimental design is a special set of saturated two level fractional factorial design that is most commonly employed for identifying important factors for further investigation (Kennedy and Krouse, 1999). Besides, Plackett-Burman design is a well-established and widely used statistical design technique for screening of medium nutrients in shake flasks (Plackett and Burman, 1946). Plackett-Burman design is an efficient tool, especially when a large number of variables are involved, for screening variables that are able to determine the influence of various factors with only a small number of trials (Gohel *et al*., 2006). Plackett-Burman design allows unbiased estimation of all main effects with the smallest possible number of experiments. The Plackett-Burman design allows reliable short listing of a small number of ingredients for further optimization and allows unbiased estimates of linear effects of all the factors with maximum accuracy for a given number of observations (Krishnan *et al*., 1998).

Plackett and Burman (1946) described the special set of fractional factorial design in which up to N-1 variables (at two levels) can be examined in only N experiments, where N is a multiple of 4. In this design, generally a multiple of four, i.e., 4, 8, 12, 16,…, 4n, experiments are required to screen 3, 7, 11, 15,…,4n-1, components, respectively, where 'n' is an integer. The design is orthogonal in nature, implying that the effects of each variable are pure in nature and not confounded with interactions among variables (Gohel *et al*., 2006)

Son *et al*. (1998) studied the production of a Ras farnesyl protein transferase inhibitor (CBR004) from *Bacillus licheniformis* using Plackett-Burman design. By using the Plackett-Burman design, K_2HPO_4 and tryptone were selected as medium components for the production of CBR004 among six components (K_2HPO_4, yeast extract, corn steep powder, $(NH_4)_2SO_4$, $NaNO_3$ and tryptone).

Naveena *et al.* (2005) also reported the application of Plackett-Burman design in the screening study on L(+) lactic acid production from wheat bran by *Lactobacillus amylophilus* GV6 in solid state fermentation (SSF). The Plackett-Burman design was used to screen 15 components. The nitrogen sources peptone, yeast extract and tri-ammonium citrate, along with $NaH_2PO_4.2H_2O$ and tween 80, were found to influence productivity. In other words, the five medium components were found as significant factors for the L (+) lactic acid production. In this study, the use of Plackett-Burman design not only helped in short listing and selecting the best medium components, but also ensured better production of lactic acid.

Srinivas *et al*. (1994) used Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system. Five nitrogen source, six minerals, six enzyme inducers, one growth promoter and one product promoter were screened, i.e., ammonium nitrate, ammonium sulphate, diammonium hydrogen phosphate, urea, ammonium chloride, corn steep liquor, guar flour, soy flour, bengal gram flour, french bean flour, black bean flour, lactose, citric acid, sodium nitrate, potassium chloride, magnesium sulphate, ferrous sulphate, sodium chloride and calcium chloride. The

studies allowed the selection of urea, corn steep liquor, guar flour, soy bean flour and citric acid as most promising sources for further optimization studies.

Besides, the Plackett-Burman design not only applied the improvement of medium for enzyme or protein production, but also used it to evaluate the nutritional requirements within a complex medium on mycelium growth and sporulation. Yu *et al.* (1997) reported the application of Plackett-Burman design in the evaluation of nutritional requirements on the production of *Colletotrichum coccodes* spores. Sucrose, soy protein, KNO_3, KH_2PO_4, $MgSO_4$, $CaCl_2$ and $CuSO_4$ were selected as the new combination of medium components for further study in the development of a low-cost and effective medium for *Colletotrichum coccodes* spore production.

2.7.2 Response Surface Methodology: Central Composite Design

The Response Surface Methodology (RSM), formerly known as Box-Wilson methodology, is a comprehensive methodology employing factorial designs to optimize production processes developed by Box and Wilson (Kennedy and Krouse, 1999; Lee and Chen, 1997). RSM is the most widely used statistical technique for bioprocess optimization (Francis *et al*., 2003; Liu *et al*., 2003; Kunamneni *et al*., 2005). It can be used to evaluate the relationship between a set of controllable experimental factors and observed results. Optimization of medium by classical method, or known as OVAT method, is extremely time-consuming and expensive for a large number of variables and also may result in the wrong conclusion. The application of RSM is time saving and cost saving due to the interaction among the possible influencing parameters that can be evaluated with limited number of experiments (de Souza *et al*., 2006; Francis *et al*., 2003). Therefore, RSM is a good sense application of statistical techniques for designing experiments, building models, evaluating the effects of several factors in order to obtain high productivity in bioprocess.

The basic strategy of RSM has four steps: procedures to move into the optimum region, behaviour of the response in the optimum region, estimation of the optimum condition and verification (Tanyildizi *et al*., 2005). According to Gunaraj and Murugan (1999), RSM is a set of techniques that include the designing of a set of experiments for adequate and reliable measurement of the true mean response of interest, and determining the mathematical model with best fits. Besides, RSM capable to find the optimum set of experimental factors that produce the maximum or minimum value of response and represent the direct and interactive effects of process variables through two dimensional and three dimensional graphs.

Central Composite Design (CCD) is one of the most useful designs of Response Surface Methodology (RSM). CCD was introduced by Box and Wilson. CCD is composite design formed from two-level factorials by the addition of just enough points to estimate curvature and interaction effects. The design can be considered as partial factorials with factors at five levels (Kennedy and Krouse, 1999).

Liu *et al*. (2003) reported the optimization of nisin production by *Lactococcus lactis* in a whey-based medium using experimental design. Plackett-Burman design was applied to identify the significant factors that influence the nisin production. In this study, yeast extract, KH_2PO_4 and $MgSO_4$ were identified from seven candidate factors to have significant effects on nisin production by *Lactococcus lactis*. Subsequently, these three significant factors were optimized using central composite design (CCD).

de Souza *et al*. (2006) also reported the application of statistical experimental design in optimization of medium composition for the production of transglutaminase (MTGase) by *Bacillus circulans* BL32 isolated from the Amazon basin. Plackett-Burman design was applied to find the key ingredients for the best medium composition among glycerol, sucrose, peptone, tryptone, Na_2HPO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$. Results indicated that sucrose and peptone have significant effects, negative and positive effects respectively, on MTGase production. Then, CCD was applied for further optimization of these two

components. Under suggested optimized conditions, the model predicted the MTGase activity of 0.280 U/ml, is very closely matched to the experimental activities of 0.306 U/ml. The optimization of the medium resulted in 60 % higher MTGase production. Besides, the optimized medium composition has reduced the costs of constituents and also solved the problem of foam production.

The application of Plackett-Burman design as a screening step before the optimization procedure was also reported in the study of optimizing riboflavin production by a UV-mutant of *Eremothecium ashbyii* (Pujari and Chandra, 2000). Initially, seven components were screened using Plackett-Burman design and four components, i.e., molasses, SSC, KH_2PO_4 and $MgSO_4.7H_2O$ were identified as significant factors in the riboflavin production. Then, RSM was used to optimize the four significant factors for enhanced riboflavin production using CCD. The medium composition for the enhanced production of riboflavin was 30.85 g/l of molasses, 39.80 g/l of SSC, 1.484 g/l of KH_2PO_4 and 0.072 g/l of $MgSO_4.7H_2O$. Consequently, the riboflavin production by *Eremothecium ashbyii* UV-mutant was increased by 35 % using the Plackett-Burman design and CCD.

Abdel-Fattah *et al*. (2005) performed the optimization of process parameters for the production of *Pseudomonas aeroginosa* uricase through statistical experimental designs. Firstly, the Plackett-Burman design was used to screen the bioprocess parameters that may significantly influence uricase production. Fifteen variables were tested in the Plackett-Burman design. Three factors, which are pH, $CuSO_4$ and $FeSO_4$ were found as significant factors. Subsequently, a three-level Box-Behnken design was employed to acquire the best process conditions. As a result, the optimal combination of major media components for uricase production was obtained, i.e., pH 5.5, 10^{-3}M $CuSO_4$ and 10^{-2}M $FeSO_4$, with predicted uricase activity of 7.051 U/ml/min. The optimal value of the enzyme activity is 10-fold increment than the basal conditions.

In addition, Naessens *et al*. (2004) employed the classical and statistical optimization methods in the study of intracellular dextran dextrinase production by *Gluconobacter oxydans*. Various carbon sources and nitrogen sources were screened

using classical or OVAT method. Among the seven carbon sources and seven nitrogen sources, glycerol and mycological peptone resulted in highest enzyme yields. After that, the effect of glycerol concentration, mycological peptone concentration and initial pH on dextran dextrinase production was investigated using CCD. The optimal fermentation parameters obtained from the CCD were 20.59 g/l of glycerol, 6.67 g/l of mycological peptone and initial pH at 6.14. The predicted dextran dextrinase production was 0.207 U/ml and the actual experimental value was 0.208±0.025 U/ml. The optimized fermentation process, which resulted from classical screening method and statistical experimental design, showed a three-fold increase in the dextran dextrinase production by *Gluconobacter oxydans*.

Nawani and Kapadnis (2005) also reported the optimization of chitinase production by *Streptomyces sp*. NK1057, NK528 and NK951 using statistics based experimental design. Firstly, preliminary studies based on OVAT method were carried out and eight variables were identified to be important to chitinase production. Then, 2-level fractional factorial design was applied to identify significant factors. As a result, chitin, yeast extract, ammonium sulphate, trace elements, pH and temperature were identified to significantly affect chitinase production. The optimal values of important variables were determined by CCD. This optimization strategy led to an increase in chitinase production in the strains *Streptomyces sp*. NK1057, NK528 and NK951 by 29 %, 9.3 % and 28 %, respectively.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial Strains and Plasmids

A total of 19 strains of bacteria, namely 9, 2, 14, 90, 39, NA, 26, 89, 7, 89, 8, 23, 19, 1, 90, 48, P2, P3, and S7 that were isolated by previous researchers, were screened by using pullulan agar, AZCl-pullulan agar and red-pullulan agar. All bacteria were incubated at 50 ^{o}C, except bacteria P2 and P3, which were incubated at 37 ^{o}C, and bacteria S7 that was incubated at 45^{o}C. *Eschericia coli* JM109 endA1, recA1, gyrA96, *thi*, *hrd* R17 (r_k-,mk+), *rel*A1, *sup* E44, Δ (*lac-pro*AB), [F', *tra* D36, *pro* AB, *lacIqZ* Δ M15] purchased from Promega were used as host strains. Plasmid pUC 19 purchased from MBI Fermentas and plasmid pGEM-T-easy supplied by Promega were used as vectors.

3.2 Bacterial Stocks Preparation

Bacterial stocks were prepared in PROTECT Bacterial Preservers beads and kept at -80 ^{o}C. The beads were purchased from Technical Service Consultants Limited. Pure culture of bacterial colonies was inoculated into broth medium. The culture broth was incubated at the desired temperature with constant shaking at 200 rpm. After overnight incubation, 10 ml culture broth was concentrated into 1ml. The concentrated bacterial culture was pipetted into beads tube. The tube was inverted several times. The bacteria stocks were then stored at -80 ^{o}C in the freezer.

3.3 Chemicals

All of the chemicals used throughout experiments were purchased from several chemical supplier companies, such as GCE, Sigma, Amresco, Fluka, Merck, Promega and Riedel-deHaen. The purity of chemicals was analytical and molecular biology grade. All of the enzymes used in molecular biology research, such as restriction enzymes, DNA modifying enzymes, DNA molecular weight marker, DNA loading dye, were purchased from Promega and MBI Fermentas. The primers for PCR were synthesized by 1st BASE Laboratories Biosyntech.

3.4 Medium Preparation

3.4.1 Modified Peptone-Yeast Extract (PYE) Medium

Exiguobacterium sp. MAAC-1 was grown in modified PYE medium (Reddy *et al*., 1999a,b). Formulation of PYE medium comprises of 0.1 %(w/v) NH_4Cl, 0.267 %(w/v) $Na_2HPO_4.12H_2O$, 0.03 %(w/v) KH_2PO_4, 0.3 %(w/v) yeast extract, 1.0 %(w/v) peptone, 0.02 %(w/v) $MgCl_2.6H_2O$, 0.2 %(w/v) starch, 2.0 %(w/v) agar (omitted in broth medium). The medium was boiled before autoclaving.

3.4.2 Screening Medium

3.4.2.1 Pullulan-PYE Agar

The pullulan-PYE agar formulation was prepared similar as mentioned in section 3.2.1 with starch replaced by 0.5 % pullulan.

The pullulanase producer was determined by pullulan precipitation technique using methanol (Bibel *et al*., 1998). The colonies on the plate was scraped from the agar surface and flooded twice with methanol for 1 hour. A clear halo around the

bacteria colony was resulted against a turbid background if the bacteria produced pullulanase.

3.4.2.2 Red-Pullulan Agar

Red-pullulan agar contained 1 %(w/v) peptone, 0.1 %(w/v) NH_4Cl, 0.5 %(w/v) red pullulan (Megazyme), and 2 % agar. The medium was boiled before autoclaving. A clearing halo around bacteria colony indicated the pullulanase producer.

3.4.2.3 AZCl-Pullulan Agar

AZCl-pullulan agar was prepared in the form of two layers agar. The composition of the bottom layer contained 1.0 %(w/v) peptone, 0.1 %(w/v) NH_4Cl and 1.5 %(w/v) agar. The composition of the upper layer comprised of 1.0 %(w/v) peptone, 0.1 %(w/v)NH_4Cl, 0.01-0.02 %(w/v) AZCl-pullulan and 1.0 % agar. Both layers of agar medium were boiled before autoclaving. The bottom agar layer was firstly poured on a plate. When the bottom agar layer was solidified, the upper AZCL-pullulan layer was poured as a thin layer onto the solidified bottom layer.

The AZCl-pullulan was remained as small blue solid form on the agar. The spread of blue colour from the solid AZCl-pullulan particle was an indicator for degradation of pullulan by pullulanase.

3.4.3 Luria-Bertani (LB) Medium

The formulated LB medium comprised of 1 %(w/v) tryptone, 0.5 %(w/v) yeast extract, 1 %(w/v) NaCl, 2 %(w/v) agar (omitted in broth medium). The pH of

LB medium was adjusted to pH 7.0 using NaOH and HCl before autoclaving. Ampicillin was added at a final concentration of 100μg/ml for antibiotic containing LB media after the autoclaved medium was cooled down to below 55 ^{o}C. Ampicillin stock was prepared at 50 mg/ml. Ampicillin stock was filter-sterilized before kept at -20 ^{o}C.

3.4.4 SOB and SOC Medium

SOB medium was used during transformation of ligation products into plasmid. The medium comprised of 2.0 %(w/v) tryptone, 0.5 %(w/v) yeast extract, 0.01 %(v/v) of 1M NaCl, 0.0025 %(v/v) of 1M KCl and 0.01 %(v/v) of filter-sterilized 2M Mg^{2+}. For the SOC medium, the 0.01 %(v/v) of filter-sterilized 2M glucose was added to the SOB medium. All components except Mg^{2+} and glucose were dissolved in distilled water and autoclaved. The filter-sterilized Mg^{2+} and glucose were added after the autoclaved medium was cooled down to room temperature. The complete medium was filter-sterilized through a 0.22 μm filter unit.

3.5 DNA Manipulation Techniques

3.5.1 Genomic DNA Isolation

The method from Ish-Horowitz and Burke (1981) was used to isolate genomic DNA from *Exiguobacterium sp.* MAAC-1. About 1.5 ml overnight bacterial cell culture was centrifuged at 13000 rpm for 5 minutes to obtain cell pellet. The pellet was resuspended in 100 μl of 10X Tris-EDTA buffer. 10 μl of 10 mg/ml lysozyme and 10 μl of 10 mg/ml of DNase-free RNase were added into the suspension and incubated at 37 ^{o}C for 30 minutes to one hour.10 μl of 10 mg/ml proteinase K was then added and incubated at 37 ^{o}C for 30 minutes to one hour. This

followed by the addition of 10 µl of 10 % sodium deodecyl sulphate (SDS) and incubated at 37 ^{o}C for 30 minutes to one hour.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was then added and mixed by inverting gently for five to ten times. The mixture was centrifuged at 13000 rpm for 5 minutes. The upper layer of supernatant was then drawn into a new eppendorf tube. The above phenol chloroform extraction was repeated with chloroform:isoamyl alcohol (24:1). Then, 1/10 volume of 3M sodium acetate (pH 5.2) was added to the resulting supernatant and mixed by inverting gently for several times. Two volume of ice-cold 100 % ethanol were added and the mixture was inverted gently for several times until DNA threads were observed. The DNA threads were collected by centrifugation at 13000 rpm for five minutes. The obtained DNA pellet was washed with 70 % ethanol. The DNA pellet was air-dried and finally resuspended in 50 µl sterile deionised water. The eluted DNA sample was stored at 4 ^{o}C or at -20 ^{o}C for longer storage periods.

3.5.2 Plasmid DNA Isolation: Alkaline Lysis Method

The bacteria cultures were grown in LB broth containing ampicillin of final concentration of 100 µg/ml and incubated at 37 ^{o}C for overnight with continuously shaking at 200 rpm. The bacteria culture was spun at 14, 000 rpm for 2 minutes in order to pellet down bacteria cells. The pellet was resuspended in 200 µl lysis solution [50 mM glucose, 25 mMTris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)]. 300 µL of 0.2M NaOH/ 1 % SDS was added and mixed well by gently inversion and incubated on ice for 5 minutes. 300 µl of 3M sodium acetate (pH 5.2) was added and mixed well by gently inversion and incubated on ice for 5 minutes. Then, the sample was spun at 14, 000 rpm for 14 minutes at room temperature. The resulting supernatant was transferred to a clean eppendorf tube. RNAse A was added to a final concentration of 10 µl/ml and incubated at 37 ^{o}C for 30 minutes.

After the cell lysis and RNAse treatment, phenol extraction was carried out twice. Firstly, 50 % volume of chloroform and 50 % volume of phenol was added and vortexed. The sample was spun for 3 minutes for 14, 000 rpm. The top layer was collected and the phenol chloroform extraction steps repeated. One volume of chloroform was added in order to remove residual phenol. A total volume of 600 µl isopropanol was then added and incubated at room temperature for 5 minutes. The DNA was pelleted down at 13, 000 rpm for 10 minutes at room temperature. The pellet was washed using 500 µl of 70 % ethanol and respun. The ethanol was drained and the tube was air-dried at room temperature. Finally, the precipitated plasmid DNA was resuspended in sterile double-distilled water. The purified plasmid was stored at -20 ^{o}C.

3.5.3 Agarose Gel Preparation and Electrophoresis

Around 1 %(w/v) of agarose powder was dissolved in 1X Tris-Borate-EDTA (1X TBE) buffer by heating the mixture using a microwave oven. The heated agarose was cooled to around 50 ^{o}C, Ethidium Bromide (EtBr) was added into the agarose at a final concentration of 0.1 µg/ml. Then, the agarose was poured into a small gel base and a comb was placed at one end. The comb was removed when the agarose gel solidified. The gel base was placed into an electrophoresis tank containing 1X TBE as running buffer. 6X DNA loading dye was mixed with DNA sample at a final concentration of 1X. Electrophoresis was carried out with 70 volt.

3.5.4 Quantification of DNA

BioPhotometer (Eppendorf) was used to check the purity of DNA samples and to quantify the concentration of DNA samples. 5 µl of DNA sample was diluted in 45 µl of sterile distilled water. The absorbance of diluted DNA sample was read at wavelength of 230 nm, 260 nm, 320 nm and 320 nm. The ratio of 260/280 indicates

the purity of the DNA sample. A value between 1.8 to 2.0 indicates high purity of nucleic acid (Eppendorf AG, 2000).

3.5.5 Digestion of DNA Using Restriction Enzyme

All restriction enzymes and buffers used were purchased from Promega. The volume of 0.1 to 10 µl DNA was digested in 10 to 50 µl of solution with 1X appropriate buffer. The mixture solution was incubated at the appropriate temperature for several minutes or hours depending on the size of digested DNA needed and digestion efficiency of the restriction enzymes on the DNA. Multicore buffer (provided by Promega) was used in double digestion involving different enzymes with different buffers.

3.5.6 Dephosphorylation of Digested Plasmid

Digested plasmid (1.0 µg) was dephosphorylated by 1 unit Shrimp Alkaline Phosphatase (SAP) in 30 to 50 µl of 1X SAP buffer at 37 ^{o}C for 15 minutes. Dephospholylation reaction was inactivated at 65 ^{o}C for 15 minutes. The dephosphorylated plasmid was then used for further cloning experiment. The dephosphorylated plasmid was stored at -20 ^{o}C.

3.5.7 Ligation of DNA

Around 0.01-0.1 µg of vector were digested with appropriate restricted enzymes. The digested vector was ligated with DNA fragment with appropriate vector/insert ratio using T4 DNA Ligase in 1X ligation buffer. 1 to 3 units of T4 DNA Ligase was used and sterile distilled water was added up to 10 µl of final volume. Then, the mixture was incubated overnight at 4 ^{o}C. 0.5 or more ratio of

vector/insert was applied for large DNA fragments and 3 or more ratio was applied for smaller DNA fragments.

