การผลิตและพัฒนาคุณสมบัติของเอนไซม์อัลฟา-อะไมเลส
ด้วยเทคโนโลยีการสลับสับเปลี่ยนดีเอ็นเอ

นางสาวศิริมา สุขเกษม

PRODUCTION AND IMPROVEMENT OF

ALPHA-AMYLASE BY DNA SHUFFLING

Sirima Sukasem

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy of Science in Biotechnology

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PRODUCTION AND IMPROVEMENT OF ALPHA-AMYLASE BY DNA SHUFFLING

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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ศิริมา สุขเกษม : การผลิตและพัฒนาคุณสมบัติของเอนไซม์อัลฟา-อะไมเลสด้วยเทคโนโลยีการสลับสับเปลี่ยนดีเอ็นเอ (PRODUCTION AND IMPROVEMENT OF ALPHA-AMYLASE BY DNA SHUFFLING) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 139 หน้า

เทคโนโลยีการสลับสับเปลี่ยนดีเอ็นเอ เป็นวิธีที่มีประสิทธิภาพในการกำกับวิวัฒนาการเพื่อปรับปรุงคุณสมบัติของเอนไซม์ในระดับของยีน นอกจากนี้ การกำกับวิวัฒนาการต้องการวิธีการที่มีศักยภาพ เพื่อคัดเลือกคุณสมบัติใหม่ของเอนไซม์ เทคโนโลยีการสลับสับเปลี่ยนดีเอ็นเอได้ประสบผลสำเร็จในการจัดตั้งในงานวิจัยนี้ โดยใช้เอนไซม์อัลฟา-อะไมเลสจากแบคทีเรียสองสายพันธุ์ ได้แก่ *Bacillus licheniformis* สายพันธุ์ DSM13 และสายพันธุ์ DSM8785 เป็นเอนไซม์ต้นแบบ การแสดงออกและการปลดปล่อยเอนไซม์อัลฟา-อะไมเลสประสบผลสำเร็จภายใต้การชักนำของ IPTG ในระบบการแสดงออกของยีนด้วยแบคทีเรีย การวิเคราะห์คุณลักษณะเอนไซม์อัลฟา-อะไมเลสทั้ง 2 ชนิด จากโคลน rpFL13-10xHis and rpFL8785-10xHis พบว่าแคลเซียมไอออนชักนำความเสถียรของโครงสร้างของเอนไซม์ และพัฒนากิจกรรมของเอนไซม์ให้สูงขึ้น สภาวะที่เหมาะสมต่อกิจกรรมของเอนไซม์อัลฟา-อะไมเลสทั้ง 2 ชนิด คือ ที่อุณหภูมิ 70 องศาเซลเซียส ที่ความเป็นกรด-ด่าง เท่ากับ 7 และพบว่า ทั้ง 2 เอนไซม์มีค่าครึ่งชีวิต เป็นระยะเวลา 30 นาที ที่อุณหภูมิ 60 องศาเซลเซียส ที่สภาวะความเป็นกรด-ด่างเท่ากับ 7 การวิเคราะห์จลนศาสตร์ พบว่า คุณสมบัติของเอนไซม์อัลฟา-อะไมเลสจากโคลน rpFL8785-10xHis ดีกว่าเอนไซม์อัลฟา-อะไมเลสจากโคลน rpFL13-10xHis ยิ่งไปกว่านี้ เอนไซม์ทั้ง 2 ชนิดมีความสามารถในการย่อยสลายแป้งมันสำปะหลังได้ดีกว่าการย่อยสลายแป้งที่ละลายน้ำ การสร้างห้องสมุดของยีนอัลฟา-อะไมเลสสร้างขึ้นด้วยเทคนิค error prone PCR และเทคนิคการสลับสับเปลี่ยนดีเอ็นเอ ได้ทำการคัดเลือกโคลนทั้งหมด 5,000 โคลน ด้วยวิธีการแสดงผลในปริมาณมากจากทั้งหมด 6 สภาวะ ได้เอนไซม์ 3 ชนิดที่ได้รับการสลับสับเปลี่ยนดีเอ็นเอ คือ เอนไซม์ 2a11h, 2b8b, 4d2d การกลายพันธุ์แบบจุดของเอนไซม์ที่ได้รับการเปลี่ยนแปลง คือ เอนไซม์ 1b9a เกิดขึ้นที่บริเวณอนุรักษ์และที่ตำแหน่งการจับของแคลเซียมซึ่งสำคัญต่อกิจกรรมของเอนไซม์

สาขาวิชาเทคโนโลยีชีวภาพ   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2550   ลายมือชื่ออาจารย์ที่ปรึกษา_____

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DIRECTED EVOLUTION/ DNA SHUFFLING/ ALPHA-AMYLASE/ HIGH

THROUGHPUT SCREENING*/ Bacillus licheniformis*

DNA shuffling technology is a powerful method of directed evolution to improve the property of enzymes at a gene level. Moreover, directed evolution requires an efficient method for screening new properties of enzymes. DNA shuffling technology was successfully established in this thesis by using two alpha-amylases from *Bacillus licheniformis* DSM13 and DSM8785 as a model. The expression and secretion of alpha-amylases were done under IPTG induction in a bacterial expression system. The characteristics of recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were analyzed. The results showed that calcium ion enhanced the stability of enzyme structure and increased the activity of both recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis. The optimal conditions of both alpha-amylases activity were at the temperature of 70 $^{\circ}$C, at pH 7 and their half life ($t_{1/2}$) lasted 30 mins, at 60°C, at pH 7. The kinetic analysis suggested that the property of rpFL8785-10xHis alpha-amylase was better than rpFL13-10xHis alpha-amylase and both enzymes could hydrolyze cassava starch better than soluble starch. The library construction of mutant alpha-amylase was created by the recombination of error prone PCR, followed by DNA shuffling. The screening by high throughput method was done under six different conditions from 5,000 clones. Three shuffled

alpha-amylases no. 2a11h, no. 2b8b, and no. 4d2d contained shuffled amino acids between two alpha-amylases from *B. licheniformis* DSM13 and DSM 8785. The single point mutation of variant enzyme no. 1b9a occurred in the conserved regions and the calcium binding sites, which were important for the activity and stability of the enzyme.