3.5.8 Preparation of Competence Cells

The competent cells of *E. coli* JM109 bacterial strain were prepared using Hanahan method according Sambrook *et al*. (2001). E. coli JM109 strain was streaked from a frozen stock onto SOB agar and incubated for 16 hours at 37 ^{o}C. A single colony was transferred into 5 ml of SOB medium and incubated at 37 ^{o}C overnight with orbital shaking at 200 rpm. A volume of 0.2 ml overnight culture was inoculated into 20 ml of SOB (100-fold dilution) and incubated at 37 ^{o}C with orbital shaking at 200 rpm until the cell density reach OD_{600} of ~0.4. The cells were cooled to 0 ^{o}C by chilling the cells on ice for 10 minutes. The chilled culture was centrifuged at 4000 rpm in a Sorvall GSA rotor for 10 minutes at 4 ^{o}C. The cell pellet was resuspended by swirling gently in 6.67 ml of ice-cold FSB buffer and stored on ice for 10 minutes. The cells were recovered by centrifugation at 4000 rpm in a Sorvall GSA rotor) for 10 minutes at 4 ^{o}C. The resulted pellet was resuspended in 1.6 ml of ice-cold FSB buffer and 56 µl of DMSO was added to the suspension. The suspension was mixed gently by swirling and placed on ice for 5 minutes. The resulted competent cells were aliquoted into 200 µl volume in sterile eppendorf tubes. The tubes were then immediately chilled in a bath of liquid nitrogen and stored at -80 ^{o}C.

3.5.9 Transformation

The -80 ^{o}C frozen stock of *E.coli* competent cells were thawed on ice.2-3 µl of ligation mix was added to 50 µl of the thawed competent cells in pre-chilled eppendorf tube. The mixture was mixed by swirling the eppendorf tube gently and incubating on ice for 20 minutes. The mixture was then heat-shocked at 42 ^{o}C for 45-50 seconds in order to transform the competent cells. The mixture was returned

to ice immediately for 2 minutes. 950 µl of SOC broth was added and incubated at 37 ^{o}C for 1.5 hour. Then, the transformed cells were spread on LB-ampicillin agar plates and incubated overnight at 37 ^{o}C.

3.6 Identification of Bacteria P2 at 16S rRNA Level

Ribosomal RNAs, being ancient molecules are functionally constant, universally distributed and moderately well conserved in sequences, though they have been used separately for taxonomic and phylogenetic studies. Through the comparative sequence analyses between the ribosomal sequences, the sequence data can provide quantitative measurements of genetic relatedness or the relative evolutionary relatedness.

3.6.1 PCR Amplification of 16S rDNA Gene Using Universal Primers

The 16S ribosomal DNA (rDNA) gene of bacteria P2 was amplified using universal primers of eubacterial 16S rDNA. The forward primer Forward-B27f is 5'-AGA GTT TGA TCC TGG CTC AG –3' and the reverse Reverse-U1492r is 5'-GGT TAC CTT GTT ACG ACT T –3'. Both primers are conserved regions within 16S rDNA gene and often used to amplify the full length of 16S rDNA gene from most bacteria (Weisburg *et al*, 1991). The PCR reaction was carried out in a 50 µl reaction mixture containing 1X of PCR reaction buffer, 1 µl of Taq polymerase (5U/µl), 1 µl of each forward and reverse primers (50mM), 1 µl of dNTP mix (10mM), 4 µl of $MgCl_2$ (25mM), and 10 pg-1 µg of template DNA. The PCR reaction was performed in a Perkin-Elmer Cetus Thermocycler PCR machine. 28 cycles of reactions was performed. Each cycle consists of denaturation at 94 ^{o}C for 60 seconds, annealing at 60 ^{o}C for 90 seconds and extension at 72 ^{o}C for 90 seconds, plus an additional final extension at 72 ^{o}C for 8 minutes. The amplified PCR product was purified using the PUREGENE PCR Purification Kit purchased from

Biosyntech. The purification of PCR product was carried our according to the manufacturer's protocol.

3.6.2 Cloning and Nucleotide Sequence Determination of 16S rRNA Gene

The purified 16S rRNA gene, which was isolated through PCR method, was cloned into plasmid pGEM-T-easy using *E.coli* JM109 as a host before sending for automated nucleotide sequencing service. The cloning procedure was carried out according to Promega manufacturer's protocol. The cloned plasmid was isolated and purified from the *E.coli* host using Alkaline Lysis Method as mentioned in section 3.5.2. The purified plasmid was sent to automated nucleotide sequencing service provided by 1st BASE Laboratories, Biosyntech Malaysia. The primers used for nucleotide sequencing are universal T7 and SP6 RNA polymerase primer.

3.6.3 Analysis of 16S rRNA Gene Sequence

The comparison of rRNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eukaryotic organisms (Weisburg *et al*., 1991). The 16S rRNA gene sequence of bacteria P2 was compared with other bacterial rRNA sequences in Ribosomal Database Project (RDP-II). The RDP-II obtains bacterial rRNA sequences from the International Nucleotide Sequence Database (e.g. GenBank/EMBL/DDBJ) on a monthly basis (Cole *et al.*, 2005). The RDP-II using Sequence Match program to find the closest phylogenetic relatives sequence to the 16S rRNA sequence of bacteria P2 (Cole *et al.*, 2005). Then, CLUSTAL X program, version 1.83 was used to align the 16S rRNA sequence of bacteria P2 with the close similar sequences identified earlier by RDP-II. The neighbour-joining algorithm, which was provided by CLUSTAL X program, was used to construct the phylogenetic tree. GeneDoc program was also used to generate the statistical report that showed similarity values of the sequences in the phylogenetic tree.

3.7 Synthesis of Pullulanase Gene Fragment

Pullulanase gene contains a set of conserved regions of amino acid that plays a role in the breakage of α-1,6 and α-1,4 glycosidic linkages in pullulan and polysaccharides. Primers derived from the conserved regions can be used to amplify pullulanase gene fragment.

3.7.1 PCR Amplification of Pullulanase Gene Fragment

The primers for amplification of pullulanase gene fragment were designed based on the conserved regions. The forward primer Mix1 is 5'- TAT AAT TGG GGD TAT GAT CC -3' while the reverse primer ReMix1 is 5'- CCT AGT ATD GGG GTT AAT AT-3' (refer to Hatada *et al*., 2001). PCR reaction was performed in a Perkin-Elmer Cetus Thermocycler PCR machine. The PCR reaction was carried out in a 50 µl reaction mixture containing 1X of PCR reaction buffer, 1 µl of Taq polymerase (5 U/µl), 1 µl of each forward and reverse primers (50 mM), 1 µl of dNTP mix (10mM), 4 µl of $MgCl_2$ (25 mM), and 10 pg-1 µg of template DNA. The DNA was melted at 94 ^{o}C for 60 seconds followed by annealing at 50 ^{o}C for 90 seconds and extension at 72 ^{o}C for 90 seconds. An additional final extension was carried out at 72 ^{o}C for 8 minutes. 28 cycles of reactions was performed. The amplified PCR product was purified using the PUREGENE PCR Purification Kit purchased from Biosyntech. The purification of PCR product was carried our according to the manufacturer's protocol.

3.7.2 Cloning and Nucleotide Sequence Determination of Pullulanase Gene Fragment

The purified pullulanase gene fragment was cloned into plasmid before sending for automated nucleotide sequencing service, as mentioned in section 3.6.2.

3.7.3 Analysis of Pullulanase Gene Sequence

The nucleotide sequence of pullulanase gene fragment and the deduced amino acid sequence were compared and searched for similar sequences through BLASTN and BLASTX program provided by National Center for Biotechnology Information (NCBI), which is an online service founded at http://www.ncbi.nlm.nih.gov/blast. The sequences with high similarity percentage were compiled and analyzed using DNASIS Max 1.0 software (Hitachi Software Engineering Co., Ltd.).

3.8 Inoculum Preparation

The *Exiguobacterium sp.* MAAC-1 from –80 ^{o}C frozen stock was streaked on PYE agar plate and incubated overnight at 37 ^{o}C. A few colonies of the bacteria P2 culture were picked from the plate and transferred to a flask containing 25 ml of PYE broth aseptically. The culture was then incubated at 37 ^{o}C with orbital shaking of 200 rpm for 18 hours. After that, the culture was collected by centrifugation at 5000 rpm for 5 minutes in cool condition. The cell pellet was washed with sterilized saline solution (0.85 %w/v NaCl) twice. Then, the cells were suspended in the saline solution to give an optical density (OD) reading of around 0.5 at 550nm.

3.9 Preparation of Crude Enzyme

10 %(v/v) inoculum was inoculated aseptically to 250 ml conical flask containing broth medium to give a total volume of 50 ml. The medium was then incubated with orbital shaking of 200 rpm at 37 ^{o}C for 22 hours. Subsequently, the cultured medium was centrifuged at 5000 rpm for 15 minutes in cool condition. The resultant supernatant containing crude enzyme was used as enzyme sample in pullulanase assay.

3.10 Pullulanase Assay

3, 5-dinitrosalicylic acid (DNS) method was used to determine pullulanase activity. Pullulanase activity was routinely determined by measuring the enzymatic release of reducing sugars from pullulan (Roy *et al*., 2003). The concentration of reducing sugar released was determined by the DNS method described by Miller (1959) with slight modification. The reaction mixture contained 0.25 ml of enzyme sample and 0.25 ml of 1 %(w/v) pullulan in 100 mM glycine-NaOH buffer, pH 9.0. The mixture was incubated at 60 ^{o}C for 20 minutes. Then, the reducing sugar released in the mixture during the incubation was determined by DNS method. The mixture was added with 0.5 ml of DNS solution immediately and 40 µl of 0.1M NaOH. The mixture was then incubated at 100 ^{o}C for 10 minutes and subsequently cooled to room temperature. The non-enzymatic release of sugars was corrected by setting up a separate blank for each sample. One unit (U) of pullulanase activity is defined as the amount of enzyme required to produce reducing sugar equivalent to 1 µM of glucose per minute under the assay conditions.

3.11 Preliminary Characterization of Crude Enzyme

3.11.1 Effect of pH on Enzyme Activity

The effect of pH ranging from pH 4.0 to pH 10.0 on enzyme activity was determined by performing pullulanase assay, as mentioned in section 3.9, with the glycine-NaOH buffer, pH 9.0, which was then replaced by the following buffers: 100 mM sodium acetate buffer (pH 4 - 5), 100mM potassium phosphate buffer (pH 6 - 8) and 100 mM glycine-NaOH buffer (pH 9 - 10).

3.11.2 Effect of Temperature on Enzyme Activity

The effect of temperature on enzyme activity was determined by incubating the reaction mixture containing enzyme sample and 1 %(w/v) pullulan in glycine-NaOH buffer, pH 9.0, at different temperature ranging from 37 ^{o}C to 70 ^{o}C for 20 minutes. Then, the reducing sugar released during the incubation was determined by the DNS method as described in section 3.9.

3.12 Experimental Designs

The use of statistical experimental design in the optimization of fermentation process and media is well documented (Cockshott & Sullivan, 2001). Statistical experimental design has been used for several decades and it can be adopted at various phases of an optimization strategy, such as for screening experiments or for looking for the optimal conditions for targeted responses (Abdel-Fattah *et al*., 2005). Nowadays, the statistical experimental designs have become popular as a better choice in studying medium optimization compared with the conventional practice of one-variable-at-a-time (OVAT). The conventional OVAT practice is time-consuming, tedious and incapable to find out the true optimum due to the interaction among variables. Statistical procedures provide an alternative methodology to optimize a particular process by considering mutual interactions among the variables and give an estimate of the combined effect of these variables on the final result (Murthy *et al*., 2000).

3.12.1 Plackett-Burman Design

Plackett-Burman experimental design was applied to identify the relative significant effects of various components. Plackett-Burman design is a saturated orthogonal design work at two-levels, and can be constructed on the basis of fractional replication of a full factorial design (Plackett & Burman, 1946).

Moreover, Plackett-Burman design proved to be a valuable tool for screening, and mathematically computes the significance of large number of factors and selecting ingredients with minimal number of experiments, which is time saving and maintain convincing information on each component (Krishnan *et al*., 1998, Hallett *et al*., 1997, Abdel-Fattah *et al*., 2005). Plackett-Burman Design screens the important components for medium optimization as well as their significance levels but the design does not consider the interaction effects among the components (Pujari & Chandra, 2000).

Plackett-Burman design is a well established and widely used statistical design technique for screening the medium components in shake flasks.

Plackett-Burman Design can carry out a total number of experiments of K+1, where K is number variables or medium components. In general, a multiple of four (4n) experiments are required in this design. Each independent variable is tested at two levels, a high (+) level and a low (-) level. The number of positive signs and negative signs per experiments are (K+1)/2 and (K-1)/2, respectively. Each column and row should contain equal numbers of positives and negative signs.

Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \Sigma\beta_i X_i$$

where, Y = the response (enzyme activity)

β_0= the model intercept

β = the linear coefficient

X_i= the level of the independent variable

In this study, matrix design of Plackett-Burman was developed based on the design in the Design Expert Software Version 6.0.4 (Stat-Ease Inc., Minneapolis, USA). The Plackett-Burman design was applied for screening on a total of 17 components and two dummy variables were added in the design. The 17 components

screened are soluble starch, sago starch, tapioca starch, maltose, dextrin, yeast extract, meat peptone, casein, meat extract, NH_4Cl, $MgSO_4$, $CaCl_2$, $MgCl_2.6H_2O$, KH_2PO_4, K_2HPO_4, Na_2HPO_4 and KCl. The concentration for each component was set based on literature and experience. A total number of 20 runs were constructed in the design. The actual and coded values of each component of the design matrix for screening procedure are shown in Table 3.1. The design matrix of the Plackett-Burman design for screening is shown in Table 3.2.

Table 3.1: Actual and coded values of components of the Plackett-Burman design for screening of significance factors.

Variable	Variable Code	Units	Actual Value		Coded Value	
			Low	High	Low	High
Soluble Starch	X_1	% w/v	0	1.5	-1	+1
Sago Starch	X_2	% w/v	0	1.5	-1	+1
Tapioca Starch	X_3	% w/v	0	1.5	-1	+1
Maltose	X_4	% w/v	0	1.5	-1	+1
Dextrin	X_5	% w/v	0	1.5	-1	+1
Yeast Extract	X_6	% w/v	0	1.0	-1	+1
Meat Peptone	X_7	% w/v	0	1.0	-1	+1
Casein	X_8	% w/v	0	1.0	-1	+1
Meat Extract	X_9	% w/v	0	1.0	-1	+1
NH_4Cl	X_{10}	% w/v	0	0.02	-1	+1
$MgSO_4$	X_{11}	% w/v	0	0.02	-1	+1
$CaCl_2$	X_{12}	% w/v	0	0.02	-1	+1
$MgCl_2.6H_2O$	X_{13}	% w/v	0	0.02	-1	+1
KH_2PO_4	X_{14}	% w/v	0.01	0.05	-1	+1
K_2HPO_4	X_{15}	% w/v	0.01	0.05	-1	+1
Na_2HPO_4	X_{16}	% w/v	0.05	0.3	-1	+1
KCl	X_{17}	% w/v	0	0.02	-1	+1

Table 3.2: The design matrix of the Plackett-Burman design for screening.

Factors	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}
Run 1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1
Run 2	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1
Run 3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
Run 4	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1
Run 5	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
Run 6	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1
Run 7	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1
Run 8	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
Run 9	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1
Run 10	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1
Run 11	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1
Run 12	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1
Run 13	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1
Run 14	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1
Run 15	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1
Run 16	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1
Run 17	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
Run 18	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1
Run 19	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1
Run 20	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1

3.12.2 Central Composite Design (CCD)

In the study of optimization, Response Surface Methodology (RSM) has been chosen due to its effectiveness to supply the needed information about the shape of the response surface and also expand a minimum of resources. RSM, described first by Box and Wilson, is a collection of mathematical and statistical techniques, based on the fundamental principles of statistics, randomization, replication and duplication, which simplify the optimization of different biotechnological processes by studying the mutual interactions among the variables

over a range of values in a statistically valid manner (Silva & Roberto, 2001; Murthy *et al*., 2000).

Basically, the strategy of RSM contains four steps in optimizing the variable conditions. Firstly, RSM procedure is designed to move into the optimum region. Then, RSM determines the behaviour of the response in the optimum region, estimates the optimum conditions of the process, and lastly followed by the verification step (Tanyildizi *et al.*, 2005).

A 2^5 full Central Composite Design (CCD) was adopted to optimize five components that were identified previously using Plackett-Burman design, for pullulanase production by bacteria P2. From the Plackett-Burman design, five components out of 17 components were chosen to be as significant factors in the pullulanase production, which were sago starch, NH_4Cl, Na_2HPO_4, KCl and $MgSO_4$. The actual and coded values for each component studied in the CCD are shown in Table 3.3.

According to the CCD, the total number of experiments was $2^k + 2k + CP$, where k is the number of independent variables and CP is the number of repetitions of the experiments at the centre point. In this study, the total number of experiments of the 2^5 full CCD with 8 centre points was 50 runs. There were two axial points on the axis of each design variable at a distance of 2.3784 from the design centre.

Table 3.3: Actual and coded values of each component the CCD.

Variable	Units	Actual Value		Coded Value	
		Low	High	Low	High
Sago Starch	%w/v	1.75	3	-1	+1
NH_4Cl	%w/v	0.002	0.004	-1	+1
Na_2HPO_4	%w/v	0.025	0.05	-1	+1
KCl	%w/v	0.015	0.025	-1	+1
$MgSO_4$	%w/v	0.015	0.025	-1	+1

The coding of variables was done according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad i = 1, 2, 3, \ldots, n$$

where, x_i = coded value of the ith independent variable,

X_i = actual value of the ith independent variable,

X_0 = actual value of the ith independent variable at the centre point,

ΔX_i = step change.

Table 3.4 shows the design matrix constructed using Design Expert Software Version 6.0.4 (Stat-Ease Inc., Minneapolis, USA)

Table 3.4: The design matrix of Central Composite Design (CCD) for the optimization of pullulanase production.

Run	Block	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
		A:Sago	B:NH_4Cl	C:Na_2HPO_4	D:KCl	E:$MgSO_4$
1	{ 1 }	-1	1	1	1	1
2	{ 1 }	-1	1	1	-1	-1
3	{ 1 }	1	1	1	-1	1
4	{ 1 }	0	0	0	0	0
5	{ 1 }	0	0	0	-2.3784	0
6	{ 1 }	-1	-1	-1	1	-1
7	{ 1 }	0	0	2.3784	0	0
8	{ 1 }	1	-1	1	-1	1
9	{ 1 }	0	0	0	0	-2.3784
10	{ 1 }	-1	-1	-1	-1	1
11	{ 1 }	1	-1	1	-1	-1
12	{ 1 }	-1	-1	1	-1	-1
13	{ 1 }	0	0	0	0	0
14	{ 1 }	-1	-1	1	1	-1
15	{ 1 }	1	-1	-1	-1	-1
16	{ 1 }	0	0	0	0	0
17	{ 1 }	-1	-1	1	1	1
18	{ 1 }	-1	-1	-1	-1	-1
19	{ 1 }	1	1	1	1	-1
20	{ 1 }	2.3784	0	0	0	0
21	{ 1 }	-1	1	-1	-1	1
22	{ 1 }	1	1	1	1	1
23	{ 1 }	-1	-1	1	-1	1
24	{ 1 }	0	2.3784	0	0	0
25	{ 1 }	-1	1	-1	1	-1
26	{ 1 }	-1	1	1	1	-1
27	{ 1 }	0	0	0	0	0
28	{ 1 }	1	-1	1	1	-1
29	{ 1 }	0	0	0	0	2.3784
30	{ 1 }	1	1	-1	1	1
31	{ 1 }	1	-1	-1	1	1
32	{ 1 }	0	0	0	0	0
33	{ 1 }	-1	-1	-1	1	1
34	{ 1 }	1	1	-1	-1	1
35	{ 1 }	0	0	0	0	0
36	{ 1 }	1	-1	-1	1	-1
37	{ 1 }	1	1	-1	-1	-1
38	{ 1 }	1	1	1	-1	-1
39	{ 1 }	1	-1	1	1	1
40	{ 1 }	0	0	-2.3784	0	0
41	{ 1 }	0	0	0	0	0
42	{ 1 }	0	-2.3784	0	0	0

43	{ 1 }	-2.3784	0	0	0	0
44	{ 1 }	1	-1	-1	-1	1
45	{ 1 }	0	0	0	2.3784	0
46	{ 1 }	-1	1	-1	-1	-1
47	{ 1 }	0	0	0	0	0
48	{ 1 }	-1	1	1	-1	1
49	{ 1 }	-1	1	-1	1	1
50	{ 1 }	1	1	-1	1	-1

Once the experiments were performed, the experimental results were fitted with a second-order polynomial model equation as described below:

$$Y= \beta_o + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_i X_j + \varepsilon$$

Where Y = the predicted response

β_o = the offset term

β_i = first order regression coefficient

β_{ii} = quadratic regression coefficient

β_{ij} = coefficient for the combined model

ε = error

The second-order polynomial coefficients were calculated and analyzed using the Design Expert Software Version 6.0.4 (Stat-Ease Inc., Minneapolis, USA). The Design Expert Software was also used to generate the response surface plot and optimum values.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of pullulanase producer

Among the 19 strains of bacteria screened, only Bacteria P2 exhibited the ability to degrade pullulan and dyed-pullulan (AZCl-pullulan and red pullulan). Commercial dyed-pullulan, such as AZCL-pullulan (Megazyme) and red pullulan (Megazyme), were successfully applied in agar plate to screen for pullulanase-producer. Non-dissolvable AZCL-pullulan can be hydrolysed by pullulanase produced by Bacteria P2 and blue-colour AZCL is released and spread to agar. The dissolution of AZCL-pullulan pellet on the agar plate indicated the degradation of AZCL-pullulan by pullulanase produced by Bacteria P2 grown on the AZCL-pullulan agar plate. Figure 4.1 shows the ability of Bacteria P2 to degrade AZCl-pullulan and release the AZCl to agar medium.

In addition, the disappearance of red colour indicated that the red pullulan has been hydrolysed by pullulanase (Kanno and Tomimura, 1985). The clearing zone formed around colonies on red colour agar medium indicates the degradation by pullulanase produced by Bacteria P2. Figure 4.2 shows the degradation of red pullulan by pullulanase produced by Bacteria P2. According to this figure, the disappearance of red colour is greater if Bacteria P2 was incubated for two days. This is due to the high yield of pullulanase enzyme produced and released to the agar medium. Hence, the larger clearing zones were formed.

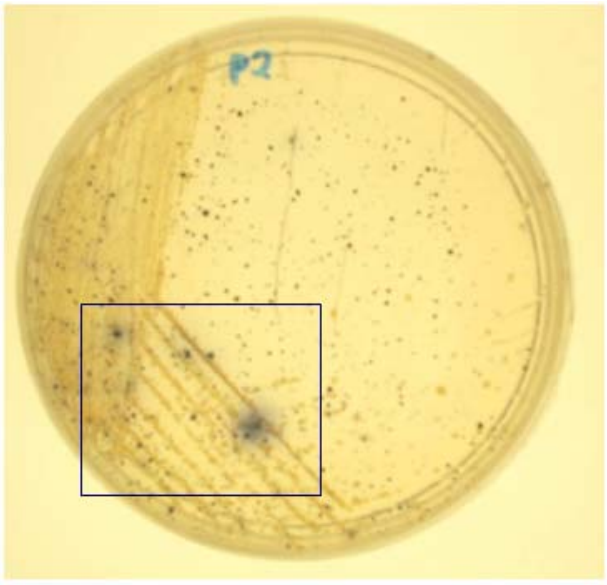


Figure 4.1: Degradation of AZCl-pullulan pellet by pullulanase produced by Bacteria P2 after 24 hours incubation at 37 ^{o}C. The blue AZCl compounds were released after the degradation and spread to agar.

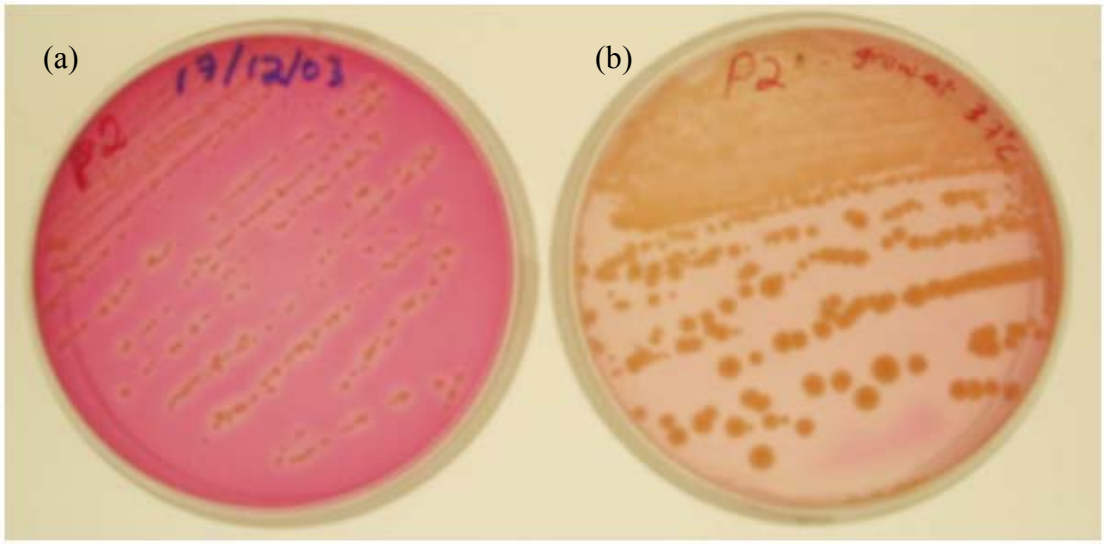


Figure 4.2: Degradation of Red-Pullulan by pullulanase produced by Bacteria P2. (a) 24 hours incubation at 37 ^{o}C; (b) 48 hours incubation at 37^{o}C. Larger clearing zones were formed around colonies when incubation time was elongated.

Figure 4.3: Degradation of pullulan by Bacteria P2 pullulanase after 24 hours incubation at 37 ^{o}C. The colonies were scraped and the plate was flooded twice with methanol or ethanol for around one hour. Clearing zones formed around the colonies were due to the degradation of pullulan in this area.

Another effective method for screening of pullulanase-producer is the pullulan precipitation method, where ethanol or methanol is used to precipitate pullulan. The precipitated pullulan on agar medium formed a turbid background. The clearing zone formed around colonies after the agar medium was flooded with ethanol or methanol revealed that pullulan around the colonies was degraded by pullulanase produced by Bacteria P2 (Figure 4.3).

The dyed-pullulan is usually applied in screening of genomic library for pullulanase gene recombinant clones. Chen *et al*., (2001) reported the application of red-pullulan in screening of the genomic library of *Bacillus stearothermophilus* TS-23. Three recombinant clones that harboured amylopullulanase gene insert exhibited halo zones on the 0.5 %(w/v) red-pullulan LB/ampicillin plate. Kim *et al.* (2000) also reported the application of red-pullulan (Megazyme, USA) in screening of genomic library of *Thermus* strain IM6501 for type I pullulanase gene recombinant clones. A transformant that is able to form clear zone around the colony on the red-pullulan plate was selected. Besides, AZCl-pullulan was applied to screen

recombinant clones for pullulanase gene isolation from *Desulfurococcus mucosus* (Duffner *et al*., 2000). Moreover, Bibel *et al*. (1998) have applied the methanol precipitation method for the screening of *Thermatoga maritime* genomic library for pullulanase. Thus, Bacteria P2 was identified as the pullulanase-producer and selected for further experimental study.

4.2 Identification of Bacteria at 16S rRNA level

The polymerase chain reaction (PCR)-based analysis of 16S rRNA genes is a powerful and essential tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eukaryotic organisms (Weisburg *et al*., 1990; Hongoh *et al*., 2003). 16S rRNA gene sequence has been used extensively for phylogenetic classification, identification, and genotypic typing of bacteria. It has enabled us to detect and identify as-yet unculturable bacteria, and in recent years has led to an enormous increase in our knowledge of bacterial ecology and taxonomy. By 1990s, the analysis has become the principal method of establishing phylogenetic relationships among the prokaryotes. Recently, it is becoming more important that a 16S rRNA sequence will be the first piece of data collected for an unknown organism (Lilburn and Garrity, 2004). According to Kiratisin *et al*. (2003), 16S rRNA gene sequencing is the most commonly used method for bacterial identification.

In 2005, Kim *et al*. reported the identification of two novel species of *Exiguobacterium* using 16S rRNA genes comparison method. The novel species formed a coherent cluster with *Exiguobacterium* species in a phylogenetic tree based on 16S rRNA gene sequences. They showed the closest phylogenetic affiliation to *Exiguobacterium aurantiacum*, with 16S rRNA gene sequence similarity values of 98.1–98.3 %.

4.2.1 PCR Amplification and Sequencing of 16S rRNA from Bacteria P2

1.6 kb of 16s rRNA gene was amplified from Bacteria P2 genomic DNA using universal primers as shown in Figure 4.4. The universal primers have been proven to produce an approximately 1.5 kb fragment of 16S rRNA gene from all bacteria (Weisburg *et al*., 1990).

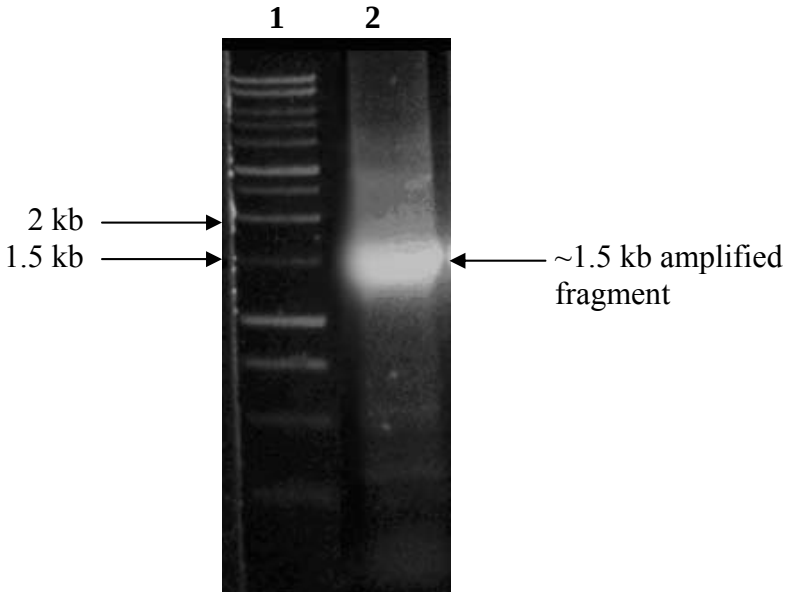


Figure 4.4: The amplified 1.5 kb of 16S rRNA gene from Bacteria P2 [Lane 1: 1 kb marker, Lane 2: 1.5 kb 16S rRNA gene fragment]

4.2.2 Analysis of 16S rRNA Gene Fragment of Bacteria P2

The 1.6 kb amplified 16S rRNA gene fragment was sequenced from both strands and the complete sequence is shown in Figure 4.5.

```
1     AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC
61    GCAGGAAGCC GTCTGAACCC TTCGGGGGGA CGAACGGTGG AATGAGCGGC GGACGGGTGA
121   GTAACACGTA AAGAACCTGC CCATAGGTCT GGGATAACCA CGAGAAATCG GGGCTAATAC
181   CGGATGTGTC ATCGGACCGC ATGGTCCGCT GATGAAAGGC GCTCCGGCGT CGCCCATGGA
241   TGGCTTTGCG GTGCATTAGC TAGTTGGTGG GGTAACGGCC CACCAAGGCG ACGATGCATA
301   GCCGACCTGA GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
361   AGGCAGCAGT AGGGAATCTT CCACAATGGA CGAAAGTCTG ATGGAGCAAC GCCGCGTGAA
421   CGATGAAGGC TTTCGGGTCG TAAAGTTCTG TTGTAAGGGA AGAACAAGTG CCGCAGGAAT
481   GGCGGCACCT TGACGGTACC TTGCGAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG
541   GTAATACGTA GGTGGCAAGC GTTGTCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGCGGC
601   CTCTTAAGTC TGATGTGAAA GCCCCCGGCT CAACCGGGGA GGGCCATTGG AAACTGGGAG
661   GCTTGAGTAT AGGAGAGAAG AGTGGAATTC CACGTGTAGC GGTGAAATGC ATAGAGATGT
721   GGAGGAACAC CAGTGGGGGA AGGCGACTCT TTGGCCTATA ACTGACGCTG AGGCGCGAAA
781   SGTGGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA
841   GGTGTTGGAG GGTTTCCGCC TTCAGTGCTG AAGCTAACGC ATTAAGCACT CCGCTGGGGA
901   GTACGGTCGM AAGCTGAAAC TCAAAGGAAT TGACGGGGGC CGCACAAGCG GTGGAGCATG
961   TGGTTTAATT CGAAGCAACG CGAAGAACCT TACCAACTCT TGACATCCCC CTGACCGGTA
1021  CAGAGATGTA CCTTCCCCTT CGGGGGCAGG GGTGACAGGT GGTGCATGGT TGTCGTCAGC
1081  TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCTTGTC CTTAGTTGCC
1141  AGCATTTGGT TGGGCACTCT AGGGAGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG
1201  ACGTCAAATC ATCATGCCCC TTATGAGTTG GGCTACACAC GTGCTACAAT GGACGGTACA
1261  AAGGGCAGCG AAGCCGCGAG GTGGAGCCAA TCCCAGAAAG CCGTTCTCAG TTCGGATTGC
1321  AGGCTGCAAC TCGCCTGCAT GAAGTCGGAA TCGCTAGTAA TCGCAGGTCA GCATACTGCG
1381  GTGAATACGT TCCCGGGTCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGCAACACC
1441  CGAAGTCGGT GAGGTAACCG TAAGGAGCCA GCCGCCGAAG GTGGGGCAGA TGATTGGGGT
1501  GAAGTCGTAA CAAGGTAACC
```

Figure 4.5: Nucleotide sequence of 16S rRNA gene of Bacteria P2. The universal primers used in PCR amplification of the 16S rRNA gene were underlined and bolded. The 16S rRNA nucleotide sequence of Bacteria P2 was submitted to GenBank with the Accession No. DQ366351.

A total of 1600 bp nucleotide of 16S rRNA gene derived from Bacteria P2 were successfully determined. The taxonomic relationships were inferred from 16S rRNA gene sequence comparison. The 16S rRNA gene sequence of Bacteria P2 were compared with other sequences from the Ribosomal Database Project (RDP-II) release 9.21 (http://rdp.cme.msu.edu/) and from Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). RDP-II is a tool for high-throughput rRNA

analysis. The RDP-II obtains bacterial rRNA sequences from the International Nucleotide Sequence Databases on a monthly basis (Cole *et al*., 2005). The BLAST programs are widely used tools for searching proteins and DNA databases for sequence similarities (Altschul *et al*., 1997).

Using the Hierarchy Browser tool found in RDP-II, Bacteria P2 that was determined, belongs to the Domain of *Bacteria*, Phylum of *Firmicutes,* Class of *Bacilli*, Order of *Bacillales* and Genus of *Exiguobacterium* (Table 4.1). The Hierarchy Browser allows rapid navigation through the RDP sequence data (Cole *et al*., 2005).

Table 4.1: Taxonomy hierarchy of Bacteria P2 resulted by using Hierarchy Browser tool of RDP-II. (Confidence threshold at 95 %)

Taxonomic Division	**Taxon**
Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Bacillaceae*
Genus	*Exiguobacterium*

The nucleotide sequence of 16S rRNA gene of Bacteria P2 was compared and matched using RDP-II and BLAST. Seventeen close-phylogenetically related 16S rRNA gene sequences were selected, including updated eight published members of genus *Exiguobacterium* that were deposited in DSMZ (German Collection for Microorganisms), which are *E. aurantiacum*, *E. acetylicum*, *E. aestuarii*, *E. antarcticum*, *E. aurantiacum*, *E. marinum*, *E. oxidotolerans* and *E. undae*. The selected sequences were completely aligned using multiple sequence alignment program Clustal X. The similarity values were also calculated (Table 4.2). The statistical report shows that 16S rRNA gene sequence of Bacteria P2 shared the

highest similarity with *Exiguobacterium sp.* JL-42, *Exiguobacterium sp.* JL-25 and *Exiguobacterium sp*. JL-24 (98 %), while *Exiguobacterium aestuarii*, *Exiguobacterium sp*. BTAH-1, *Exiguobacterium sp.* SA10 and *Exiguobacterium sp*. SA10 shared 96 % similarity. The rest of the 16S rRNA gene sequences also showed high similarity ranging from 86 % to 95 %.

A phylogenetic tree was constructed using the Neighbor-Joining method (N-J method) that applied the Clustal X program (Figure 4.6). Bootstrap analysis was used to assess the grouping reliability based on 1000 resamplings. The constructed neighbour-joining phylogenetic tree shows that the cluster was divided into two major branches. *E. acetylicum, E. artemiae, E. undae, E. antarcticum* and *E. oxidotolerans* were grouped into the same branch, while the rest 16S rRNA gene sequences were in other branch. This phylogenetic grouping pattern is in good agreement with the study conducted by Kim *et al*. (2005). Bacteria P2 was grouped in the second branch and found as the most phylogenetically-related to *Exiguobacterium sp.* JL-25 and *Exiguobacterium sp.* JL-42, with highest bootstrap value at 876.

It has been proposed that a prokaryote whose 16S rRNA sequence differs by more than 3 % from that of all other organisms (that is, the sequence is less than 97 % identical to any other sequence), should be considered a new species (Madigan *et al*., 2000). There is also some exception that leads to misclassification if 16S rRNA sequence similarities analysis is solely considered. The recommendation has been questioned recently. Drancourt *et al.* (2000) suggested that a 99 % similarity as a suitable cutoff value for identification at the species level and a 97 % similarity as a suitable cutoff value for identification at the genus level.

However, to confirm the identification at species level, DNA-DNA hybridization analysis is recommended. The Bacteria P2 was assigned as *Exiguobacterium sp.* MAAC-1.

Table 4.2: Statistical report of 16S rRNA gene sequence similarities.

	marin	lacti	aest	BTAH1	SA10	9IX	JL24	P2	JL25	JL42	AT1b	mexi	auran	arte	acety	antar	undae	oxido
marin	0	98%	99%	96%	96%	97%	96%	96%	96%	96%	93%	89%	94%	86%	88%	89%	92%	94%
lacti	1496	0	98%	95%	95%	96%	95%	95%	95%	95%	92%	88%	93%	84%	87%	88%	91%	92%
aest	1508	1498	0	97%	97%	97%	96%	96%	97%	97%	94%	89%	94%	86%	88%	89%	93%	94%
BTAH1	1471	1463	1473	0	98%	98%	96%	96%	96%	96%	96%	91%	94%	88%	90%	92%	90%	91%
SA10	1479	1469	1481	1473	0	98%	97%	96%	97%	97%	95%	90%	94%	87%	89%	91%	91%	92%
9IX	1485	1472	1487	1474	1485	0	97%	96%	97%	97%	96%	91%	95%	88%	90%	91%	92%	92%
JL24	1494	1480	1494	1471	1484	1493	0	98%	98%	98%	95%	90%	96%	87%	90%	90%	92%	92%
P2	1481	1473	1483	1466	1477	1478	1500	0	98%	98%	95%	89%	95%	86%	89%	89%	91%	91%
JL25	1487	1479	1492	1473	1484	1485	1508	1501	0	99%	96%	90%	96%	87%	90%	90%	91%	92%
JL42	1490	1480	1493	1474	1487	1486	1509	1502	1515	0	96%	90%	96%	87%	90%	90%	92%	92%
AT1b	1446	1438	1450	1451	1451	1457	1466	1458	1464	1465	0	93%	93%	90%	93%	92%	89%	89%
mexi	1370	1356	1373	1369	1372	1378	1383	1370	1380	1385	1382	0	90%	91%	89%	89%	86%	85%
auran	1462	1449	1465	1441	1456	1460	1482	1470	1478	1483	1439	1386	0	86%	89%	89%	91%	91%
arte	1327	1311	1325	1325	1324	1331	1338	1328	1336	1339	1337	1329	1335	0	94%	94%	91%	90%
acety	1364	1351	1363	1364	1365	1370	1382	1373	1379	1380	1382	1323	1377	1392	0	95%	92%	92%
antar	1373	1357	1373	1371	1372	1379	1380	1367	1376	1379	1373	1318	1370	1395	1421	0	95%	94%
undae	1422	1406	1422	1385	1398	1404	1417	1406	1413	1416	1373	1319	1406	1402	1420	1455	0	97%
oxido	1429	1413	1428	1393	1402	1407	1417	1405	1412	1415	1372	1314	1403	1390	1422	1442	1490	0

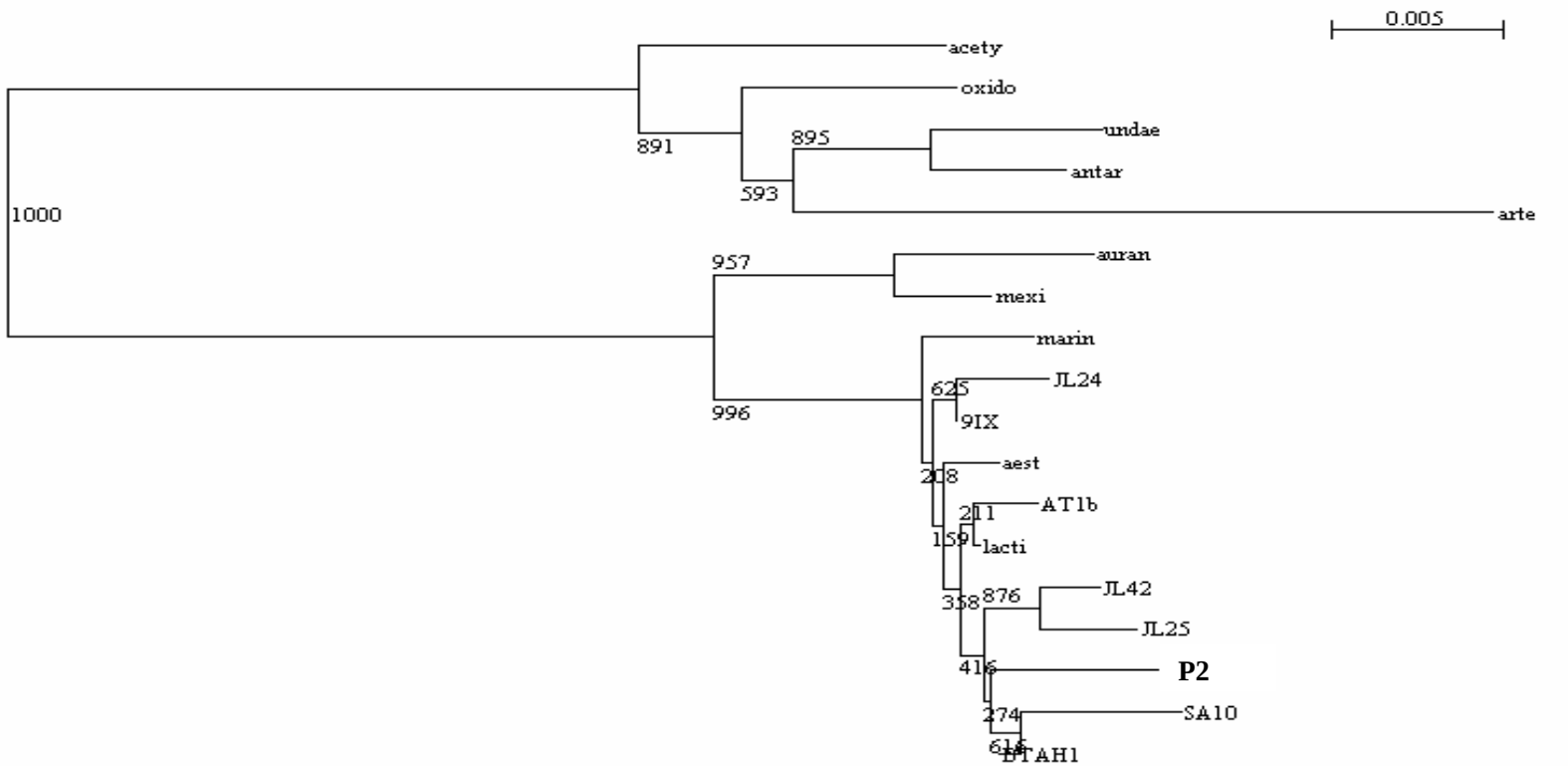


Figure 4.6: The Neighbour-joining phylogenetic tree constructed based on 16S rRNA gene sequences showing the phylogenetic positions of Bacteria P2 and 17 close-phylogenectically related sequences. Numbers on the branches indicate the bootstrap value of 1000 resamplings. Bar indicates five nucleotides substitutions per 1000 nucleotide positions. The abbreviated bacteria are as follows: **P2**: Bacteria P2; **acety**: *E. acetylicum* (X70313); **arte**: *E. artemiae* (AM072763); **undae**: E. *undae* (AJ344151); **antar**: *E. antarcticum* (AJ297437); **oxido**: *E. oxidotolerans* (AB105164); **mexi**: *E. mexicanum* (AM072764); **auran**: *E. aurantiacum* (X70316); **marin**: *E. marinum* (AY594266); **JL24**: *E. sp*. JL-24 (AY745822); **JL25**: *E. sp.* JL-25 (AY745823); **JL42**: *E. sp.* JL-42 (AY 745848); **91X**: *E. sp*. 91X/AO1/159 (AY612767); **aest**: *E. aestuarii* (AY594264); **AT1b**: *E. sp.* AT1b (DQ302410); **lacti**: *E. lactigenes* (AY818050); **BTAH1**: *E. sp*. BTAH1 (AY205564); **SA10**: *E. sp.* SA10 (AY864659).