School of Biotechnology   Student's Signature_____

Academic Year 2007   Advisor's Signature_____

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CHAPTER I

INTRODUCTION

One of the most abundant of natural polysaccharide is starch, which is the major product of agricultural crops. Plants synthesize starch as a result of photosynthesis in leave and keep starch as storage compound for respiration during dark period. Starch is also kept as long-term storage in roots and seeds. It has been harvested and used as raw materials or source of energy for chemical or enzymatical processes of variety products in manufactories such as alcohol production, paper industry, textiles, starch hydrolysate, glucose syrup, fructose syrup, etc. In the past decades, process of starch hydrolysis has been changed from acid hydrolysis to starch-converting enzymes. Nowadays, these enzymes comprise about 30% of the world's production and they have been used in a large number of industrial applications such as laundry, detergents, food industry, etc. The alpha-amylase family, which shares common structure of (α_8/β_8) barrel, has been widely used in various industries. The characterizations of starch-converting enzymes have been developed or created novel properties to increase their efficiency for industrial utilisations. Development of these enzymes can be done by screening microorganisms from various environmental sources or by modiftying at genetic level using molecular biology techniques. The genetic modification; e.g. directed evolution, is an efficient method to create new specific properties or to select the best property by potentially selection process. Directed evolution methods such as site mutation, saturated mutation, error prone

PCR, or DNA shuffling, are applied for modification in genetic level. Alpha-amylase has widely applications not only in food industry or alcohol production but also in laundry, textiles and detergents industries. The *Bacillus* species is good bacterial sources for alpha-amylases since they can produce alpha-amylase having many specific properties such as tolerance to high temperature or work well under acidic or alkaline condition. Moreover, *Bacillus* species is easy to be cultured under normal condition. The alpha-amylase from *Bacillus lichenisformis* has various properties for many industrial applications because its alpha-amylase works well under alkaline condition and at high temperature. Moreover, alpha-amylase from *B. licheniformis* is stable under high temperature. Most alpha-amylases require calcium ions for enhancement of structure stabilization and increase the activity. These are good principle properties to improve alpha-amylase for laundry or detergent. This applications require the thermostable alpha-amylase which has high activity under alkaline condition or tolerance to chelating agent such as EDTA. Because the chelating agent is always found in the detergent and it is indispensable ingredient in detergent formulation. The chelating agent removes calcium ions from structure of alpha-amylase resulting in unstable in the structure and also reduce the activity of enzyme. Therefore, the high activity of alpha-amylase under low concentration of calcium or without addition of calcium is required for laundry and detergent.

The aim of this study was to improve property of alpha-amylase by using DNA shuffling as potential method of directed evolution in order to increase the activity under alkaline condition or more thermostable at high temperature or more stable under presence of EDTA as chelating agent. Moreover, the improvement of property without addition of calcium ions was also expected. Two genes of alpha-

amylases from *B. licheniformis* DSM13 and DSM 8785 were cloned and over expressed as recombinant alpha-amylase in *Escherichia coli* expression system. Moreover, further improvement of alpha-amylase property by directed evolution using DNA shuffling technique was also investigated.

CHAPTER II

REVIEW AND LITERATURE

2.1 Starch

Starch, which is one of the most aboundantly distributed polysaccharides in natural, is the main substrate of alpha-amylase. Starch consists of two main compounds; amylose and amylopectin. Amylose is a linear chain of glucose residuces linked together by alpha-1,4-glycosidic linkage. Amylopectin is branched polymer which contains two main structures; linear and branched structure. The linear structure of amylopectin has amylose as backbone. The branched part is a short alpha-1,4-glucose residuces, which link to its backbone with alpha-1,6-glycosidic linkage (Kuriki and Imanaka, 1999).

Hydrolysis of starch can be done by exo-enzymes (i.e. beta-amylase, glucoamylase and α-glucosidase) or by endo-enzyme (alpha-amylase). The action of exo-enzyme from nonreducing end produces low molecular weight products while randomly digestion of endo-enzyme in interior chain generates linear and branched saccharides with various lengths (Kuriki and Imanaka, 1999; Richardson T.H. et al., 2002).
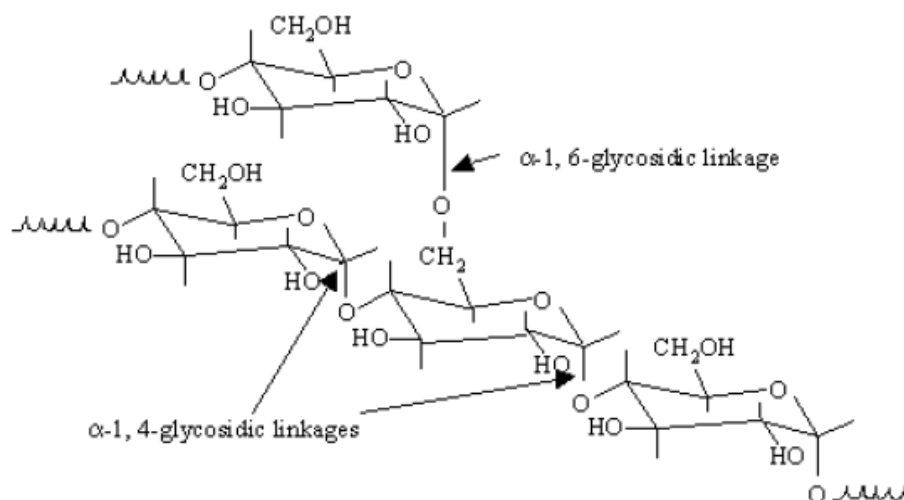
Figure 1. Structure of starch.

(http://www1.eere.energy.gov/biomass/images/graph_polymeric_structure_glucose.gif
)

2.2 Alpha-amylase

The main natural substrate of alpha-amylase is starch. Alpha-amylase or alpha-1,4-glucan-glucanohydrolase (E.C.3.2.1.1) is an endo-hydrolyzing enzyme which hydrolyzes starch, amylose, amylopectin, and various maltodextrin by randomly cleaved alpha-1,4-glycosidic linkage, resulting in production of shorter polysaccharides and dextrin. The great number of alpha-amylases have been produced from variety of eukaryotic and prokaryote organisms such as *B. amyloliquefaciens* (Machius et al., 1998a), *B. licheniformis* (Machius et al., 1995), *B. stearothermophilus* (Lutz et al., 2001; Nielsen and Borchert, 2000), *Myceliophthora thermophilia*, *Staphylothermus marinus* (Ostermeier et al., 1999), *Aspergillus oryzae*, *A.niger* (Ryu and Nam, 2000), *Pyrococcus furiosus*, *P. woesei*, *Thermococcus profundus* (Vallee et al., 1959), and etc. Generally, structure of alpha-amylase contains three domains; A, B

and C (Figure 2). The primary structure of alpha-amylase is TIM barrel or parallel $(\alpha/\beta)_8$, which is in Domain A. It consists of 8 α-sheets and 8 β-sheets in parallel direction. The central domain (domain A) forms the core of the structure and contains the active site. In the order of the structure, domain B and C are located at the opposite site of TIM barrel structure. Domain B is formed between 3rd strand and 3rd helix of TIM-barrel and it forms a large part of the substrate binding cleft. Domain B is a protrusion between the 3rd beta sheet and 3rd alpha-helix of Tim barrel and forms an irregular beta-rich structure. The size and structure of domain B varies among the various number of the alpha-amylase structure. This domain is probably responsible for the differences in substrate specificity and stability among alpha-amylase family (Nielsen et al., 1999). Domain C is C-terminal part of sequence which contains Greek key motif, beta-sandwich structure (Nielsen and Borchert, 2000). In general, the structure of alpha-amylase has four highly conserved regions (region I, II, III and IV) among alpha-amylase family (Kuriki and Imanaka, 1999; Nielsen et al., 1999). This regions are related to the catalytic and substrate-binding site of enzyme. The crystallization of alpha-amylase (pdb code; 1BLI) from *B. licheniformis* suggested that alpha-amylase consists of 512 amino acids. The first 29 amino acids are belong to *Bacillus* alpha-amylase signal peptide and 483 amino acids are in mature enzyme, which consists of domain A, B and C (Machius et al., 1998b). The amino acids in domain A are 32-140 and 236-413. Domain B and C comprise with amino acids in range of 141-235 and 414-512, respectively. Four highly conserved regions of alpha-amylase are in domain A. The region I is amimo acid at 131-136 (131-DVVINH-136). The region II, III and IV are sequences in range of 255-QLDGFRL<u>D</u>AV KHIK-264, 285-K<u>E</u>MFTVAEYWQN-296 and 354-FVDNH<u>D</u>TQP-362, respectively. Three acidic amino

acid residues responding for activity of alpha-amylase, are in region I, II and III. These are Asp262, Glu286 and Asp359. Alpha-amylase requires several ions, such as calcium ion or sodium ion for its stability or enhanced catalytic efficiency. One or more additional calcium ions have been found in several structures (Kuriki and Imanaka, 1999). Calcium ion is essential for stabitlization of enzyme structure. This ion is located between domain A and B, and it bound tightly to structure. Several alpha-amylase contain chloride ion in structure and it can be found at the active site of alpha-amylase (Kuriki and Imanaka, 1999; Nielsen and Borchert, 2000). This ion enhances catalytic efficiency. Moreover, in some alpha-amylase, sodium ion is also found.
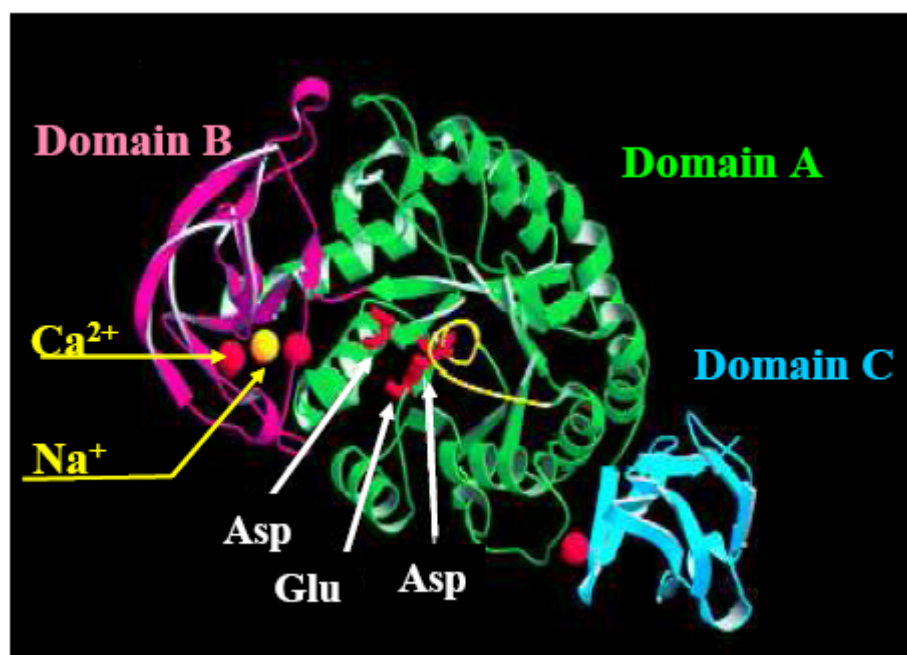
Figure 2. The domain organization of the alpha-amylases from *B. licheniformis*.
(Nielsen and Borchert, 2000).

2.3 Application of alpha-amylase

Alpha-amylases have been isolated from variety organisms such as bacteria, fungi, plants and mammalian cells. Its applications are powerful for industry since starch modification by alpha-amylase is one of the most of important steps in food processing. Moreover, alpha-amylase has application in production of maltodextrin and also in the modification of starch for resizing paper and textiles. They are of interested in clinical analysis of some diseases, such as pancreatitis.

Table 1. Application of alpha-amylase in variety of industries.

Application	Information	References
Bread and baking	Alpha-amylase not only enhances fermentation rate and reduces the viscosity of dough (resulting in improvement of the volumn and texture), but also generates sugar in dough, which improves the taste, crust color and toasting qualities of the bread.	(Nielsen and Borchert, 2000)
Starch liquefaction and saccharification	Process requires alpha-amylase which more active at high temperature and also need other amylolytic enzyme to hydrolyze short polysaccharide into monomer.	(Nielsen and Borchert, 2000)
Textile desizing	Remove starch on textile by hydrolysis resulting in dextrins, which is water-soluble compound	(Maarel et al., 2002)
Paper industry	Alpha-amylase digests starch protecting paper against mechanical force during finishing process	(Tolan, 1996)
Detergent	Application requires alpha-amylase which actives and more stability under strong alkaline condition at high temperature. Moreover, alpha-amylase should not be sensitive to oxidizing agents which are components of detergent formulation	(Maarel et al., 2002)