4.3 Partial Characterization of Crude Pullulanase Enzyme

The sample was taken at 22 h of growth at 37 ^{o}C. The supernatant of the sample was collected after centrifuging the sample and the crude enzyme was prepared for the determination of optimum pH and optimum temperature.

4.3.1 Effect of pH on Enzyme Activity

The effect of pH on the pullulanase activity produced by *Exiguobacterium sp*. MAAC-1 was tested. The pH profile depicted the relative activity versus pH is shown in Figure 4.7. The crude pullulanase enzyme produced by *Exiguobacterium sp.* MAAC-1 showed highest activity at pH 9.0 and the crude enzyme was significantly inactivated at pH 4.0 and 10.0 with lower than 50 % of relative enzyme activity observed. The crude pullulanase enzyme was active in the pH range between 7.0 –9.0 with higher than 85 % relative activity observed. This indicates that the pullulanase produced by *Exiguobacterium sp*. MAAC-1 is more active in slight alkaline condition. There are few studies that reported about the alkaline pullulanase. Lee *et al*. (1997) reported that an alkaline pullulanase produced by *Bacillus sp*. S-1 was found to be stable in a pH range of 6.0 to 10.5, with optimum pH of 9.0. Ara *et al.* (1995) also made the first report of alkaline type II pullulanase, which was produced by alkalophilic *Bacillus sp*. KSM-1378, has maximum activity at pH 9.5. Ara et al. (1995) also reported that the type II pullulanase could be suitably used as an effective additive in dishwashing and laundry detergents due to its alkaline-active property.

Basically, most microbial pullulanases are active at neutral or acidic conditions, with the optimum pH of enzyme activity between pH 5.0 to 7.0. Gomes *et al*. (2003) reported that pH optima of the crude pullulanase of *Rhodothermus marinus* were in the range of pH 6.5-7.0. The enzyme activity was significantly decreased at pH 4.0 and 9.0. Besides, Roy *et al.* (2003) reported that acidic type II pullulanase produced by *Bacillus*

sp. US149 was active between pH 4.5 and 5.6, with an optimum pH of 5.0. Another acidic pullulanase was reported by Kriegshäuser and Liebl (2000). The acidic pullulanase produced by *Thermotoga maritima* showed higher than 50 % of maximum activity was observed between pH 4.5 to 7.5, with an optimum at pH 5.9. In addition, Brunswick *et al.* (1999) and Reddy *et al*. (1998) reported that pullulanase of *Bacillus sp*. DSM 405 and *Clostridium thermosulfurogenes* SV2, respectively, was optimally active at pH 6.0. Moreover, pullulanase from *Clostridium thermohydrosulfuricum* was stable at pH 3.0-5.0 and the activity was gradually inactivated at pH above 5.5. The pullulanase showed maximal activity at pH 5.5, with about 95 % and 75 % activity at pH 5.0 and 4.5 respectively (Saha *et al*., 1988).

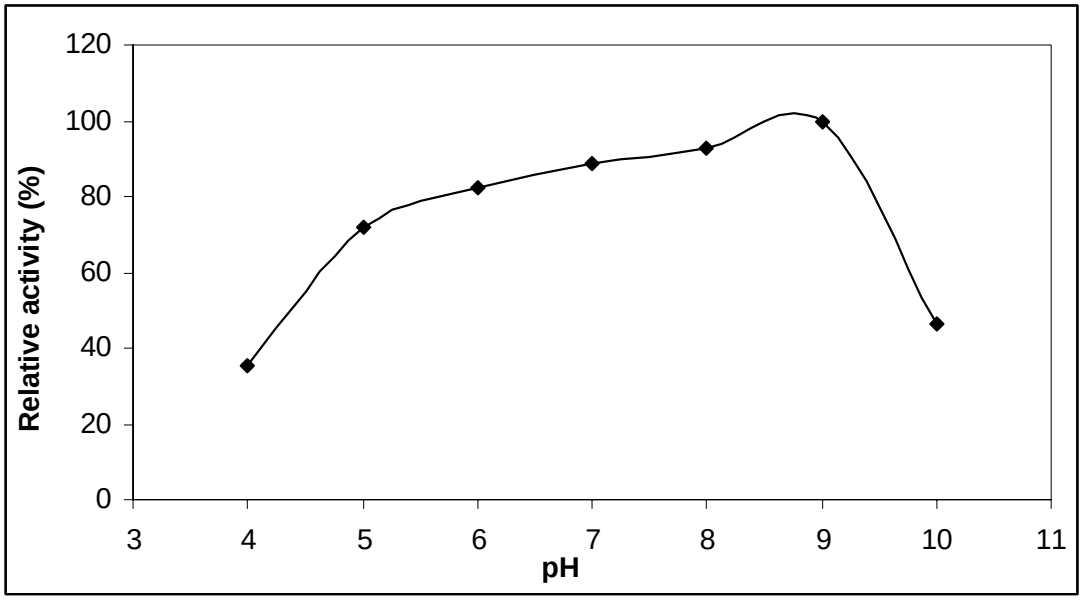


Figure 4.7: Optimum pH of crude pullulanase enzyme from *Exiguobacterium sp.* MAAC-1. The crude pullulanase was found most active at pH 9.0.

4.3.2 Effect of Temperature on Enzyme Activity

The temperature profile that depicted the effect of temperature on pullulanase activity is shown in Figure 4.8. The effect of temperature in the range of 37- 70 ^{o}C on enzyme activity was tested. The relative activity versus temperature was drawn. The pullulanase produced by *Exiguobacterium sp*. MAAC-1 was found active in a wide range of temperature from 50 ^{o}C to 70 ^{o}C. The optimum temperature of pullulanase from *Exiguobacterium sp*. MAAC-1 was found at 60 ^{o}C, and slight decrease of the enzyme activity was observed at 55 ^{o}C and 65 ^{o}C, with relative activity of 85.9 % and 87.4 % respectively. The activity was inactivated at relative low temperature; only 29.3 % and 49 % of relative activity was observed at 37 ^{o}C and 45 ^{o}C respectively.

Several studies have reported the same optimum temperature observed in different microorganisms. For example, Devi and Yogeeswaran (1999) reported that the pullulanase produced by mesophile *Micrococcus halobius* OR-1 was optimally active at 60^{o}C. Roy *et al*. (2003) and Stefanova *et al*. (1999) also reported that the maximal activity of pullulanase from thermophiles, *Bacillus sp*. US149 and *Bacillus acidopullulyticus* respectively, were observed at 60 ^{o}C. This optimum temperature should be suitably used for saccharifying enzymes in starch processing industry. This is because of the temperature usually applied in saccharification process is around 60 ^{o}C (Lévêque et al., 2000; Devi and Yogeeswaran, 1998).

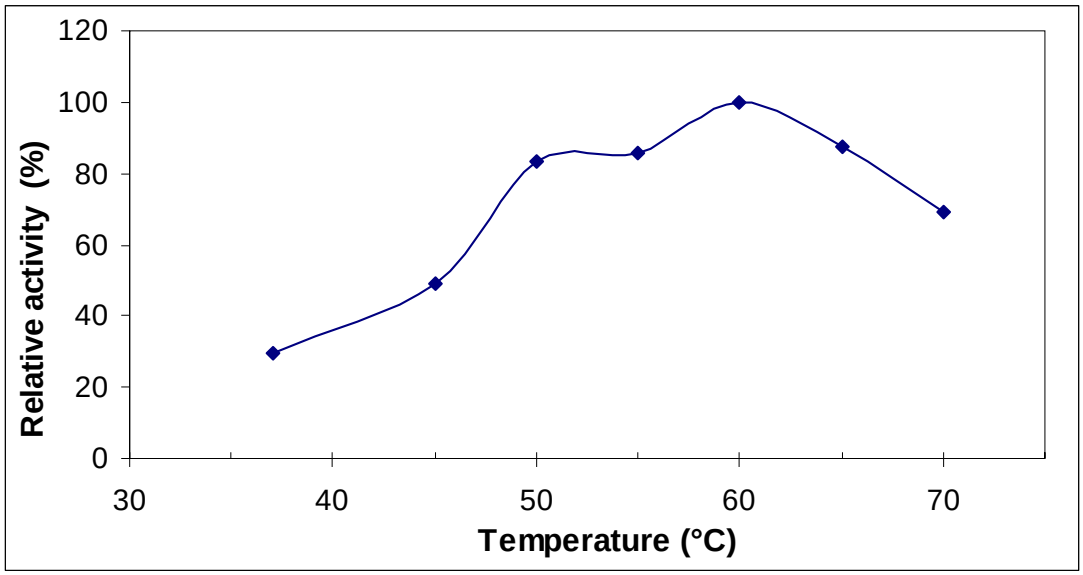


Figure 4.8: Optimum temperature of crude pullulanase enzyme from *Exiguobacterium sp.* MAAC-1. The crude enzyme was found most active at 60 ^{o} C.

4.4 Growth Curve of *Exiguobacterium sp.* MAAC-1

The time course for growth of *Exiguobacterium sp.* MAAC-1 and pullulanase production is shown in Figure 4.9. *Exiguobacterium sp*. MAAC-1 was cultured using modified PYE medium containing 2.0 % soluble starch in a conical flask. The bacteria was incubated at 37 ^{o}C with constant orbital shaking at 200 rpm for 36 h. The trend of pullulanase production was influenced by the growth of the cell. The incubation time that gave the highest pullulanase activity was determined and this point was used as the sampling point in the following optimization study of pullulanase production.

The growth curve of bacteria usually consisted of four distinct phases, which are lag phase, exponential phase, stationary phase and death phase. When a microorganism is inoculated into a fresh medium, the growth usually does not begin immediately but after a lag phase due to the culture requiring time to resynthesis essential constituents or new enzymes for the synthesis of the essential metabolites (Madigan *et al*., 2000). The

length of the lag phase can be shortened or extended depending on the type of the culture and growth conditions. The growth curve of *Exiguobacterium sp*. MAAC-1 began with the exponential phase immediately once the culture was inoculated into fresh medium and there is no lag phase. This may be due to healthy growing cells that were inoculated into the same medium under the same conditions of growth (Madigan *et al*., 2000). The same growth conditions may facilitate the growth of bacteria cells and skip the lag phase.

The culture grew extensively for the first 6 h of incubation. This period is the exponential phase of *Exiguobacterium sp.* MAAC-1 growth. The exponential phase was terminated after 6 h of incubation and then the stationary phase began. The length of the stationary phase of *Exiguobacterium sp.* MAAC-1 was considerably extended under the growth condition.

Pullulanase production was increased gradually during the exponential phase and achieved an optimum point (0.126 U/ml) at 22 h. The pullulanase production decreased during the death phase of growth curve. From the pullulanase production trend and growth curve of *Exiguobacterium sp.* MAAC-1, pullulanase production is a mixed-growth associated. It was found that the pullulanase production increased during the exponential growth of cells and the increment continued in stationary phase and reached a maximal production after 22 h incubation at 37 ^{o}C.

According to Gantelet and Duchiron (1998), extracellular pullulanase synthesis was mainly associated with the exponential growth phase and with the late exponential growth phase. However, most microorganisms showed similar pullulanase production trend as *Exiguobacterium sp.* MAAC-1. Legin *et al*. (1998) reported that *Thermococcus hydrothermalis* reached the end of the exponential growth phase at 6 h. However, the *Thermococcus hydrothermalis* produced 80 % of total pullulanase after 6 h growth and the pullulanase production increased and reached a maximum level after 24 h. Besides, Swamy and Seenayya (1996) also stated that the production of pullulanase by

Clostridium thermosulfurogenes SV9 was closely related to bacterial growth. The maximal production of pullulanase was achieved at the beginning of the stationary phase, which is after 12 h of incubation. From the studies shown above, it can be proposed that the production of pullulanase is paralleled with the bacterial growth and the pullulanase production increased continuously, and reached a maximal level during stationary phase. This production trend may be due to the pullulanase required to degrade the highly branched oligosaccharides that are hardly degraded by other amylases, such as α-amylase and α-glucosidase.

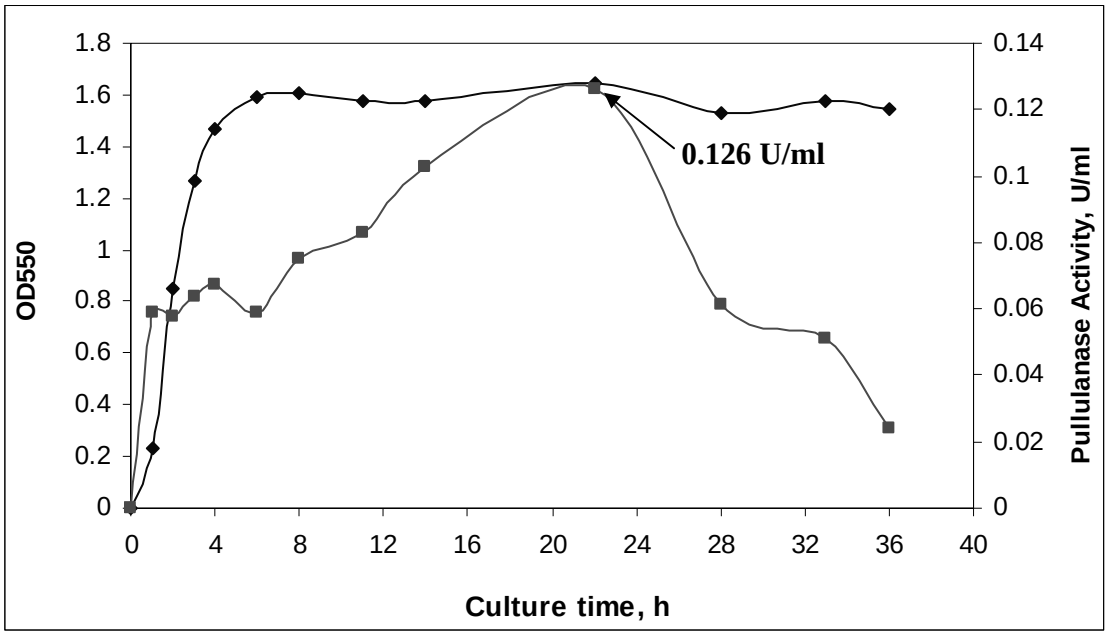


Figure 4.9: Growth profile of *Exiguobacterium sp.*MAAC-1 and its relationship with pullulanase production. ■, pullulanase activity; ◆, culture optical density at OD550 nm.

4.5 Medium Enhancement for Pullulanase Production Using Experimental Design

A statistical approach was applied in order to improve the production of pullulanase by *Exiguobacterium sp.* MAAC-1. The first step of this approach deals with screening of various medium components that affect the pullulanase production by using Plackett-Burman Design. The second step is to optimize the selected components that have significant effects on the pullulanase production.

4.5.1 Screening of Nutrients for pullulanase production by *Exiguobacterium sp.* MAAC-1 Using Plackett-Burman Design

The Plackett-Burman design was applied to evaluate the relative importance of various medium components for pullulanase production by *Exiguobacterium sp.* MAAC-1. The matrix design of the experiment was developed based on the design in the Design Expert Software Version 6.0.4. The 17 components screened, which included carbon source, nitrogen source, phosphorous source and mineral salts, are soluble starch, sago starch, tapioca starch, maltose, dextrin, yeast extract, meat peptone, casein, meat extract, NH_4Cl, $MgSO_4$, $CaCl_2$, $MgCl_2.6H_2O$, KH_2PO_4, K_2HPO_4, Na_2HPO_4 and KCl. For each component, a high (+) and a low (-) concentration were tested. A total of 20 runs were designed, which two dummy factors included in the design. Table 4.3 represents the results of Placket-Burman experiment with respect to pullulanase activity. The pullulanase activity of each run was measured after 22 h of incubation at 37 ^{o}C with constant orbital shaking at 200 rpm. The analysis of the data from the Plackett-Burman experiments involved a first order (main effects) model.

Table 4.3: Pullulanase production from the result of Plackett-Burman experiment. Pullulanase activity of each run was measured after 22 hours of incubation at 37 ^{o}C with constant shaking at 200 rpm.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	X_{19}	Pullulanase activity, U/ml
1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	1.6712
2	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	0.2696
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0
4	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1.0780
5	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	0.9884
6	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	0.7008
7	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	0.8088
8	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	0.7908
9	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	0.8444
10	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	0.1436
11	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	1.7968
12	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	0.9344
13	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	0.3772
14	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	0.3772
15	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	0.3592
16	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1.2940
17	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	0.8624
18	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	0.3056
19	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1.1860
20	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	0.6828

X_1: soluble starch; X_2: sago starch; X_3: tapioca starch; X_4: maltose; X_5: dextrin; X_6: yeast extract; X_7: meat peptone; X_8: casein; X_9: meat extract; X_{10}: NH_4Cl; X_{11}: $MgSO_4$; X_{12}: $CaCl_2$; X_{13}: $MgCl_2.6H_2O$; X_{14}: KH_2PO_4; X_{15}: K_2HPO_4; X_{16}: Na_2HPO_4; X_{17}: KCl; X_{18} & X_{19}: dummy.

The medium components were screened at the confidence level of 90 % on the basis of their effects, either positive or negative effects. Table 4.4 shows the result of ANOVA of first model in the Plackett-Burman design. The Model F-value of 12.39 implies that the model is significant. There is only 7.71 % chance that a "Model F-Value" could occur due to noise. This shows that the model can be used to explain the design.

The coefficient of determination, $R^2 = 0.9900$, and correlation coefficient, $R = 0.9950$. The determination coefficient (R^2) value provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions (Haaland, 1989). This value ranges from zero to one. A value of $R^2 > 0.75$ indicates the aptness of the model. Thus, the $R^2 = 0.9900$ implies a good agreement between the experimental and predicted values.

Table 4.4: ANOVA of first order model in Plackett-Burman design

Source	Sum of Squares	Mean Square	F Value	Prob > F
Model	4.2569	0.2661	12.3944	0.0771
Residual	0.0429	0.0215	-	-
Block	0.1407	0.1407	-	-

$R^2 = 0.9900$; $R = 0.9950$; confidence level = 90 %

The main effects of the examined factors on the enzyme activity were calculated and presented in Table 4.5. Values of "Prob > F" less than 0.1000 indicate that model terms are significant. Values greater than 0.1000 indicate that the model terms are not significant. In this case soluble starch, sago starch, tapioca starch, dextrin, NH_4Cl, $MgSO_4$, KH_2PO_4, Na_2HPO_4, KCl are significant.

Table 4.5: The analysis result of Plackett-Burman experiment for pullulanase production by *Exiguobacterium sp*. MAAC-1

Source	Sum of Squares	Mean Square	F Value	Prob > F
Soluble starch	0.4186	0.4186	19.4996	0.0476 [a]
Sago starch	0.4238	0.4238	19.74397	0.0471 [a]
Tapioca starch	0.2669	0.2669	12.43554	0.0719 [a]
Maltose	0.0915	0.0915	4.263105	0.1750
Dextrin	0.3088	0.3088	14.38567	0.0630 [a]
Meat peptone	0.1187	0.1187	5.529279	0.1430
Casein	0.0076	0.0076	0.351751	0.6133
Meat extract	0.0333	0.0333	1.549693	0.3393
NH_4Cl	0.3276	0.3276	15.26161	0.0597 [a]
$MgSO_4$	0.7360	0.7360	34.28815	0.0279 [a]
$CaCl_2$	0.0949	0.0949	4.421513	0.1702
$MgCl_2.6H_2O$	0.0051	0.0051	0.239701	0.6729
KH_2PO_4	0.3357	0.3357	15.63873	0.0584 [a]
K_2HPO_4	$5.88e^{-05}$	$5.88e^{-05}$	0.002741	0.9630
Na_2HPO_4	0.4783	0.4783	22.27963	0.0421 [a]
KCl	0.6318	0.6318	29.43082	0.0323 [a]

a: sigfinificant factor (value of "Prob>F" less than 0.1000)

The dummy variables are used to measure variability of Plackett-Burman design. It gives a direct estimate of standard error for a factor effect.