2.4 Directed evolution and DNA shuffling

Directed evolution is a powerful tool for improvement or modification of protein properties. This evolution experiments are created *in vitro* by mimic the selection of natural process. This method does not require long time for evolution when compares with natural selection, which generates only small mutation points (Arnold and Volkov, 1999; Kurtzman et al., 2001). The natural evolution has selected the most suiTrial enzyme that ensures the survival of its original organism in its environments. On the other hand, directed evolution can be advanced in gene level in the test tube, and expression of modified gene in suitable microorganism host can be achieved under mild condition of microbial growth. This method leads to avoid the consideration of survival of microorganism by directly modification of cell metabolism for protein development under extreme environment. Directed evolution involves generation of diversity of library by DNA recombination of various sets of parent nucleotide sequences and/or random mutagenesis. Nowadays, the most exiting procedures of directed evolution precess are based on PCR technique (error prone PCR) and DNA shuffling. Because directed evolution advances in creation of diversity of genetic or diversity of protein properties, this technique, therefore, offers a way to improve protein properties rapidly for unknown structure. Functionally improved variants are first isolated by high-throughput selection, and then used as parents for other rounds of recombination or mutagenesis. Successfully, evolution experiments require three considerations. First is the suitable microorganism host for expression of improvement functional enzymes. Second is the avaibility of selection or screening sensitive to desire properties of protein, and the last is the identification of workable evolution strategy (Kuchner and Arnold, 1997). Selection and screening

are major challenges of directed evolution. Normally, selection involves intracellular enzymes which required for cell survival. Activity of desired protein is easy to detect, and it does not interfere with cellular metabolism (Arnold and Volkov, 1999). Moreover, target activity can be distinced from background of other cellular activities. Screening is required when desired proteins cannot be linked easily to cell survival. High throughput, generally, is not high sensitivity to the desired properties. A quantitative method such as colorimetric assay is one of methods that were improved for screening to get rid off this limitation. The potential of selection or screening technology should reduce the times and cost of experiments and they also should increase the possibility of difficult problems involving in multiple enzymes, multicomponent enzymes and creation of new novol functional molecules.

Principle steps of directed evolution of proteins and example keys in which multiple generations of mutagenenis and screening or selection have been described in Figure 3 and Table 2, respectively.
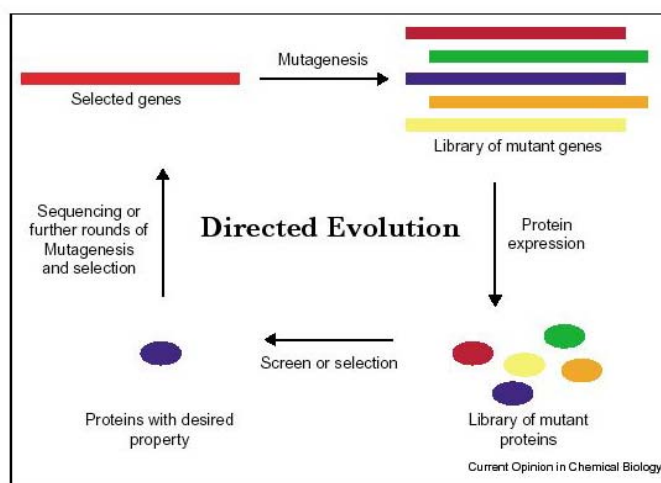
Figure 3. The improvement of gene properties by directed evolution.

(Whalen et al., 2001).

Table 2. Summary of key directed-enzyme-evolution experiments utilizing sequential generations of random mutagenesis and recombination with selection or screening.

Enzyme	Altered property	Mutagenesis method	Screened or selected	References
Subtilin E	Activity in organic solvents	Error-prone PCR	Screened	(Chen and Arnold, 1993)
β-Lactamase	Total activity and substrate specificity	DNA shuffling	Selected	(Stemmer, 1994)
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-PCR and recombination	Screened	(J. C. Moore and Arnold, 1996)
Subtilin BPN	Stability	Cassette	Screened	(Strausberg et al., 1995)
β-galactosidase	Substrate specificity	DNA shuffling	Screened	(Zhang et al., 1997)
Arsenate detoxification pathway	Arsenic resistance	DNA shuffling	Screened	(Crameri et al., 1997)
Paranitrobenzyl esterase	Substrate specificity and activity in organic solvents	Error-PCR and DNA shuffling	Screened	(J. C. Moore et al., 1997)

In the recent years the possibility of creation the diversity of genetic has been expanded enormously by application of PCR technique such as error prone PCR, and DNA shuffling (Crameri et al., 1997; Zhao et al., 1998). DNA shuffling which was recently developed by Stemmer, is one of directed evolution to accelerate the genetic diversity *in vitro* (Stemmer, 1994). The key advantage of DNA shuffling is not

only recombine DNA fragments, but also introduce point mutation at very low rate (Zhao and Arnold, 1997) or create multiple crossover in reassembled sequences (G. L. Moore et al., 2001). The sources of parental genes not only from a set of related genes, but also generate the related sequnces by error prone PCR, which random mutagenesis occurs. Error prone PCR is achieved by *Taq* DNA polymerase under low concentration of DNA template and various concentration of dNTP. *Taq* DNA polymerase lacks proofreading activity as found in *pfu* DNA polymerase. This property leads to amplification of PCR under low fedility. Nevertheless, increment of concentration of Mn^{2+} enhances intensity of mutation points. The recombination of random fragmentation of a pool of parental genes which performed by DNaseI is most important for DNA shuffling. The hybridization of homologous sequences prime each others, after denaturation of different parent templates, resulting in crossovers are locked in by polymerase extension. Multiple cycles of PCR resulting in an enhancement of increase of multiple crossover and resulting in a library of full length sequences. After construction of library into an expression vector and transfer into microorganism host for expression, potential selection and/or screening indentify the best combination clones and, then, used as parent genes in the next round.
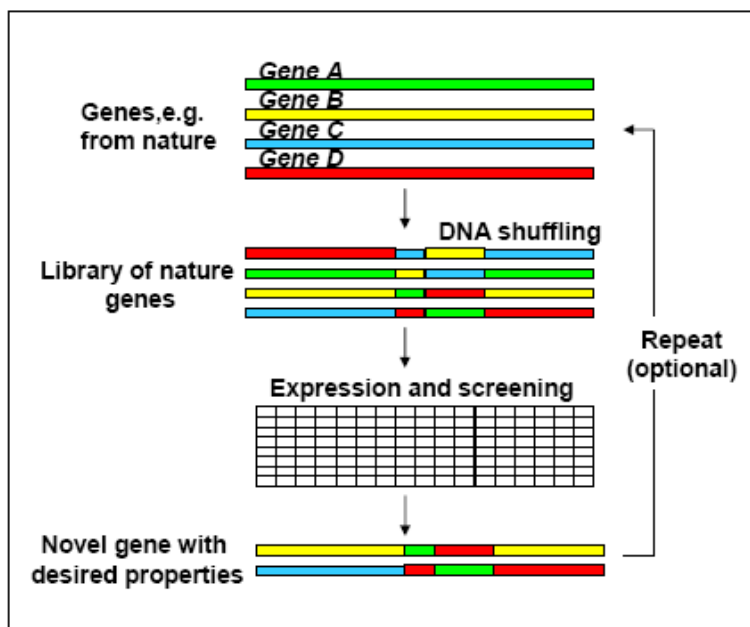
Figure 4. The molecular breeding of directed molecular evolution process.

(Powell et al., 2001).

Nowadays, numerous alpha-amylases which have been developed to desire properties by directed evolution or other methods, have been established in many applications as described in Table 3.

Table 3. Summary of properties development of alpha-amylases by directed evolution and other methods.

Microorganism	Applications	Mutagenesis methods	Altered properties	References
B. licheniformis	Detergent	Error prone PCR and gene shuffling	Opimun pH of BAA42 mutant shifed from pH6 to pH 7, and 5 folds of activity increase at pH 10	(Bessler et al., 2003)
Bacillus sp. strain TS-23 alpha-amylase (BLA)	Understand mechanism of starch binding domain	Site directed mutagenesis	Single mutant (Thr-527, Trp-545, Trp-561,or Lys-576) reduced binding activity, while combinational mutations did not lead to a complete loss of the activity	(H. F. Lo et al., 2004)
Barley alpha-amylase	Protein secretion	degenerate oligonucleotide gene shuffling (DOGS)	-Mutants (8 clones from 843 clones) showed large halo ratio on starch agar. -Mutants (F21M/Q44H, A42P/A47S and A42P rAMY2) showd the better secretion of enzyme outside the cells.	(Fukuda et al., 2005)
B.licheniformis		site-directed mutagenesis	Enhancement of thermostability by deletion of Arg78 and Gly177 in region I, and by substitutions of aspartic acid for Asn" in region I1	(Suzuki et al., 1989)

Table 3. (Continued).

Microorganism	Applications	Mutagenesis methods	Altered properties	References
B. licheniformis MTCC 6598	Starch hydrlysis for food industry	Site directed mutagenesis	-Mutant (N104D) improved specific activity at pH 5 and 70°C -Mutant (D161N) retained activity at 30°C degrees C but had significantly less activity at 70°C -Mutant (D430N) was not changed at 30 degrees C but had an improved activity at 70°C	(Priyadharshini and Gunasekaran, 2007)
Thermus sp. strain IM6501 (maltogenic alpha-anylase)	Starch hydrolysis	DNA shuffling	The optimal temperature of recombinant enzyme was 75°C, which was 15°C higher than wild-type, and the melting temperature was increased by 10.9°C. The half-life of ThMA-DM was 172 min at 80°C, a temperature at which wild-type ThMA was completely inactivated in less than 1 min	(Y. W. Kim et al., 2003)

Table 3. (Continued).

Microorganism	Applications	Mutagenesis methods	Altered properties	References
B. licheniformis	Acidic resistance for starch liquefaction	DNA shuffling	Mutants showed improvement of starch binding under low pH values and showed higher activity of starch hydrolysis at pH 4.5 by using phage display	(Verhaert et al., 2002)
Barley alpha-amylase	Starch hydrolysis	Error prone PCR and DNA shuffling	Mutant (Mu322) showed 1000 times the total activity and 20 times the specific activity thad wild type	(Wong et al., 2004)
B. licheniformis	Acidic resistance	Site-directed mutagenesis	Mutant was more acidic resistance than native enzyme. The optimum pH and stable range of pH with the mutagenised protein was 4.5 and 4.0 to 6.5, respectively, compared with pH 6.5 and 5.5 to 7.0 as the favorite pH and pH stability range of the native protein	(Liu et al., 2008)

Table 3. (Continued).

Microorganism	Applications	Mutagenesis methods	Altered properties	References
Isolation of a group of thermostable alpha-amylase (2000 genes) from different geographical deep sea enrichments and acid soil	Starch liquefaction	Gene reassembly	The 34 clones showed higer activity of liquefaction and improved thermostability at pH 4.5	(Richardson. T.H. et al., 2002)
B. substilis	Cyclodextrin production	Error prone PCR and saturation mutagenesis	Conversion of our model enzyme, cyclodextrin glucanotransferase (CGTase), into an alpha-amylase like hydrolytic enzyme by saturation mutagenesis close to the catalytic core yielded a triple mutant (A231V/F260W/F184Q) with the highest hydrolytic rate ever recorded for a CGTase, similar to that of a highly active alpha-amylase, while cyclodextrin production was virtually abolished.	(Kelly et al., 2007)

CHAPTER III

RESEARCH OBJTCTIVES

The goal was to establish and apply DNA shuffling technology for improvement of properties of industrial enzyme by using alpha-amylase as a model.

The objectives of this research were summarized as belowed

1. To establish DNA shuffling technology for improvement of alpha-amylase property.

2. To analyze characteristics of alpha-amylase such as optimum pH and temperature, pH and temperature stability and kinetics.

CHAPTER IV

MATERIALS AND METHODS

4.1 Bacterial strains and cultivation conditions

Two bacterial strains used in this experiment, were *B. licheniformis* DSM 8785 and DSM 13. Both strains were supplied from Division of Food Biotechnology, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Science, Vienna, Austria. These bacteria were cultured on M1 medium at 30°C and 37°C for *B. licheniformis* DSM 8785 and DSM 13, respectively. *E. coli* DH5αF' was used as host for cloning. *E. coli* BL21 (DE3) and *E. coli* TOP10 were used to express alpha-amylase expression vector. Three bacteria were grown in Luria-Bertani (LB) medium at 37°C.