When the effect of the tested component, $E(x_i)$ is positive, the influence of the component upon pullulanase activity is greater at a high concentration, whereas if the effect is negative, the influence of the component is greater at low concentration.

Carbon components such as starches, oligosaccharides and sugars are major sources of cellular carbon and energy source (Shuler and Kargi, 1992; Madigan *et al.*, 2000). Based on the analysis result, all of the starches and dextrin, which are high in Degree of Polymerization (DP) value and consisting of many branching points, have greater effect on pullulanase production compared to low DP components, i.e. maltose in this study. This result shows that reducing sugars gave less inducing effect on pullulanase production.

Krishnan *et al*. (1998) reported that the positive effect of liquefied starch indicated that starch hydrolysing enzymes may be produced by the organism. Starch is a biopolymer composed of amylopectin and amylase. Amylopectin is a highly branched and large macromolecule that consists of short chains of 1,4- linked-α-D-glucose with 1,6-α- linked branches. Amylose is a macromolecule of linear 1,4-linked-α-D-glucan. The high amylopectin contents of starch may induce the microbes to produce debranching enzymes in order to degrade and utilize starch. Maltose showed the lowest significance value indicating that the production of pullulanase, the debranching enzyme, is affected by maltose which does not contain branch points.

The inducing effect of starch on pullulanase production was also reported in the study conducted by Reddy *et al*. (1999). Various carbon sources for pullulanase production by *Clostridium thermosulfurogenes* SV2 were screened using Plackett-Burman design. Among the carbon sources (lactose, potato starch, maltose and dextrin) screened, potato starch was found to be the best for the enzyme production. Potato starch was found to be the best for the enzyme production and maltose had positive effect on the cells growth but not on the pullulanase production. This study suggested that the potato starch had the inducing effect on the pullulanase production.

Antranikian *et al.* (1987) also reported the investigation on the effect of various carbohydrates on pullulanase production by *Clostridium sp*. strain EM1. They found that starch had the highest inducing effect on the production. Maltose and glucose was found to be less effective inducer compared to starch and dextrin.

However, there are also studies that reported that maltose had inducing effect on pullulanase production. Gomes *et al*. (2003) reported the investigation on the effects of various carbon sources on the pullulanase production by *Rhodothermus marimus*. Among the carbon sources, maltose was reported to increase the pullulanase production where the highest pullulanase production was induced in the presence of maltose.

Among the carbon sources used in this study, sago starch showed the highest significant level (prob>F = 0.0471). This indicates that the pullulanase production is greatly enhanced by sago starch. Marlida *et al*. (2000) reported that carbon from different sources would significantly influence the secretion of starch degrading enzymes activity into the growth media. The study also stated that the enzyme activity is strongly influenced by the properties of carbon sources and capability of the microorganisms to utilize them.

The nitrogen source also plays a very important role in enzyme production. Nitrogen source serves as an important constituent of proteins, nucleic acids, and coenzymes. In the investigation on the effects of nitrogen source on pullulanase production by *Exiguobacterium sp*. MAAC-1, all the organic nitrogen sources showed comparatively higher value of prob>F (greater than 0.1) than the inorganic nitrogen source (NH_4Cl) which indicated that all the organic nitrogen sources were not significant factors for the pullulanase production. The effect of yeast extract was ignored in the analysis of the design. This may be due to the effect of yeast extract which is unstable since yeast extract is a complex medium component that not only contains nitrogen, but even contains many other nutrients and salts.

In this study, NH_4Cl was found to be the best nitrogen source. NH_4Cl showed negative effect on the pullulanase production, thus low concentration is preferred to enhance the pullulanase production.

Significant effect of inorganic source for pullulanase production was also reported in the study of Reddy *et al.* (1999a). They reported the investigation on the

screening of nutrients for pullulanase production by *Clostridium thermosulfurogenes* SV2, $(NH_4)_2HPO_4$ was found to be the most significant nitrogen factors compared to organic nitrogen sources. The results of the data analysis using Plackett-Burman found that $(NH_4)_2HPO_4$ showed the highest t-value (8.06) among nitrogen sources used. The high t-value indicates the effect impact of the corresponding factor on the pullulanase production. This may be due to the inorganic nitrogen in ammonium (NH_4) form required to induce the pullulanase production.

Among the three phosphorous sources screened (KH_2PO_4, K_2HPO_4 and Na_2HPO_4), Na_2HPO_4 was found to be the most significant factor, with prob>F = 0.0421. The negative effect indicates that the influence of Na_2HPO_4 on the pullulanase production is greater at low concentration. Phosphorus is a key element in the regulation of cell metabolism. Phosphorus is also present in nucleic acids and in the cell wall of some gram-positive bacteria as teichoic acids (Shuler and Kargi, 1992). Moreover, phosphorus is also involved in the energy generating pathway and biosynthesis of nucleic acids and phospholipids (Madigan *et al*., 2000) Thus, phosphorous source is a major factor affecting the production of enzyme.

Phosphorus is added in most medium preparation. Gomes *et al.* (2003), Reddy *et al*. (1999b) and, Swamy and Seenayya (1996) reported the addition of two types of phosphorous source, Na_2HPO_4 and KH_2PO_4, in the pullulanase production medium. Brunswick *et al*. (1999) and Madi *et al*. (1987) also reported the addition of KH_2PO_4 in medium for production of amylopullulanase and pullulanase from *Bacillus sp.* DSM 405 and *Clostridium* strain, respectively. Reddy *et al*. (1999c) used NaH_2PO_4 for the production of pullulanase from *Clostridium thermosulfurogenes* SV2. Phosphorus is an important component as energy source for growth of culture, thus phosphorous sources were used in most studies of pullulanase production although in different phosphorous form. The addition of various phosphorous sources has been reported in the production of α-amylase, uricase, chitinase, xylanase, etc. (Francis *et al.,* 2003; Abdel-Fattah *et al*., 2005; Gohel *et al*., 2006; Ghanem et al., 2000).

The effects of four mineral salts, which are $MgSO_4$, $CaCl_2$, $MgCl_2.6H_2O$ and KCl, were analyzed. It was found that $MgSO_4$ and KCl showed very low value of prob>F, 0.0279 and 0.0323 respectively. This indicates that both are significant factors for pullulanase production by *Exiguobacterium sp*. MAAC-1. Mineral salts are essential to microbial nutrition. Most of the mineral salts function as cofactors for some enzymes, and some of the mineral elements are required in some important cell metabolism.

Data analysis showed that $MgSO_4$ has greater effect on the pullulanase production compared with $MgCl_2.6H_2O$ (see Table 4.5, p. 75). According to Madigan *et al.* (2000), sulfur is required because of its structural role in the amino acids cysteine and methionine. Sulfur is present in some proteins and coenzymes (Shuler and Kargi, 1992). Sulfur undergoes a number of chemical transformations in nature carried out exclusively by microorganisms. Magnesium functions to stabilize ribosomes, cell membranes, and nucleic acids and is required in carbohydrate metabolism. Magnesium is also a cofactor for many enzymes.

Besides, KCl also showed a very high degree of significance on pullulanase production. This indicates that potassium (K^+) and chloride (Cl^-) are important factors that affect the pullulanase production. Potassium is essentially required by all organisms. Potassium is a cofactor for some enzymes including the enzymes involved in protein synthesis. Meanwhile, chloride is involved in stabilizing most enzymes.

The addition of several mineral salts for the production of pullulanase has also been reported. Reddy *et al*. (1999a) reported the statistical screening of few mineral salts including $FeSO_4.7H_2O$, $MgCl_2.6H_2O$, $CaCl_2.H_2O$ and KCl for pullulanase production by *Clostridium thermosulfurogenes* SV2. The study found that $FeSO_4.7H_2O$ and $MgCl_2.6H_2O$ are the significant factors affecting the pullulanase yields. Swamy and Seenayya (1996) also used $FeSO_4$ and $MgCl_2.6H_2O$ in their studies on optimization of pullulanase production by *Clostridium thermosulfurogenes* SV9. In the study of Gomes *et al*. (2003), the addition of $FeSO_4.7H_2O$ and $MgCl_2$ was also reported for the production of pullulanase from

Rhodothermus marinus. Besides both salts, NaCl and $FeSO_4.7H_2O$ were also supplemented into the production medium. Meanwhile, $MgCl_2.6H_2O$ was added into the production medium for pullulanase production by a strain of Clostridium (Madi *et al*., 1987).

Different mineral salts are preferred by different microorganism for pullulanase production. In the study of Melasniemi (1987), $FeSO_4.7H_2O$, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were added in to the medium for the production of pullulanase by *Clostridium thermohydrosulfuricum* strain E101-69. However, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ were supplemented in the complex medium for pullulanase production from *Fervidobacterium pennavorans* Ven5 (Koch *et al*., 1997).

4.5.2 Optimization of Pullulanase Production Using Central Composite Rotatable Design (CCRD)

Five medium components, which were identified as significant factors for pullulanase production from the screening process using Plackett-Burman method (see chapter 4.5.1), were selected for further optimization. It comprises of a carbon sorce, a nitrogen source, a phosphorous source and two mineral salts. The five components selected are sago starch, NH_4Cl , Na_2HPO_4, $MgSO_4$ and KCl. The concentration of these five components were optimized to enhance the pullulanase production by *Exiguobacterium sp*. MAAC-1 by using response surface methodology.

A 2^5 full factorial Central Composite Rotatable Design (CCRD) was employed to search for the optimal concentration of the five significant components. In order to ensure the design is rotatable, star points were set at +/- alpha (α) value at 2.37841 in the design. The value for alpha is calculated in the design for both rotatability and orthogonality of blocks. A total of 50 experiments were carried out based on the design developed using Design Expert Software Version 6.0.4 (Stat-

Ease, Inc., Minneapolis USA). Table 4.6 represents the design matrix of the five significant factors in coded level, and along with the experimental pullulanase activity. Pullulanase activity from *Exiguobacterium sp.* MAAC-1 was measured after 22 h of incubation at 37 ^{o}C with constant orbital shaking at 200 rpm.

Table 4.6: Design matrix of 2^5 full factorial central composite design with corresponding experimental pullulanase activity

Run	A	B	C	D	E	Response 1
	X_i: Sago	X_{ii}: NH_4Cl	X_{iii}: Na_2HPO_4	X_{iv}: KCl	X_v: $MgSO_4$	* Pullulanase activity, U/ml
1	-1	1	1	1	1	0.7394
2	-1	1	1	-1	-1	0.7864
3	1	1	1	-1	1	0.9977
4	0	0	0	0	0	0.8216
5	0	0	0	-α	0	0.9272
6	-1	-1	-1	1	-1	0.7512
7	0	0	α	0	0	0.6925
8	1	-1	1	-1	1	1.0211
9	0	0	0	0	-α	0.5047
10	-1	-1	-1	-1	1	0.8568
11	1	-1	1	-1	-1	0.9624
12	-1	-1	1	-1	-1	0.7042
13	0	0	0	0	0	0.7394
14	-1	-1	1	1	-1	0.7981
15	1	-1	-1	-1	-1	0.9038
16	0	0	0	0	0	0.8451
17	-1	-1	1	1	1	0.8685
18	-1	-1	-1	-1	-1	0.6925
19	1	1	1	1	-1	0.8685
20	α	0	0	0	0	0.8568
21	-1	1	-1	-1	1	0.8920
22	1	1	1	1	1	0.6808
23	-1	-1	1	-1	1	0.8333
24	0	α	0	0	0	1.1620
25	-1	1	-1	1	-1	0.9859
26	-1	1	1	1	-1	0.9272
27	0	0	0	0	0	0.7746
28	1	-1	1	1	-1	0.6690

29	0	0	0	0	α	0.7042
30	1	1	-1	1	1	0.8568
31	1	-1	-1	1	1	0.8099
32	0	0	0	0	0	0.7629
33	-1	-1	-1	1	1	1.0211
34	1	1	-1	-1	1	1.0329
35	0	0	0	0	0	0.8803
36	1	-1	-1	1	-1	0.6808
37	1	1	-1	-1	-1	0.9859
38	1	1	1	-1	-1	0.9624
39	1	-1	1	1	1	0.8451
40	0	0	-α	0	0	0.7160
41	0	0	0	0	0	0.8216
42	0	-α	0	0	0	0.9507
43	-α	0	0	0	0	0.7864
44	1	-1	-1	-1	1	1.1268
45	0	0	0	α	0	0.7864
46	-1	1	-1	-1	-1	0.8216
47	0	0	0	0	0	0.9390
48	-1	1	1	-1	1	0.7160
49	-1	1	-1	1	1	1.0211
50	1	1	-1	1	-1	0.8099

* Pullulanase activity was measured after 22 h of incubation.

Table 4.7 and Table 4.8 represent the sequential model Sum of Square (SS) and the result of Lack of Fit test of each model, respectively. The model characteristics were evaluated to determine if the models could be efficiently used. Both tables show the significance of each model. The quadratic model (second-order polynomial model) was found to be significant with prob>F = <0.0001. Moreover, the quadratic model also found that the lack of fit was not significant (prob>F = 0.7226). These indicate that the design generated fits the second-order model with a good agreement. Hence, the quadratic model (second-order polynomial model) was clearly found to fit well with the experimental values and was suggested for the analysis of the optimization.

Table 4.7: Sequential Model Sum of Squares. The quadratic model was found to be as significant model with prob>F = <0.0001.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Mean	35.78659	1	35.78659		
Linear	0.156045	5	0.031209	2.066812	0.0877
2FI	0.328384	10	0.032838	3.322722	0.0042
Quadratic	**0.235355**	**5**	**0.047071**	**13.56028**	**< 0.0001**
Cubic	0.030894	15	0.00206	0.413264	0.9496

Table 4.8: Results of lack of fit test. Quadratic model shows insignificant in "lack of fit" test with prob>F = 0.7226.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Linear	0.634253	37	0.017142	3.97962	0.0314
2FI	0.305869	27	0.011328	2.629982	0.0942
Quadratic	**0.070514**	**22**	**0.003205**	**0.744104**	**0.7226**
Cubic	0.03962	7	0.00566	1.314008	0.3639

The model also shows the coefficient of determination, R^2 at 0.8773, correlation coeffiecient, R = 0.9366 and coefficient of variation, C.V. = 6.96 %. The R^2 value indicates that only 12.27 % of the total variation is not explained by the second-order polynomial model.

According to Murthy *et al*. (2000), the correlation coefficient and the coefficient of variation are generally used to provide correlation measures for the estimation of the regression model. The closer the value of correlation coefficient to unity the better is the correlation between the observed and predicted values. The value of correlation coefficient (R) = 0.9366 indicates a high degree of correlation between the observed and predicted values of the pullulanase production obtained from the model. Meanwhile, the coefficient of variation (C.V.) indicates the degree of precision with which the experiments are compared. The lower the value of the

greater is the reliability of the experiments. The value of C.V. = 6.96% is considered adequately low and indicates the experiments carried out were found to be very reliable.

The adequate precision value of this design was 15.468. The adequate precision value is used to measure the signal to noise ratio. A ratio greater than 4 is desirable. The value of adequate precision at 15.468 indicates an adequate signal in the design. This model can be used to navigate the design space.

The results obtained after the CCD experiments were then analyzed by standard analysis of variance (ANOVA). Table 4.8 represents the summary of ANOVA for the model, all main effects and its two-factor interactions.

Table 4.9: ANOVA table for response surface quadratic model of pullulanase production by *Exiguobacterium sp*. MAAC-1

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	0.7198	20	0.0360	10.3678	< 0.0001*
A	0.0215	1	0.0215	6.2015	0.0187*
B	0.0251	1	0.0251	7.2268	0.0118*
C	0.0197	1	0.0197	5.6831	0.0239*
D	0.0389	1	0.0389	11.1959	0.0023*
E	0.0508	1	0.0508	14.6464	0.0006*
A2	0.0020	1	0.0020	0.5723	0.4554
B2	0.1253	1	0.1253	36.0824	< 0.0001*
C2	0.0121	1	0.0121	3.4919	0.0718
D2	0.0083	1	0.0083	2.3841	0.1334
E2	0.0584	1	0.0584	16.8106	0.0003*
AB	0.0011	1	0.0011	0.3175	0.5775
AC	0.0069	1	0.0069	1.9843	0.1696
AD	0.2084	1	0.2084	60.0249	< 0.0001*
AE	0.0001	1	0.0001	0.0198	0.8890
BC	0.0108	1	0.0108	3.1005	0.0888
BD	0.0039	1	0.0039	1.1162	0.2995
BE	0.0641	1	0.0641	18.4589	0.0002*
CD	0.0014	1	0.0014	0.4018	0.5311
CE	0.0289	1	0.0289	8.3390	0.0073*
DE	0.0029	1	0.0029	0.8384	0.3674
Residual	0.1007	29	0.0035	-	-
Lack of Fit	0.0705	22	0.0032	0.7441	0.7226
Pure Error	0.0302	7	0.0043	-	-

- " * " indicates significant term with significant threshold at 0.0500.
- $R^2 = 0.8773$; R = 0.9366; C.V. = 6.96 %

The F value is the ratio of the mean square due to regression to the mean square due to the real error. The F value of the quadratic model in ANOVA for the optimization of pullulanase production was 10.3678. In general, the calculated F value should be several times greater than the tabulated value for a good model. The F value of the quadratic model equals to 10.3678 implies the model is significant. The value also indicates that only a 0.01 % chance that the F value could occur due to noise. The value of prob>F less than 0.0001 also indicates the quadratic model is significant in an excellent agreement.

The values of prob>F greater than 0.0500 indicate the model terms are not significant. As shown in Table 4.9, the coded terms of A, B, C, D, E, B^2, E^2, AD, BE, CE are significant model terms with prob>F value less than 0.0500. The mathematical model equation relating the production of pullulanase is given in the second-order polynomial shown below:

$$Y = 0.83 + 0.022A + 0.024B - 0.021C - 0.030D + 0.034E + 5.979e^{-003}A^2 + 0.047B^2 - 0.015C^2 + 0.012D^2 - 0.032E^2 - 5.869e^{-003}AB + 0.015AC - 0.081AD + 1.467e^{-003}AE - 0.018BC + 0.011BD - 0.045BE - 6.602e^{-003}CD - 0.030CE - 9.536e^{-003}DE \quad [eq.1]$$

where, Y was pullulanase activity (U/ml) and A, B, C, D and E are sago starch, NH_4Cl, Na_2HPO_4, KCl, and $MgSO_4$ respectively.

The adequacy and validity of the design can be inspected using various diagnostic plot provided by Design Expert software. The normal probability plot of the studentized residual is the most important diagnostic plot to determine the validity of design. Figure 4.10 shows the normal probability plot of the studentized residuals. The normal probability plot indicates whether the residuals follow a normal distribution. The data points should be approximately linear or follow a straight line. A non-linear pattern like an S-shaped curve indicates the non-normality in the error term, which may be corrected by a transformation. The plot in Figure 4.10 shows that the residuals are normally distributed along the line. This indicates the design is valid for estimation of pullulanase production.

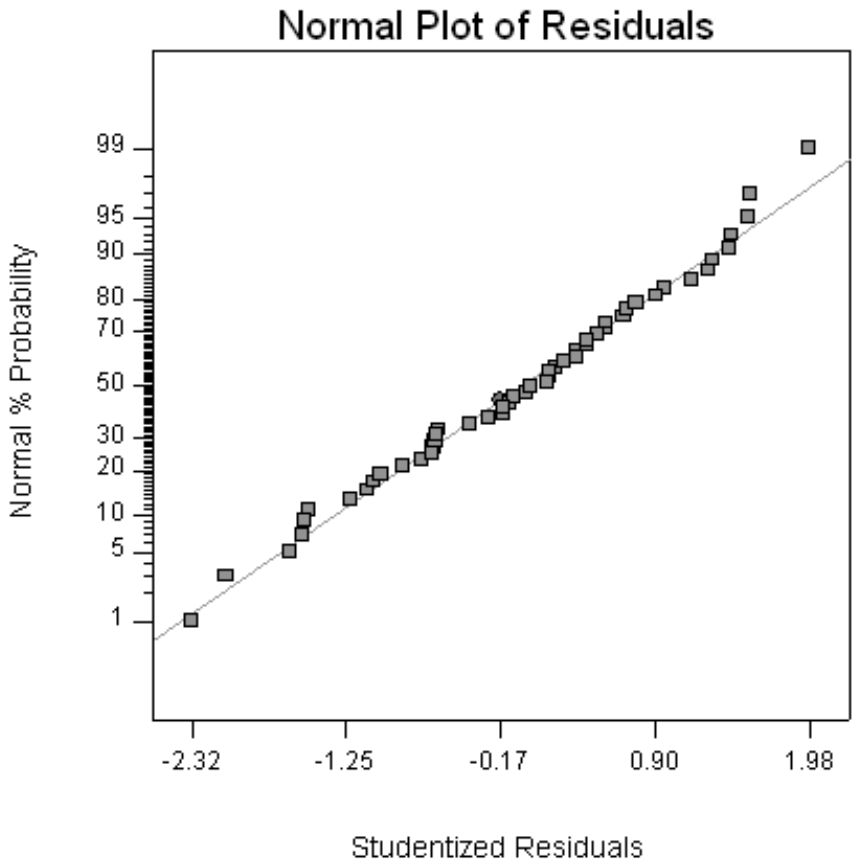


Figure 4.10: Normal probability plot of the studentized residuals. The distribution of studentized residuals is in a linear pattern. This indicates the residuals are distributed normally.