4.2 Construction of alpha-amylase expression vector

The genomic DNA of each *Bacillus* strain was used as DNA template. Genomic DNA was extracted directly from each single colony by boiling in ultra pure water at 95°C for 5 min, and then, put on ice to cool down immediately. Two primer sets, flanked with two appropriated restriction sites were designed based on DNA sequence from Genbank no. X03236 to create 1.5 kb of alpha-amylase gene with/without *Bacillus* signal peptide. First set of primers (3'-*BspH*I site (5'-ggA ggA TCA TgA AAC AAC AAA AAC ggC TTT ACg-3') and 5'-*Xho*I site (5'-gCA CAg CTC gAg TCT TTg AAC ATA AAT TgA AAC CgA CCC-3')) generated alpha-amylase with *Bacillus* signal peptide. This gene was inserted into pET21d (+)

expression vector (named rpET8785 and rpET13). Other set of primer was used for amplification of alpha-amylase without signal peptide containing 3'-*EcoR*I site (5'-CTg TgC gAA TTC gCA AAT CTT AAT ggg ACg CTg ATg C-3') and 5'-*Xho*I site (5'-gCA CAg CTC gAg TCT TTg AAC ATA AAT TgA AAC CgA CCC-3'), then the amplified gene was inserted into pFLAG-CTS (or pFLAG-CTS plus 10xHis) expression vector to construct rpFL8785 (or rpFL8785-10xHis) and rpFL13 (or rpFL13-10xHis) expression vector. Mature alpha-amylase gene was fused to *Omp*A signal peptide of *E. coli* on pFLAG-CTS vector after construction. The PCR amplifications of two primer sets were done under same condition. PCR reaction consisted of 1x *pfu* DNA polymerase buffer, 2.9 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 μM of primer, and 3 unitof *pfu* DNA polymerase (Promega). Fist denaturation was done at 96°C for 2 min. Thirty cycles of PCR were performed by following step of denature temperature at 95°C for 45 second, annealing temperature at 55°C for 1 min, and extension temperature at 72°C for 4 min. Last extension was done to complete gene amplification at 72°C for 10 min. The PCR products were cloned into pET21d (+) or in pFLAG-GTS (or pFLAG-CTS plus 10xHis), then, resultant plasmids were transformed to *E. coli* strain BL21 (DE3) or TOP10, respectively, for gene expression.

4.3 Sequence analysis

Nucleotide sequences were analyzed by DNA sequencing service unit of Macrogen Inc (World Meridian Venture Center, Seoul, Korea).

Multiple alignment of nucleotide sequences were analyzed by using vector NTI program whereas the percentage of similarity was determined by ClustalW2 from EMBL-EBI software on-line. The secondary structure of alpha-amylases were done by

Espript2.2 program. The phylogenic tree was analyzed from amino acid equences by using vector NTI program.

4.4 Expression and production of recombinant alpha-amylase

4.4.1 Expression on LB agar containing soluble starch

Sigle colony of *E. coli* BL21 (DE3) and/or *E. coli* TOP10 harboring recombinant alpha-amylase expression vector were grown on screening media consisting of LB agar containing 1%(w/v) soluble starch (Sigma-Aldrich), 100 μg/ml amplicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 16-18 hr at 37°C (optional). The starch hydrolysis was shown as clear zone around colony after overlaid starch plate with I_2 solution. Negative controls were done by growing each single *E. coli* carring original pET21d (+) or pFLAG-CTS (or pFLAG-CTS-10xHis) expression vector under same condition.

4.4.2 Production and purification of alpha-amylase

Overnight cultivatuion of each single colony of *E. coli* carrying recombinant alpha-amylase expression vector was done in 5 ml LB broth plus 100 μg/ml amplicillin at 37°C and, then, transferred into 250 ml LB broth. Induction of IPTG at final concentration of 1 mM was performed when the optical density (at 600 nm) of the culture medium reached 1.2 to 1.4 and further cultivation at 25°C, shaking speed of 150 rpm for 18 hr. Cells were harvested by centrifugation at 4°C for 20 min, then followed by cell lysis and use as crude enzyme. Alpha-amylase from recombinant alpha-amylase in pET21d (+) or in pFLAG-CTS-10xHis were purified by Nickel column. To remove imidazole, purified alpha-amylase was washed 3 times with 50 mM potassium phosphate (pH 7.0) by ultra-centrifugation with molecular weight cut off 30,000 Da. Molecular weight was determined by SDS-PAGE analysis.

4.4.3 SDS-PAGE and zymogram analysis

Molecular weight analysis of purified alpha-amylases and in-gel assay were done by SDS-PAGE analysis. Purified alpha-amylase band was separated by 12% (w/v) polyacrylamide gel for SDS-PAGE or by 8% (w/v) polyacrylamide gel containing 0.22% (w/v) of soluble starch as final concentration. For SDS-PAGE analysis, purified alpha-amylase was mixed with protein sample buffer (0.0625 M Tris-HCl (pH 6.8), 0.01% (w/v) bromophenol blue, 2% (w/v) SDS, 20% (w/v) glycerol) containing 0.1 M dithiothreitol (DTT) and then, boiled at 100°C for 3 min. Electrophoresis was performed at 100 volt for 2 hr by usig BioRad Mini Protein II Cell (BioRad). The gel, after electrophoresis, was washed with DI and then, stained with Coomassie brilliant blue-G250. Molecular weight was determined with *Rf* value by comparing with standard protein marker. In gel assay by zymogram was done at the same time with SDS-PAGE analysis. Mixture of purified enzyme and protein sample buffer without DTT was heated at 100°C for 3 min before applying into soluble starch-polyacrylamide gel. Electrophoresis at 4°C was done as condition as SDS-PAGE analysis. In gel assay was achieved by following steps of (1) washed starch-polyacrylamide gel with distrilled water, (2) soaked gel with 2.5% (w/v) Triton X-100 at 4°C for 1hr for SDS removal, (3) incubated gel in 50 mM potassium phosphate buffer (pH7) at 45°C for 3 hr with slowly rotation, (4) soaked gel with 0.1 M acetic acid to terminate activity, and (5) stained gel with I_2 solution. The activity of alpha-amylase on gel was distinctly shown as clearing band from brown background.

4.5 Library construction of shuffled alpha-amylase

Shuffeld protocol was performed by following published protocol with some modifications (Zhao and Arnold, 1997). Two libraries were generated in pET21d (+) and pFLAG-CTS expression system.

4.5.1 Error prone PCR

Random mutagenesis was created by error prone PCR. Four amplifications were done directly from four expression vectors. Expression vectors rpET13 and rpET8785 were used as template for amplification by primers 3'-*BspH*I and 5'-*Xho*I. PCR products were name ep13 and ep8785. Vectors rpFL13 and rpFL8785 were used for PCR amplification by primers 3'-*EcoR*I and 5'-*Xho*I, and products were defined as epF13 and epF8785, respectively. All reactions of error prone PCR consisted of 6.24 ng of DNA template, 1x*Taq* DNA polymerase buffer, 0.2 mM each of dATP and dGTP, 1.0 mM each of dCTP and dTTP, 2.9 mM MgCl$_2$, 0.5 mM MnCl$_2$, 5U of *Taq* DNA polymerase (New England Lab) and 0.3 µM of primer. Thermocycle was achieved by following step of initial denaturation at 95°C for 2 min, and 45 cycles of 95°C, 45 second, 55°C 1 min and 72°C for 2 min, and 72°C for 10 min as last extension. Molecular size of error prone products were verified on agarose gel and purified by QIA PCR purification kit (Quiagen).

4.5.2 DNaseI digestion

Digestion by DNaseI was prepared for all error prone products under same condition. Products ep13 and ep8785 were mixed together (or epF13 and epF8785) Creation of random fragments DNA was performed at 15°C for 7 min by reaction consisted of DNaseI (Fermentas) at 0.1 to 1 U/µg DNA (error prone PCR), 50 mM Tris-HCl pH 7.4 and 10 mM MnCl$_2$. Fragments size from 50 bp to 250 bp were cut

from 15% (w/v) polyacrylamide gel and sliced into small pieces by steriled pipette tip. Fragments were removed out from gel by incubating gel in 3M ammonium acetate at 37°C for overnight, and then, filtrated with glass wool. The phenol:choloform extraction and ethanol precipitation were done in order to purified DNA fragments. The pellet was dissolved in 30 μl of ultra pure water. The purified product from mixture of ep13 and ep8785 was named DpETM, whereas mixture of epF13 and epF8785 was named DpFLM.

4.5.3 Reassembled PCR into full length

To reassemble small fragments into full length of alpha-amylase gene, PCR amplification without primers was undertaken. DpETM and DpFLM were reassembled by same condition and designated PCR product as ReETM and RpFLM, respectively. Fifty microliters of reassembled reaction consisting of 30 μl of purified short fragments (from previous step), 1x *pfu* DNA polymerase, 25 mM of each dNTP and 2.5 U of *pfu* DNA polymerase. Amplification was applied by intinital temperature at 96°C for 2 min and followed by 30 cycles of 95°C, 45 second of denaturation step, 55°C for 1.30 min of annealing step and 72°C for 2 min+5 sec/cycle of extention step, and by 72°C for 10 min as last extension.

4.5.4 Amplification of shuffled alpha-amylase

Two amplificaitons of shuffled alpha-amylase were performed by using two different primer sets, (1) 3'-*BspH*I and 5'-*Xho*I and (2) 3'-*EcoR*I and 5'-*Xho*I, by applied ReETM and RpFLM as template under same condition of amplification. 50 μl of PCR amplification consisting of 1 μl of DNA template (ReETM or RpFLM), 1x *pfu* DNA polymerase buffer (promega), 0.25 mM each of dNTP, 2.9 mM MgCl$_2$, 3 U of *pfu* DNA polymerase and 2.5 U of *Taq* DNA polymerase (New England Lab) and

0.25 μM of primers. The amplification was performed by initial denaturation at 96°C for 2 min, followed by 35 cycles of 95°C for 45 second, 55°C for 1 min, and 72°C for 4 min. Last extension was done to complete gene amplification at 72°C for 10 min. To verify the correct size of shuffle alpha-amylase, molecular size of shuffled gene was determined by 1% (w/v) agarose gel. Two constructions of shuffled amylase (named as rpET-SH and rpFL-SH) were achieved by insertion of SHeTM (primer 3'-*BspH*I and 5'- *Xho*I) and SHeFM (3'-*EcoR*I and 5'-*Xho*I) into pET21d (+) and pFLAG-CTS, respectively.

4.6 Screening of library of shuffled alpha-amylases

The screening of shuffled alpha-amylase genes was done into two steps First step was primary screening. It was performed by selecting clones that produced clear zone around colony. Alpha-amylase activity analysis in microtiter plate was then performed in a secondary screening.

4.6.1 Primary screening

This step was required for the selection of shuffled clone from recombinant rpET-SH library by tooth pick method. Single clone of *E. coli* harboring shuffled alpha-amylase was grown on screening medium consisting of LB agar containing 1% (w/v) soluble starch (Sigma), 1mM IPTG and 100 μg/ml of ampicillin at 37°C for overnight. *E. coli* carrying original pET21d (+) was used as negative control. Positive control was *E. coli* harboring native alpha-amylase expression vector (rpET8785). The clear zone was observed by I_2 staining. Clones which showed clearing zone (positive clones) were selected for secondary screening. The shuffled clones from library of rpFL-SH could show clear zone after electroporation without induction of IPTG on screening medium. Recombinant rpET13 and rpET8785 or rpFL13 and rpFL8785

were used as positive control whereas pET21d (+) and pFLAG-CTS were used as negative control.

4.6.2 Secondary screening by activity assay of alpha-amylase

The condition for secondary screening of library of rpET-SH and rpFL-SH was different from each others. The expression of shuffle alpha-amylase was performed in 96 wells plate (for rpET-SH library) or in 96 deep wells plate (for rpFL-SH library). The single colony of positive clones were cultured separately in 120 µl or 500 µl of LB in each well for rpET-SH library and rpFL-SH library, respectively, for overnight at 37°C, and shaking at 200 rpm. The induction of 1mM IPTG of rpET-SH library was done at 25°C, at 200 rpm for 4 hr. Induction of ITPG was not necessary for library of rpFL-SH. Single colony from rpFL-SH library was grown in 500 µl LB broth containing 100 µg/ml amplicillin for 22 hr, 200 rpm at 37°C. To recover culture broth (enzyme sample), centrifugation was done for 20 min, at 4000 rpm, 4°C.

4.6.2.1 Screening condition of rpET-SH library

Screening analysis under high temperature was performed on 96 wells microtiter plate at two different pH coditions; pH 3.5 by using 50 mM sodium acetate buffer and pH 9.5 by using 50 mM potassium phosphate buffer. Control experiment of screening was performed by detection of activity of shuffled alpha-amylase at pH 6.5 by using 50 mM potassium phosphate buffer. Reaction (200 µl) consisting of 20 µl enzyme sample and 180 µl of 0.025% (w/v) cassava starch in each pH buffer. Activity assay was started by incubating the reaction at 90°C for 10 min, and then, stopped reaction immediately by adding 20 µl of 0.5 M glacial acetic acid. Remaining cassava starch in reaction after starch hydrolysis was determined by observing an absorbance values at 595 nm after development of color in reaction by adding I_2 solution (20 µl).