Another key diagnostic plot is the plot of residuals versus predicted values. This is a plot of the residuals versus the ascending predicted response values. The plot is used to test the assumption of constant variance. The plot should show a pattern of random scatter, and the points should be scattered inside the constant range of residuals across the graph (-3.00 to 3.00). If megaphone pattern ("<") occur, this indicates the problem of non-constant variances of the residuals in the design and a transformation of the response is needed to improve the fit of model. The Figure 4.11 shows the points are randomly scattered. This indicates that no transformation is needed.

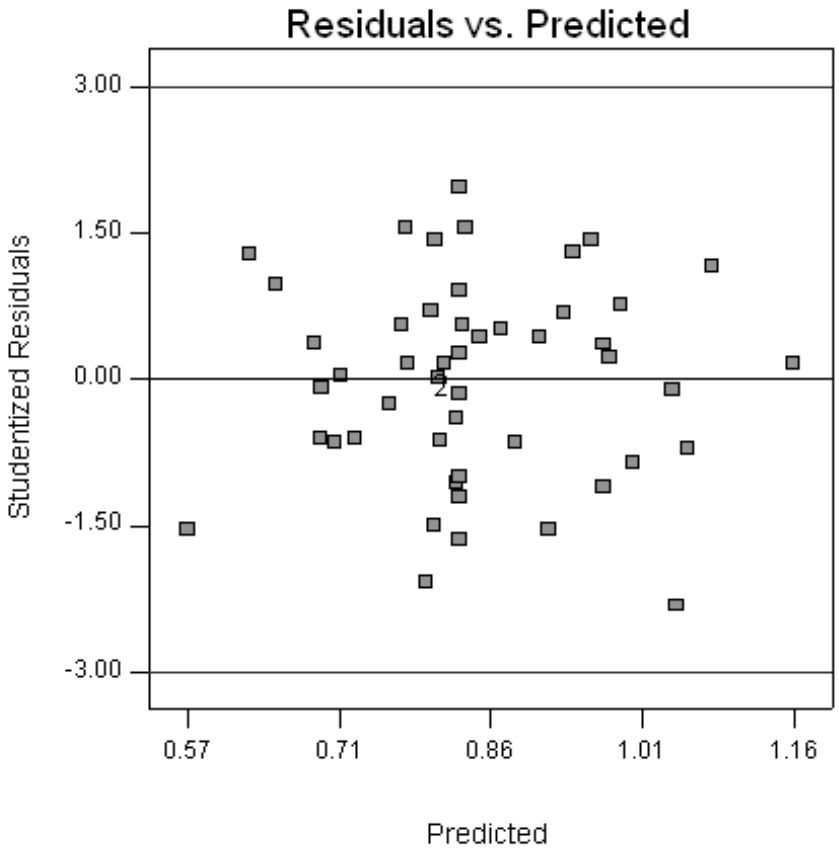


Figure 4.11: Plot of residuals versus predicted values. All the points are randomly scattered inside the constant range of residuals.

The outlier T plot provides an easier way to identify abnormal runs if any points stand out, which fall outside the +3.5 and -3.5 standard deviation limits. The outlier T statistic shows how that data point fits in with the other points for this model.

Outlier T is a measure of how much standard deviation the actual value deviates from the value predicted after deleting the point in question. Sometimes outlier T is also called externally studentized residual. Figure 4.12 represents the outlier T plot. This plot shows that all the points fall well within the limit range of standard deviation. This indicates no outliers or abnormal experiments were found in the design.

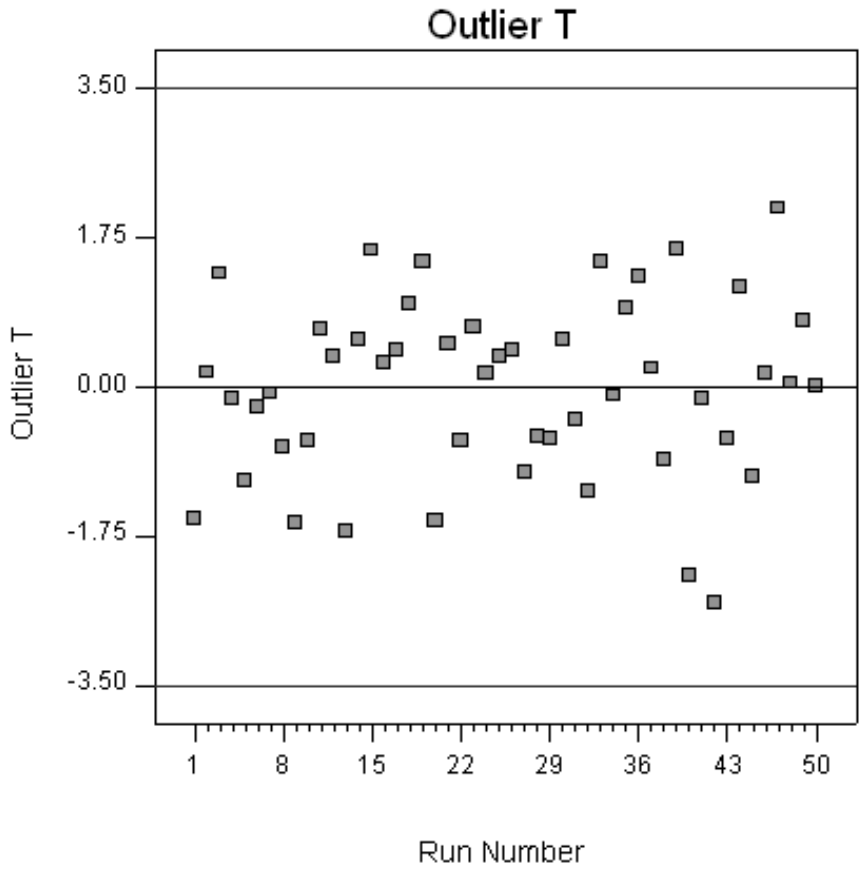


Figure 4.12: Outlier T plot. All points fell within the limits.

The last diagnostic plot is the Box-Cox plot. The Box-Cox plot is a tool that provides a guideline for selecting the correct power law transformation. The red lines indicate the 95 % confidence limits. The blue line shows the lambda value of current transformation. If the blue line points to a lambda value at 1, it symbolizes no transformation of the response. The green line indicates the best lambda value. A recommended transformation is listed based on the best lambda value, which is found at the minimum point of the curve generated by the natural log of the sum of squares of the residuals. If the 95 % confidence interval around this lambda includes 1, then no specific transformation is recommended by the Design Expert software. Figure 4.13 depicts the Box-Cox plot for power transformation. The plot shows that the current lambda value of 1 fell within the 95 % confidence interval. Thus, no power transformation is recommended. The plot also shows the current lambda

value (1) was very close to the best current value suggested (1.17). Thus, the non-transformed model is well accepted in a good agreement.

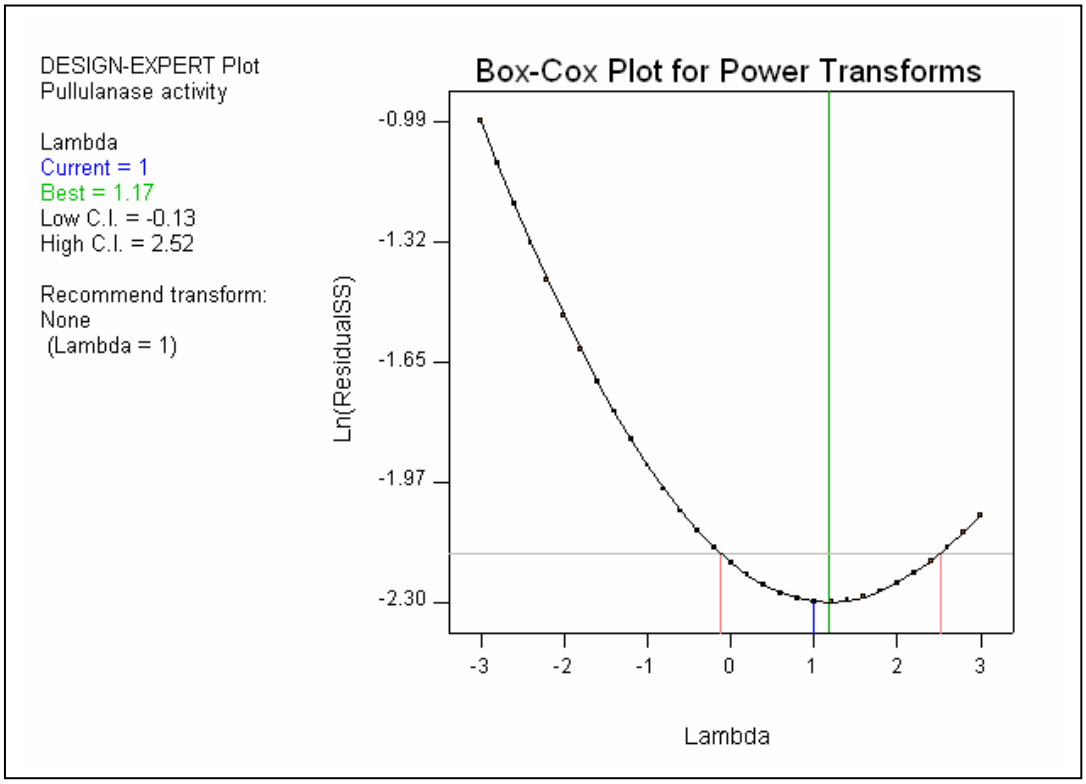


Figure 4.13: Box-Cox plot for power transformation.

4.5.3 Optimization Conclusion

Five significant medium components were selected among 17 components using a statistical screening method, the Plackett-Burman design. These five components were further applied in the optimization of pullulanase production using Central Composite Design (CCD). Both experimental designs successfully identified the significant factors that enhanced the production of pullulanase by *Exiguobacterium sp.* MAAC-1.

From the result of the diagnostic plots, the second-order polynomial model or quadratic model that explained the response of the pullulanase production is in an

excellent agreement. The value of prob>F of each independent factor, which are sago starch, NH_4Cl, Na_2HPO_4, KCl and $MgSO_4$, was less than 0.05 which implies they have significant effect on the pullulanase production. This result also supported the analysis result of Plackett-Burman design that the five components are significant factors.

Besides, the ANOVA table also shows that some significant two-factor interactions in the CCD design model. Figure 4.14 depicts the graph of two-factor interaction between sago starch and KCl. The prob>F value of this interaction was less than 0.0001. This indicates the interactions have a great effect on the pullulanase production by *Exiguobacterium sp*. MAAC-1. The interaction graph shows the two lines are not parallel and this implies that there is an interaction between those two factors (Haaland, 1989). The interaction graph reveals that higher pullulanase production was obtained when higher concentration of sago starch was applied at lower concentration of KCl, or vice versa. The highest pullulanase was achieved when 0.015 % w/v of KCl and 3 % w/v of sago starch were applied.

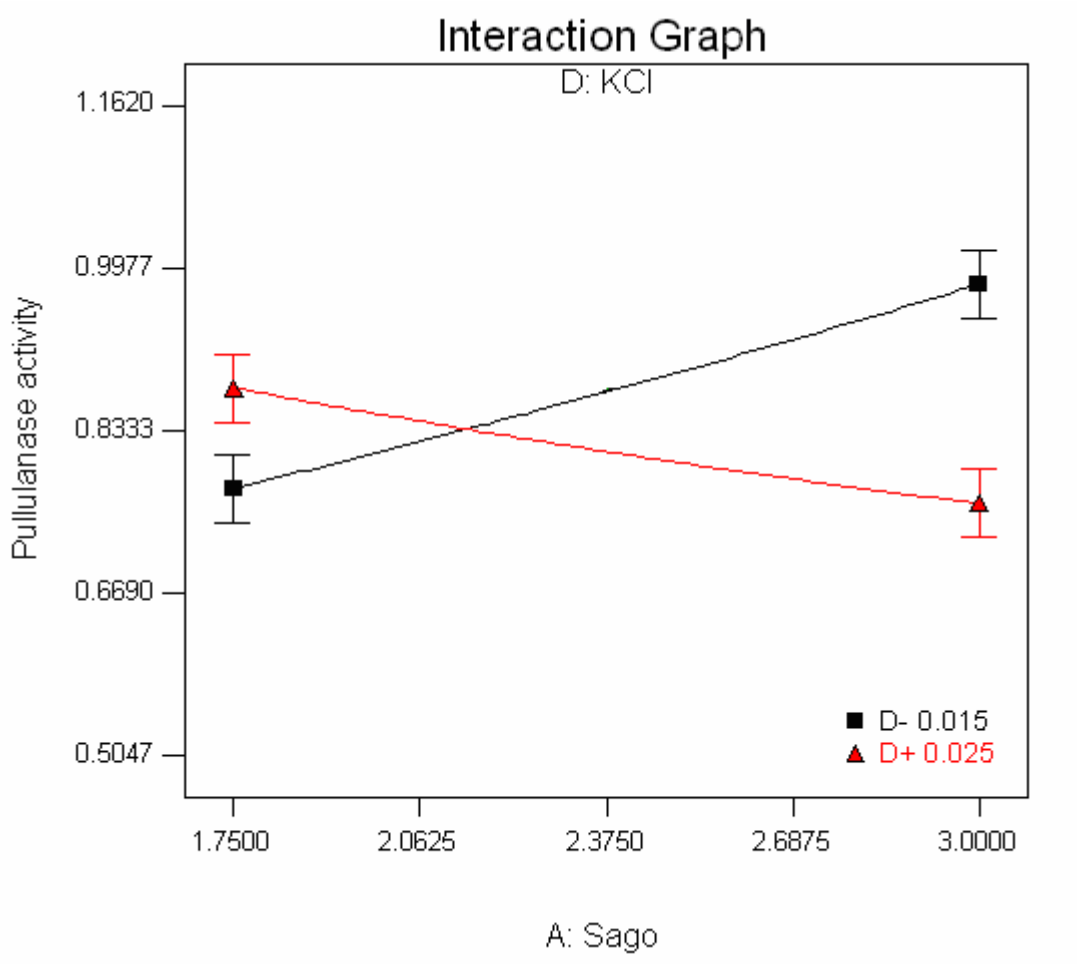


Figure 4.14: Two-factor interaction graph between sago starch and KCl

Figure 4.15 depicts the graph of two-factor interaction between $MgSO_4$ and NH_4Cl. The prob>F value of the two-factor interaction was 0.0002. This value indicates the interaction have great impact on the pullulanase production. The interaction graph shows that lower concentration of NH_4Cl and higher concentration of $MgSO_4$ produced higher pullulanase, or vice versa. The graph also shows that highest pullulanase was produced when 0.025 %w/v of $MgSO_4$ and 0.002 %w/v of NH_4Cl were applied.

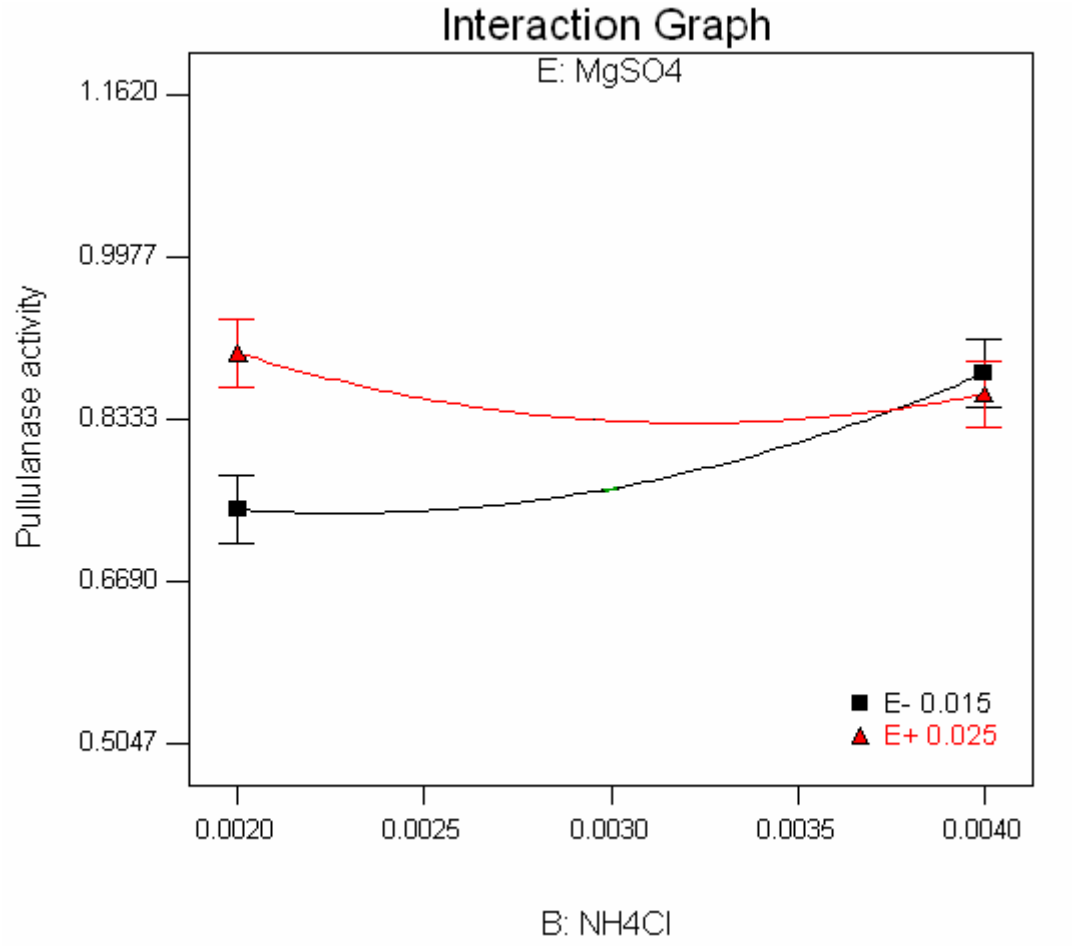


Figure 4.15: Two-factor interaction graph between NH_4Cl and $MgSO_4$

The interaction between $MgSO_4$ and Na_2HPO_4 also has high impact on pullulanase production by *Exiguobacterium sp*. MAAC-1. The ANOVA test revealed the two-factor interaction has prob>F value of 0.0073. This value implies the two-factor interaction has significant effect on the pullulanase production. Figure 4.16 depicts the interaction graph between $MgSO_4$ and Na_2HPO_4. The graph reveals higher pullulanase production was obtained at lower concentration of Na_2HPO_4 and higher concentration of $MgSO_4$ or vice versa. However, highest pullulanase production was achieved at 0.025 %w/v of Na_2HPO_4 and 0.025 %w/v of $MgSO_4$.

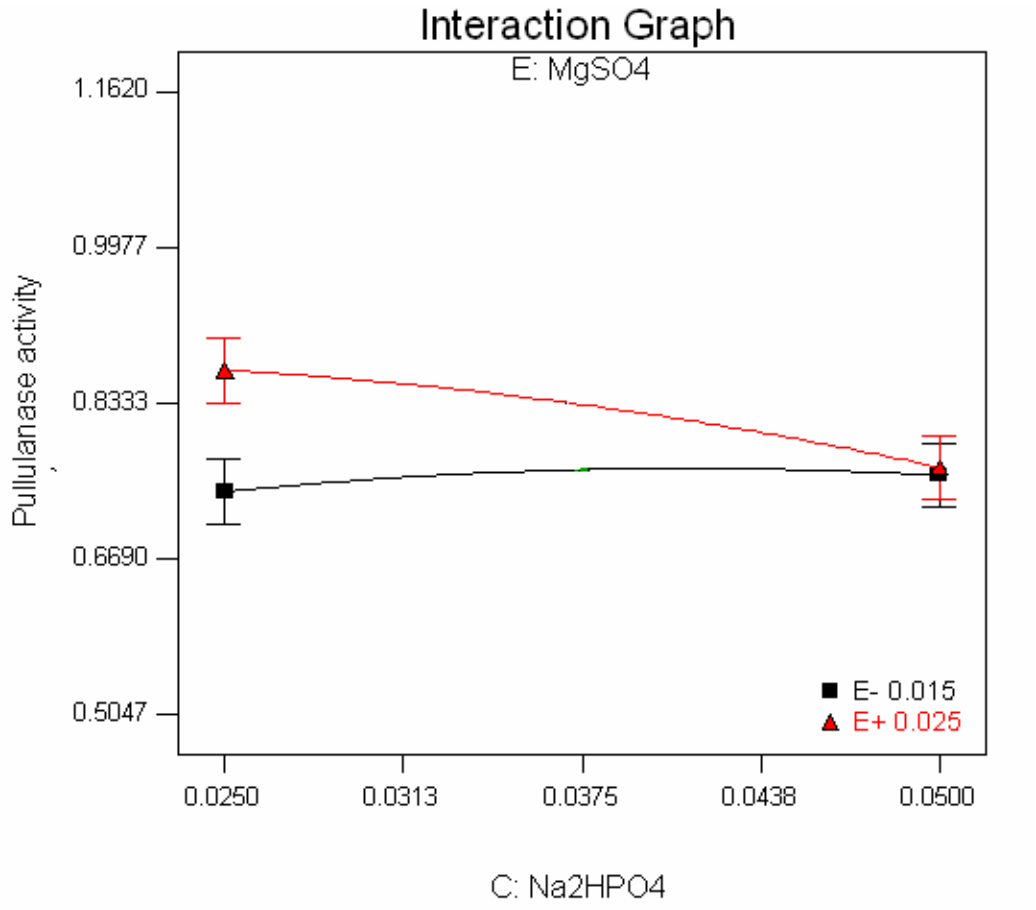


Figure 4.16: Two-factor interaction graph between Na_2HPO_4 and $MgSO_4$.

4.5.4 Optimization Point Prediction and Verification

The second-order polynomial model resulted from the CCD has successfully fitted the response of pullulanase production by *Exiguobacterium sp*. MAAC-1. In other words, the model can be used to predict the pullulanase production. An optimization point was predicted based on the resulted model by the Design Expert software. Figure 4.17 depicts the medium formulation suggested by the model. The suggested formulation was 3.86 %w/v of sago starch, 0.002 %w/v of NH_4Cl, 0.05 %w/v of Na_2HPO_4, 0.015 %w/v of KCl and 0.025 %w/v of $MgSO_4$. The desirability is an objective function that ranges from zero outside of the limits to one at the goal. It reflects the desirable ranges for each response. The desirable ranges are from zero to one which indicates least to most desirable, respectively. The desirability value of

the model was 0.995. The predicted pullulanase activity of this suggested medium formulation obtained from the equation (eq.1) was 1.252 U/ml.

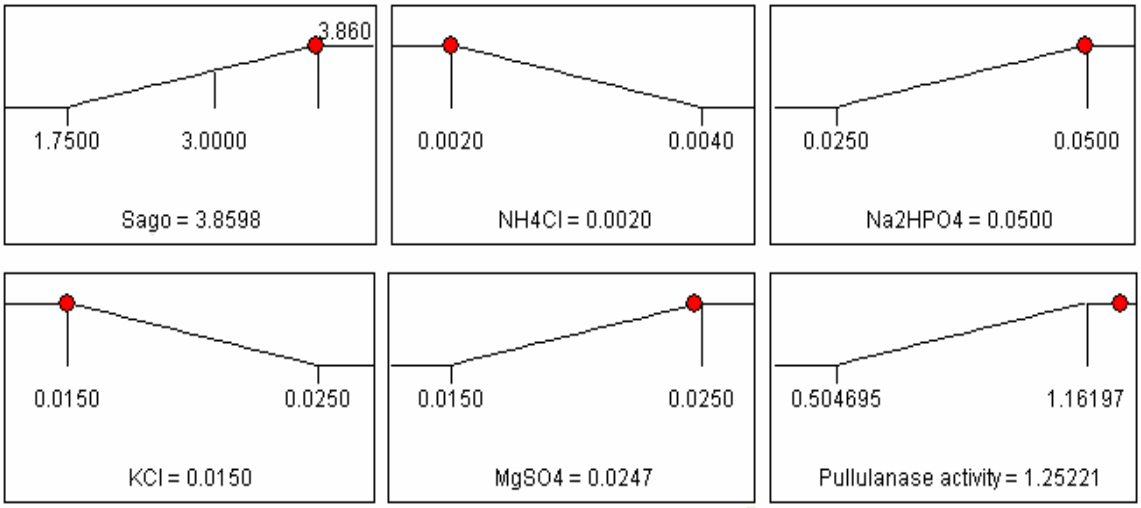


Figure 4.17: Medium formulation suggested by the model

The adequacy of the model for predicting the optimum pullulanase production was proven experimentally using the recommended medium formulation. The experimental pullulanase activity obtained from the recommended medium formulation was 1.208 U/ml, which is very close to the predicted value. The experimental value is obviously in good agreement with the model prediction. By employing the statistical approach, the pullulanase production by *Exiguobacterium sp*. MAAC-1 was successfully increased approximately 9.6-fold compared to the initial medium.

The application of statistical approach in pullulanase production was also reported by Reddy *et al.* (1999b). The study reported the optimization of nutrients using CCD and 16.5 %w/w of potato starch, 2.5 %w/w corn steep, 0.015 %w/w ferrous sulphate and 14 %w/w pearl millet flour have been found optimal for the pullulanase production by *Clostridium thermosulfurogenes* SV2. The medium formulation was found to produce 10 % more pullulanase.

4.6 Partial Pullulanase Gene Isolation

4.6.1 PCR Amplification and Sequence Determination

Around 1.2 kb of fragment was successfully amplified from *Exiguobacterium sp*. MAAC-1 using forward primer Mix1 and reverse primer ReMix1 (Figure 4.18). Both primers were depicted from Hatada *et al.* (2001). The amplified fragment was expected to be the pullulanase gene fragment.

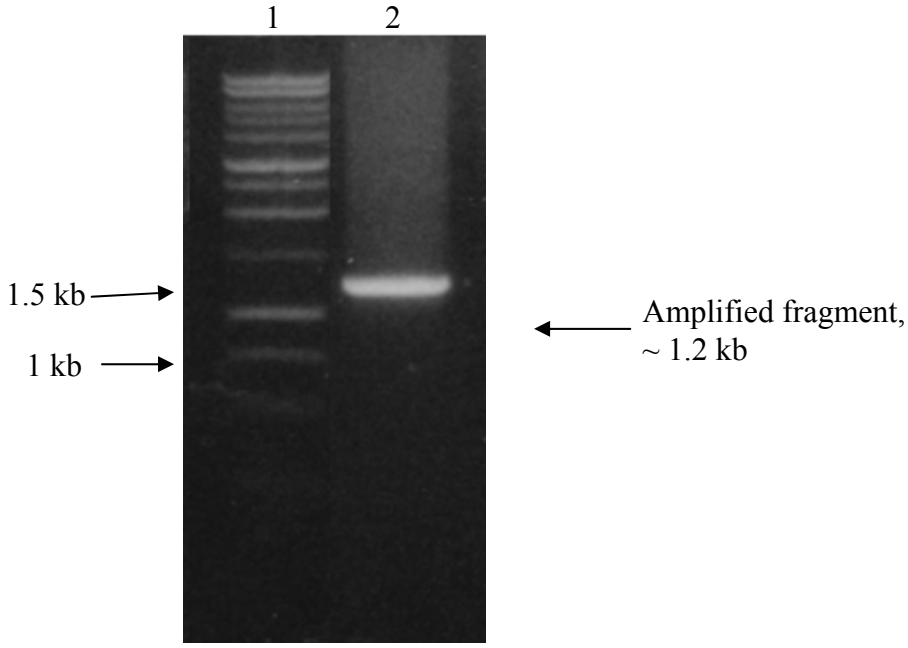


Figure 4.18: PCR amplified fragment using forward primer Mix1 and reverse primer ReMix1

The amplified PCR fragment was ligated into pGEM-T-easy vector. The ligated plasmid was transformed into *E.coli* JM109 competent cells. Recombinant clones were identified by blue/white selection using LB-ampicillin plates containing X-Gal and IPTG. The insert of the selected recombinant was confirmed by restriction endonuclease analysis. The clone with correct insert was sequenced bidirectionally. A total of 1177 bp nucleotides were determined form the insert. The nucleotide sequence was deduced into amino acid sequence. Figure 4.19 shows the nucleotide sequence and deduced amino acid sequence of the insert.

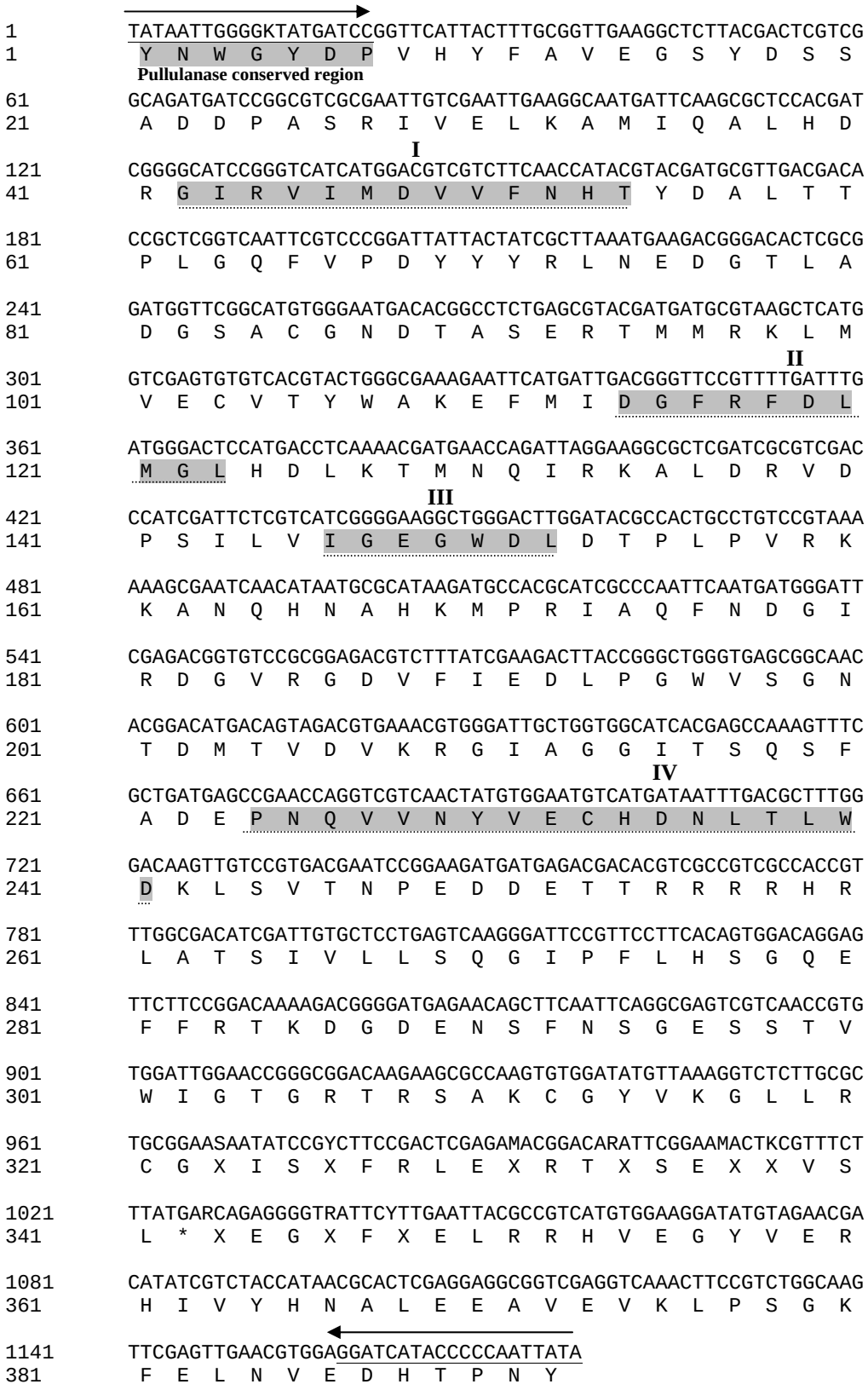
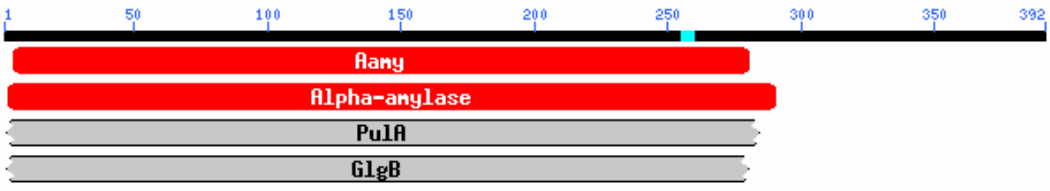


Figure 4.19: Nucleotide and amino acid sequence of amplified product. [Primers are underlined; the conserved regions are highlighted.]

4.6.2 Analysis of PCR Fragment

According to the result of multiple alignments, the sequence of amplified product contains all the four conserved regions of amylolytic enzyme and a highly conserved region of pullulanase type I. The partial gene sequence was subjected to a search against all known sequences in databases using BLASTN and BLASTX provided by NCBI (http://www.nih.gov). The search result shows that the gene fragment is close similar with known pullulanase gene to-date. Both searches resulted the highest identities of the amplified partial gene fragment with hypothetical pullulanase gene from *Exiguobacterium sibiricum* 255-15, with 61 % and 57 % from BLASTN and BLASTX search, respectively.

The deduced amino acid sequence was also compared with protein sequences in the secondary database by using a search service called Conserved Domain Database or CDD provided by NCBI. CDD is a new search service introduced by NCBI aimed at identifying conserved domains within a protein sequence. The search against CDD produced four significant alignments (Figure 4.20). The domains in the alignments belong to Family 13 of glycosyl hydrolases. This result shows that the partial gene is derived from one on the member from the Family 13 of glycosyl hydrolases or α-amylase family.

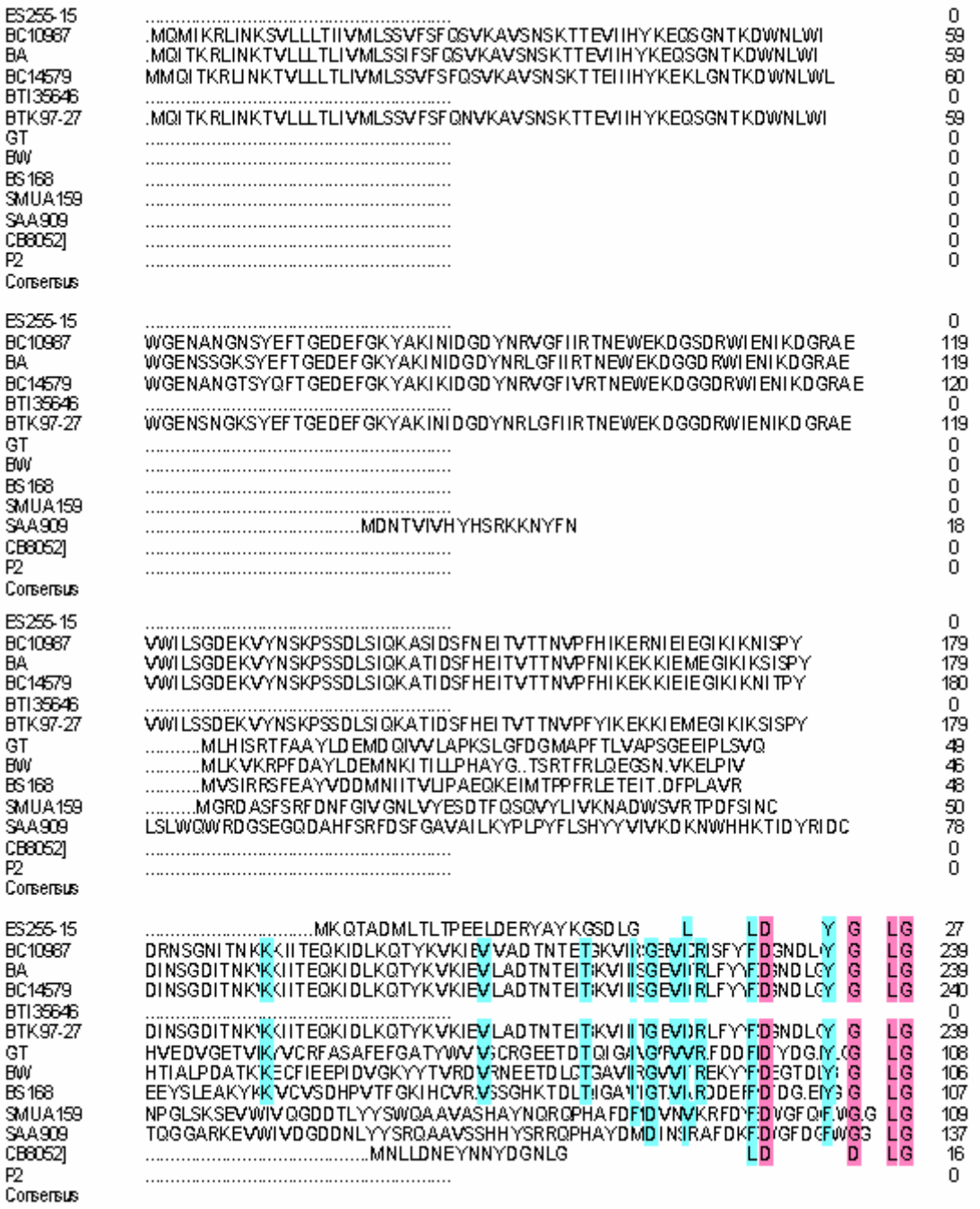


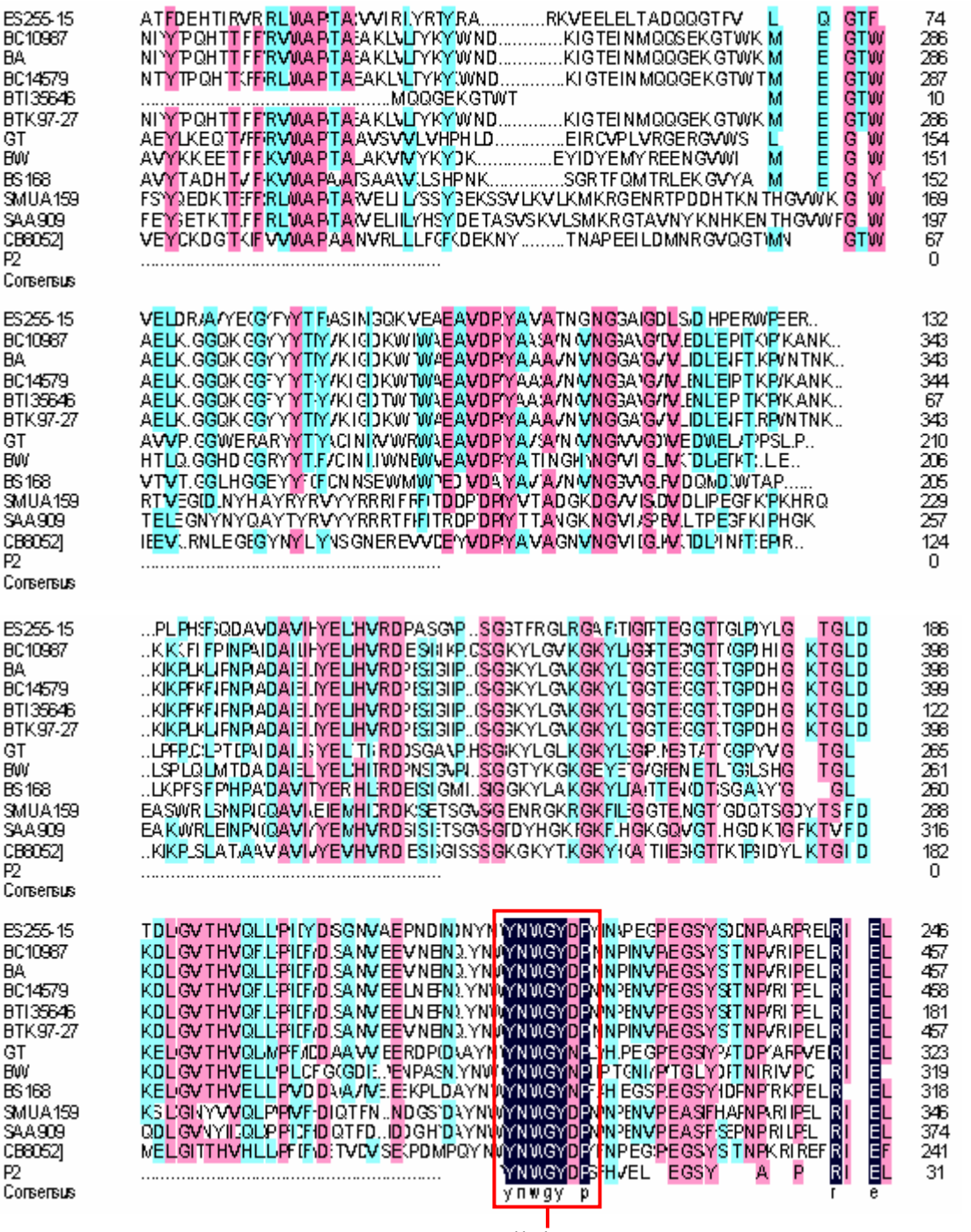
	Score (bits)	E value
gnl|CDD|24317 smart00642, Aamy, Alpha-amylase domain; .	65.6	1e-11
gnl|CDD|25407 pfam00128, Alpha-amylase, Alpha amylase, catalytic domain. Alp...	92.8	7e-20
gnl|CDD|11237 COG1523, PulA, Type II secretory pathway, pullulanase PulA and...	282	4e-77
gnl|CDD|10170 COG0296, GlgB, 1,4-alpha-glucan branching enzyme [Carbohydrate...	86.1	6e-18

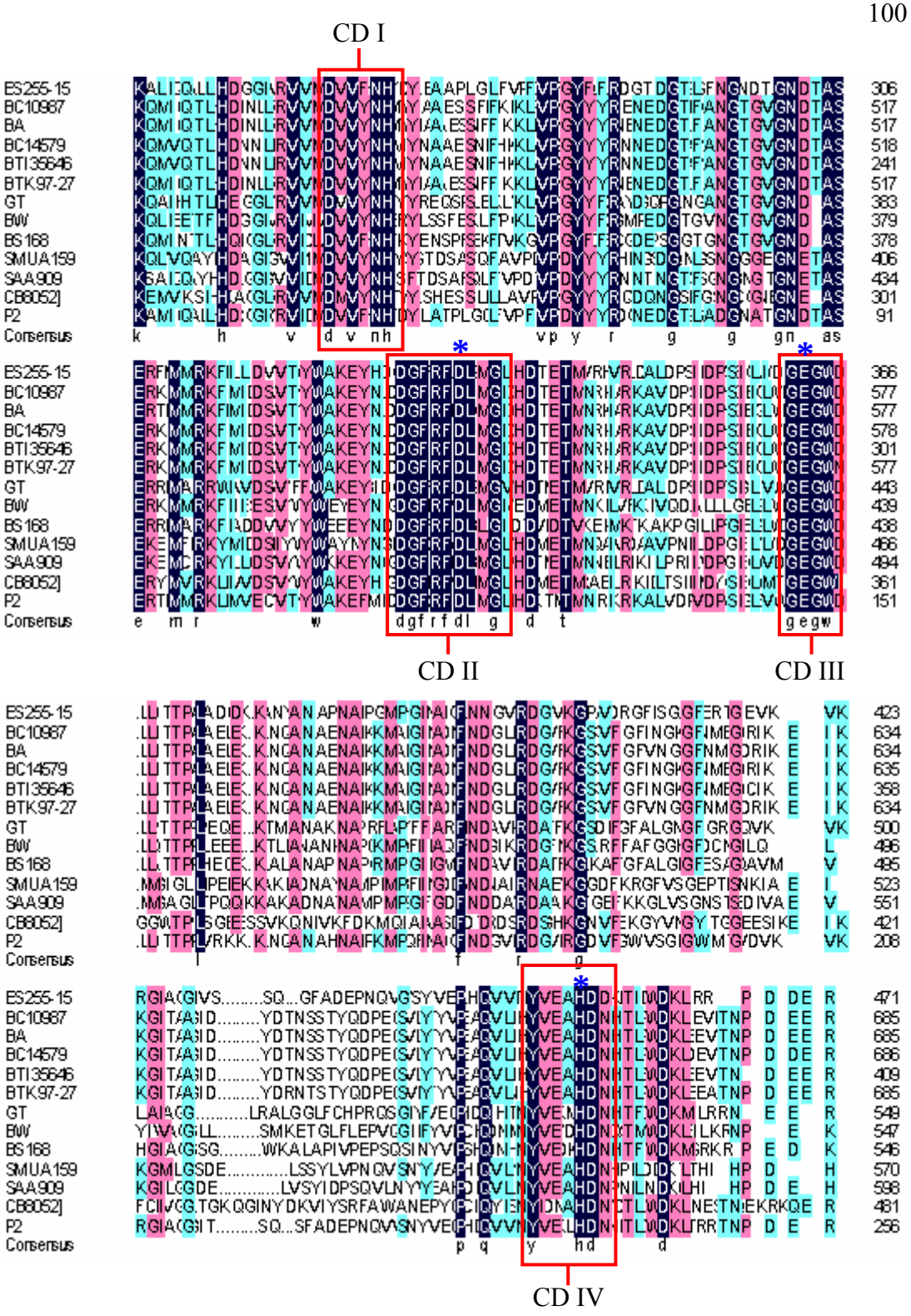
Figure 4.20: The search result against Conserved Domain Database (CDD)

The CDD search result also shows the deduced amino acid sequence of partial gene is most similar with pullulanase which gave the highest score (282) and lowest E value ($4E^{-77}$) among the alignments. This reveals that the amplified partial gene is part of the pullulanase gene.

The deduced amino acid of partial pullulanase gene amplified from *Exiguobacterium sp*. MAAC-1 genomic DNA was aligned with 12 amino acid sequences of pullulanase obtained from the BLASTX search result. The multiple alignment was carried out using the ClustalX software (Figure 4.21). A highly conserved region consisting of seven amino acids, YNWGYDP, is found in all Type I pullulanases (Bertoldo *et al*., 2004). Four conserved regions were observed in all enzymes from α-amylase family and identified as important motifs responsible in substrate binding and catalytic action. The alignment reveals the partial pullulanase gene contains the conserved region of pullulanase and the four conserved regions of α-amylase family.




Pullulanase
conserved region


CD I
CD II
CD III
CD IV

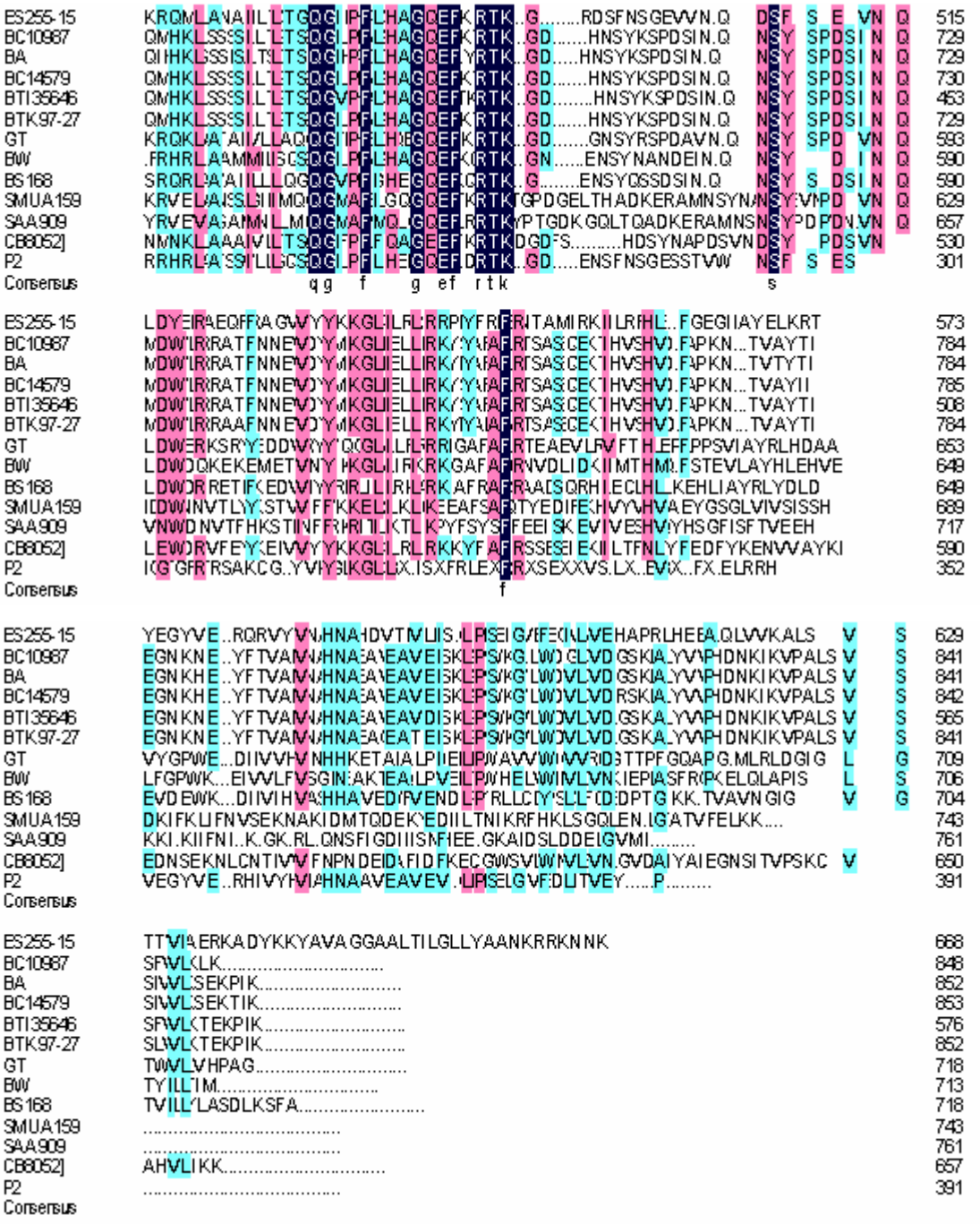


Figure 4.21: Multiple alignment for comparison of partial pullulanase gene of *Exiguobacterium sp*. MAAC-1 with other 12 pullulanase genes. The red box indicates the conserved region (CD: conserved region). * : Highly conserved amino acids involved in substrate binding and enzyme catalytic action.
(ES255-15: *Exiguobacterium sibiricum* 255-15; BC10987: *Bacillus cereus* ATCC 10987; BA: *Bacillus anthracis* str. 'Ames Ancestor'; BC14579: *Bacillus cereus* ATCC 14579; BTI35646: *Bacillus thuringiensis* serovar israelensis ATCC 35646; BTK97-27: *Bacillus thuringiensis* serovar konkukian str. 97-27; GT: *Geobacillus*

thermoleovorans; BW: *Bacillus weihenstephanensis* KBAB4; BS168: *Bacillus subtilis* subsp. subtilis str. 168; SMUA159: *Streptococcus mutans* UA159; SAA909: *Streptococcus agalactiae* A909; CB8052: *Clostridium beijerincki* NCIMB 8052; P2: *Exiguobacterium sp*. MAAC-1)

Table 4.10 depicts the conserved regions for some enzyme from α-amylase family. All the enzymes from α-amylase family contain three conserved amino acids, which are Asp 206 (Taka-amylase A numbering), Glu230 and Asp297. The three amino acids are bolded and underlined in the table. The conserved amino acid residues (Asp206, Glu230 and Asp297) were identified to play an important role in the catalysis mechanism of α-amylase family enzymes, and found to be at the centre of the active site (Kuriki and Imanaka, 1999; Janeček, 1997; MacGregor *et al*., 2001). The Glu230 residue is believed to be the proton donor, while the Asp206 functioned as the nucleophile in the catalysis mechanism. Although the role of Asp297 has not been clearly confirmed yet, but it has been proposed to be involved in substrate binding. (Janeček, 1997; MacGregor *et al*., 2001).

The partial pullulanase gene also contains the YNWGYDP motifs which only can only be found in Type I pullulanase. This very strong evidence reveals that the partial gene is a fragment of Type I pullulanase from *Exiguobacterium sp*. MAAC-1.

Table 4.10: Conserved regions of enzymes from α-amylase family. CD: conserved region; the highly conserved amino acids is bolded and underlined.

Enzyme	YNWGYDP	CD I	CD II	CD III	CD IV
α-amylase	-	DVVANH	GLRI**D**TVKH	**E**VLD	FVENH**D**
CGTase	-	DFAPNH	GIRF**D**AVKH	**E**WFL	FIDNH**D**
Isoamylase	-	DVVYNH	GFRF**D**LASV	**E**PWA	FIDVH**D**
Branching enzyme	-	DWVPGH	ALRV**D**AVAS	**E**EST	LPLSH**D**
α-glucosidase	-	DLVINH	GFRI**D**TAGL	**E**VAH	YIENH**D**
Cyclodextrinase	-	DAVFNH	GWRL**D**VANE	**E**VWH	LIGSH**D**
Oligo-1,6-glucosidase	-	DLVVNH	GFRM**D**VINF	**E**MPG	YWNNH**D**
Amylomaltase	-	DMWAND	IVRI**D**HFRG	**E**ELG	YTGTH**D**
Type II pullulanase	-	DGVFNH	GWRL**D**VANE	**E**NWN	LLGSH**D**
Type I pullulanase	YNWGYDP	DVVYNH	GFRF**D**LMGY	**E**GWD	YVSKH**D**
Partial pullulanase	YNWGYDP	DVVFNHT	GFRF**D**LMGL	**E**GWD	YVECH**D**

A phylogenetic tree was constructed using ClustalX software to show the relatedness of the deduced amino acid of the partial pullulanase from *Exiguobacterium sp.* MAAC-1 with other pullulanases (Figure 4.22). The relatedness tree reveals that the partial pullulanase from *Exiguobacterium sp.* MACC-1 has the closest relationship with the pullulanase from *Exiguobacterium sibiricum* 255-15.

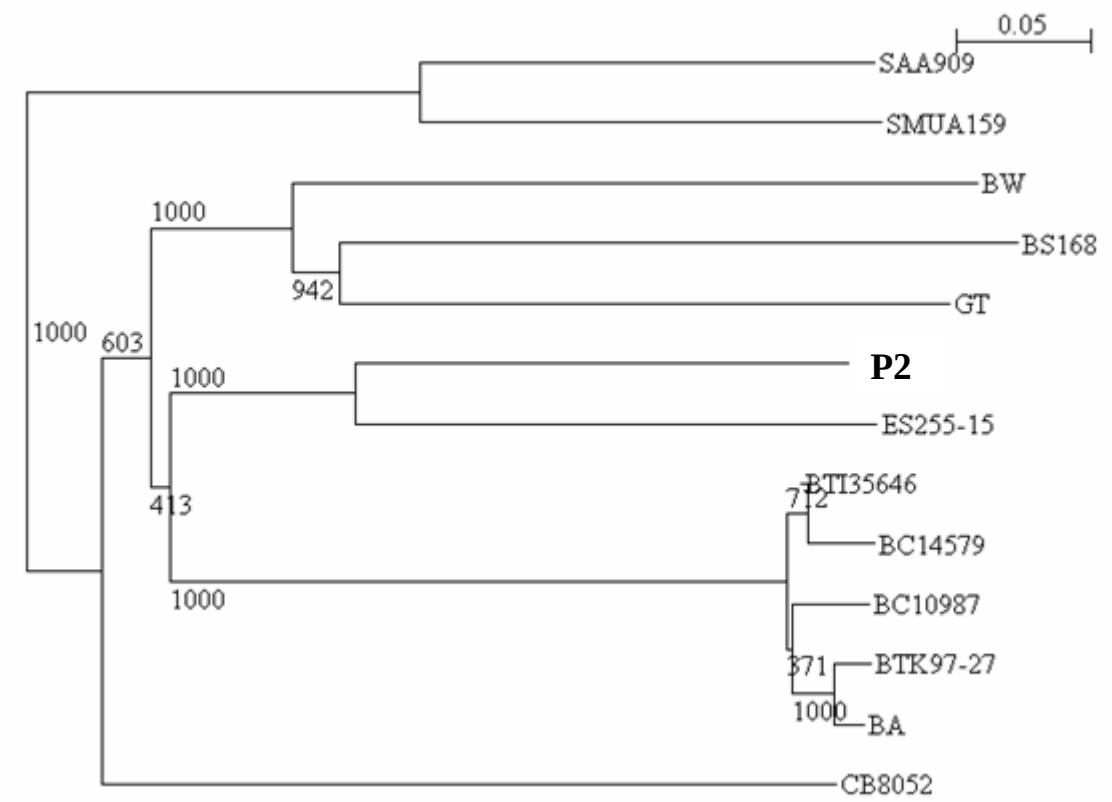


Figure 4.22: Relatedness tree of partial pullulanase from *Exiguobacterium sp.* MAAC-1 with other known pullulanases.

(ES255-15: *Exiguobacterium sibiricum* 255-15; BC10987: *Bacillus cereus* ATCC 10987; BA: *Bacillus anthracis* str. 'Ames Ancestor'; BC14579: *Bacillus cereus* ATCC 14579; BTI35646: *Bacillus thuringiensis* serovar israelensis ATCC 35646; BTK97-27: *Bacillus thuringiensis* serovar konkukian str. 97-27; GT: *Geobacillus thermoleovorans*; BW: *Bacillus weihenstephanensis* KBAB4; BS168: *Bacillus subtilis* subsp. subtilis str. 168; SMUA159: *Streptococcus mutans* UA159; SAA909: *Streptococcus agalactiae* A909; CB8052: *Clostridium beijerincki* NCIMB 8052; P2: *Exiguobacterium sp*. MAAC-1)

CHAPTER V

CONCLUSIONS

5.1 Conclusions

A total of 19 strains bacteria (Bacteria 9, 2, 14, 90, 39, NA, 26, 89, 7, 89, 8, 23, 19, 1, 90, 48, P2, P3, and S7) were screened using pullulan, AZCl-pullulan and Red-pullulan. Through the observation of clearing zones on pullulan plate and Red-pullulan plate, and the release of blue colour on AZCl-pullulan plate, Bacteria P2 was found to be the pullulanase-producer.

Bacteria P2 was identified using the 16S rRNA gene sequence analysis technique. A total of 1600 bp nucleotide of 16S rRNA gene derived from Bacteria P2 were successfully determined and compared with other 16S rRNA gene sequences from RDP-II and NCBI database. The comparison result shows that Bacteria P2 belongs to the Domain of *Bacteria*, Phylum of *Firmicutes,* Class of *Bacilli*, Order of *Bacillales* and Genus of *Exiguobacterium.* Seventeen close-phylogenetically related 16S rRNA gene sequences were selected and aligned with 16S rRNA gene sequence of Bacteria P2. The result reveals that Bacteria P2 shared the highest similarity (98 %) with *Exiguobacterium sp.* JL-42, *Exiguobacterium sp.* JL-25 and *Exiguobacterium sp*. JL-24. The 16S rRNA nucleotide sequence of Bacteria P2 was submitted to GenBank with the Accession No. DQ366351 and assigned as *Exiguobacterium sp.* MAAC-1.

The crude pullulanase enzyme from *Exiguobacterium sp*. MAAC-1 showed the highest activity at pH 9.0 and significantly inactivated at pH 4.0 and 10.0 with lower than 50 % of relative enzyme activity. The crude pullulanase enzyme was active in the pH range between 7.0 and 9.0 with higher than 85 % relative activity was observed. Besides, the pullulanase is active at a wide range of temperature from 50 ^{o}C to 70 ^{o}C, with the optimum temperature at 60 ^{o}C. A slight decrease of the enzyme activity was observed at 55 ^{o}C and 65 ^{o}C, with relative activity of 85.9 % and 87.4 % respectively and highly inactivated at temperature below 45 ^{o}C. The initial enzyme characterization revealed that the pullulanase is stable in slightly alkaline condition and stable at relatively high temperature.

The pullulanase production by *Exiguobacterium sp.* MAAC-1 was enhanced using statistical approach. The Plackett-Burman design was employed to screen the significant nutrients for the pullulanase production by *Exiguobacterium sp.* MAAC-1. The statistical analysis of Plackett-Burman design reveals that the model obtained is significant with the F-value at 12.39. Five nutrients, which are sago starch, NH_4Cl, $MgSO_4$, Na_2HPO_4 and KCl were identified as significant factors for the pullulanase production and selected out from 17 nutrients. The five nutrients proceeded to the optimization step.

The concentration of sago starch, NH_4Cl, $MgSO_4$, Na_2HPO_4 and KCl was optimized to enhance the pullulanase production by *Exiguobacterium sp*. MAAC-1 using 2^5 full factorial Central Composite Design (CCD). The quadratic model was found to be significant with prob>F = <0.0001 and the “lack of fit” was not significant (prob>F of “lack of fit” = 0.7226). This implies that the design generated was fitted to the second-order model with a good agreement. Based on the statistical analysis done by CCD, the model obtained shows the correlation coefficient (R) of 0.9366 which indicates a high degree of correlation between the observed and predicted values of the pullulanase production obtained from the model. The value of C.V. of 6.96 % is considered adequately low and indicates that the experiments carried out were found to be very reliable. Besides, the model F value of 10.3678 implies the model is significant.

Based on the ANOVA results from CCD, all the five nutrients have prob>F value less than 0.1. This result implies that the nutrients are significant factors, which is the same as the results obtained from Plackett-Burman design. The ANOVA result also shows some significant two-factor interactions occurred. The two-factor interaction between sago starch-KCl, $MgSO_4$-NH_4Cl and $MgSO_4$-Na_2HPO_4 has the prob>F value less than 0.05. This indicates those interactions have a great effect on the pullulanase production by *Exiguobacterium sp*. MAAC-1. An optimized condition was predicted by the design, which at 3.86 %w/v of sago starch, 0.002 %w/v of NH_4Cl, 0.05 %w/v of Na_2HPO_4, 0.015 %w/v of KCl and 0.025 %w/v of $MgSO_4$, with predicted pullulanase activity of 1.252 U/ml. The experimental pullulanase activity obtained was 1.208 U/ml, which is obviously in good agreement with the model prediction. The pullulanase production by *Exiguobacterium sp*. MAAC-1 was successfully enhanced approximately 9.6-fold compared to the initial medium.

A total of 392 deduced amino acid residues of partial pullulanase gene from *Exiguobacterium sp*. MAAC-1 were determined and subjected to search in NCBI database. The search result shows highest identities with hypothetical pullulanase gene from *Exiguobacterium sibiricum* 255-15, with 61 % and 57 % from BLASTN and BLASTX search, respectively. The deduced amino acid sequence of the partial pullulanase gene was aligned with 12 closely similar amino acid sequences of pullulanase, four conserved regions of α-amylase family and a highly conserved region among pullulanases type I, YNWGYDP motif, were observed.

5.2 Further Works

The pullulanase enzyme has wide applications in industry, especially in starch processing industry, and the application of pullulanase in the production of branched-CDs has very high potential to be exploited in pharmaceutical area. Hence, more extensive studies should be carried out in order to obtain greater enzyme production and better enzyme characteristic.

The pullulanase production can be further enhanced through the optimization of physical parameters, such as pH of the medium, agitation speed, temperature of incubation, inoculum size, and etc. The powerful experimental design approach is an excellent tool to conduct the optimization study. Then, the optimization of pullulanase production in larger scale using fermenter can be subsequently carried out.

In order to study the mechanism of pullulanase enzyme, the purification of pullulanase enzyme is an essential study. Once the pullulanase enzyme is purified, the molecular mass and isoelectric point of the enzyme can be determined. Besides, the metal effects on the enzyme stability and kinetic properties of the pullulanase enzymes also can be determined.

To enhance the properties of pullulanase enzyme, some approaches of molecular biology should be employed. The full length of pullulanase gene has to be isolated from *Exiguobacterium sp*. MAAC-1. Subsequently, the best expression system for pullulanase production can be identified. This is crucial for future studies of crystallization and mutation. Besides, the production of pullulanase from expression can be enhanced by the optimization of inducer dosage, temperature of incubation and agitation speed. Again, the useful experimental design can be applied in the optimization study. Moreover, the mutational study, such as site-directed mutagenesis, can be carried out in order to increase the optimum temperature and thermostability of the enzyme, and also to study the function and impact of essential amino acid residues in active regions on the catalytic action and substrate binding of the pullulanase enzyme.

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