4.6.2.1 Screening condition of rpFL-SH library

The conditions of screening analysis were devided into 6 sub-experiments by using 0.025% (w/v) or 0.5% (w/v) cassava starch and 20 μl of enzyme samples. Two first sub-screening analyses was done at 37°C for 30 min by using 0.025% (w/v) cassava starch in 50 mM sodium acetate (pH 3) and 0.5% (w/v) cassava starch in 50 mM glycine-NaOH (pH 11). The third assay was done at 37°C for 30 min by using 0.5% (w/v) cassava starch in 50 mM potassium phosphate (pH 7). Stability of alpha-amylase (pH 7) at 60°C for 1 hr under presence of 10 mM EDTA was performed for forth experiment. The remained activity was determined by using 0.025% (w/v) cassava starch in 50mM potassium phosphate (pH 7) at 37°C for 30 min. The fifth screening performed in 50 mM potassium phosphate (pH 7) at 75°C for 2 hr before determination of the remaining activity. The activity at 0.025% (w/v) cassava starch in 50 mM potassium phosphate (pH 7) was done as positive control for all sub-experiments. Remaining cassava starch was determined by optical density at 595 nm after hydrolysis of starch at each condition, and development of color by adding I_2 solution.

4.7 Activity analysis of alpha-amylase

Determination of activity of alpha-amylase was performed by colorimetric method using 3,5-dinitrosalisylic acid. The assay reaction consisting of 20 μl of purified alpha-amylase in 1 ml of 1% (w/v) soluble starch in 50 mM potassium phosphate (pH 7) containing 2 mM $CaCl_2$. Hydrolysis was done at 37°C for 20 min controlling temperature by thermomixer (Eppendrof AG, Hamburg, Germany) with 1000 rpm shaking speed. Activity of alpha-amylase was terminated by addition of 3,5-dinitrosalisylic acid, then, heated at 100°C for 20 min, and observed optical density at

540 nm. One unit of alpha-amylase activity was defined as 1 μmol reducing sugar (glucose) produced per min under assay condition.

4.8 Enzyme characterization

The purified alpha-amylases rpFL13-10xHis and rpFL8785-10xHis were used for analysis of enzyme characterizations and kinetics. The analysis was besed on equal amount of proteins in the assays.

4.8.1 Optimal pH determination

The effects of pH on the activity of alpha-amylase were analyzed as described on step 4.7 by varying pH values from pH 2 to 12. The pH conditions were controlled by 50 mM sodium acetate for pH 2 to 6, 50 mM potassium phosphate for pH 6 to 9, and 50 mM glycine-NaOH for pH 9 to12.

4.8.2 Optimal temperature determination

The effect of temperature on the activity of alpha-amylase was assayed as described on step 4.7 by increasing temperature from 30 to 100°C, with increment of 10°C.

4.8.3 Analysis of temperature stability of alpha-amylase

Purified alpha-amylase was incubated in 50 mM potassium phosphate buffer (pH 7) with/without 2mM $CaCl_2$ for 30 min at 30 to 100°C, with increment of 10°C. Critical temperature was observed from relationship of temperature and (%) relative activity. Two or three points of temperature over/under critical temperature were taken for stability assay for a period of 4 hr, by sampling the sample at 5, 15, 30, 60, and 240 min. The remaining activity was assayed as described in step 4.7.

4.8.4. Analysis of pH stability of alpha-amylase

Purified alpha-amylase was incubated at 37°C for 30 hr under 7 different pH values. There were pH at 3, 4, 6, 7, 8, 9, and 10. The pH conditions were controlled by 50 mM sodium acetate for pH 2 to pH 6, 50 mM potassium phosphate for pH 6 to pH 9, and 50 mM glycine-NaOH for pH 9 to pH 12. Critical pH value was observed from relationship of pH and (%) relative activity. Two or three points of pH value over/under critical point of each temperature were selected for pH stability assay for a period of 4 hr, by sampling reaction every 30 min. The remaining activity was assayed as described on step 4.7.

4.8.5 Kinetic analysis

Concentrations of soluble starch (pH 7) ranging from 0 to 2.5% (w/v) were prepared and incubated with equal amount of protein of purified alpha-amylase at 37°C for 20 min in order to obtain steady-state kinetic constant. The values of the kinetic constant were calculated from the Michaelis-Menten plot using a non-linear curve fitting method (SigmaPlot 2000), and turnover number (k_{cat}) and catalytic efficiency values (k_{cat}/K_m) were further calculated.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Construction of recombinant expression vector of alpha-amylase

Recombinant expression vectors of alpha-amylases from two strains of *B. licheniformis* DSM 13 and DSM8785 were created as shown in Figure 5. All recombinant vectors were named rpET13, rpET8785, rpFL8785, rpFL13, rpFL8785-10xHis, and rpFL13-10xHis. Expression of recombinant rpET13 and rpET8785 vectors were controlled by T7 promoter on pET21d (+) and promoter on DE3 domain of host *E. coli* BL21 (DE3), resulting in production of alpha-amlylase. Both genes carrying *Bacillus* signal peptide, resulting in the secretion of alpha-amylase outside host cell as it could be observed as clearing zone on LB agar containing 1% (w/v) soluble starch after staining with I_2 solution as shown in Figure 6. The expression of other recombinant vectors were controlled by *tac* promoter on pFLAG-CTS or pFLAG-CTS-10xHis vector. The native signal peptide of these enzymes were replaced by *E. coli* OmpA signal peptide. Fusion of *Omp*A signal pepetide of *E. coli* with mature alpha-amylase gene led to secretion of alpha-amylase into environment as shown in Figure 6. Because pFLAG-CTS vector is a leaky plasmid, hydrolysis of starch by alpha-amylase from four recombinant vectors, rpFL13, rpFL8785, rpFL13-10xHis, and rpFL8785-10xHis, could be observed as cleare zone around *E. coli* TOP 10 without IPTG induction on LB agar containing 1% (w/v) of soluble starch (Figure 6) after transformation. Expression of alpha-amylase of single colony showed nearly

halo ratio value after observation of clear zone by tooth pick with/without IPTG induction (Figure 7). The constructions in pET21d (+) vector or pFLAG-CTS-10xHis vector resulted in the fusion of 6xHis or 10xHis at C-terminal of alpha-amylase, respectively. Because hydrolysis of starch on soluble starch agar plate could be detected (Figure 6 and 7), it confirmed that fusion of multiple histidine sequences did not inhibit activity of alpha-amylase. The result was correlated to fusion of thermostable alpha-amylase to 6 histidine sequence at C-terminus. The alpha-amylase could be observed (Hmidet et al., 2008). In case of fusion of 10 histidine to kinase, the enzyme was also active (Belin et al., 2006).
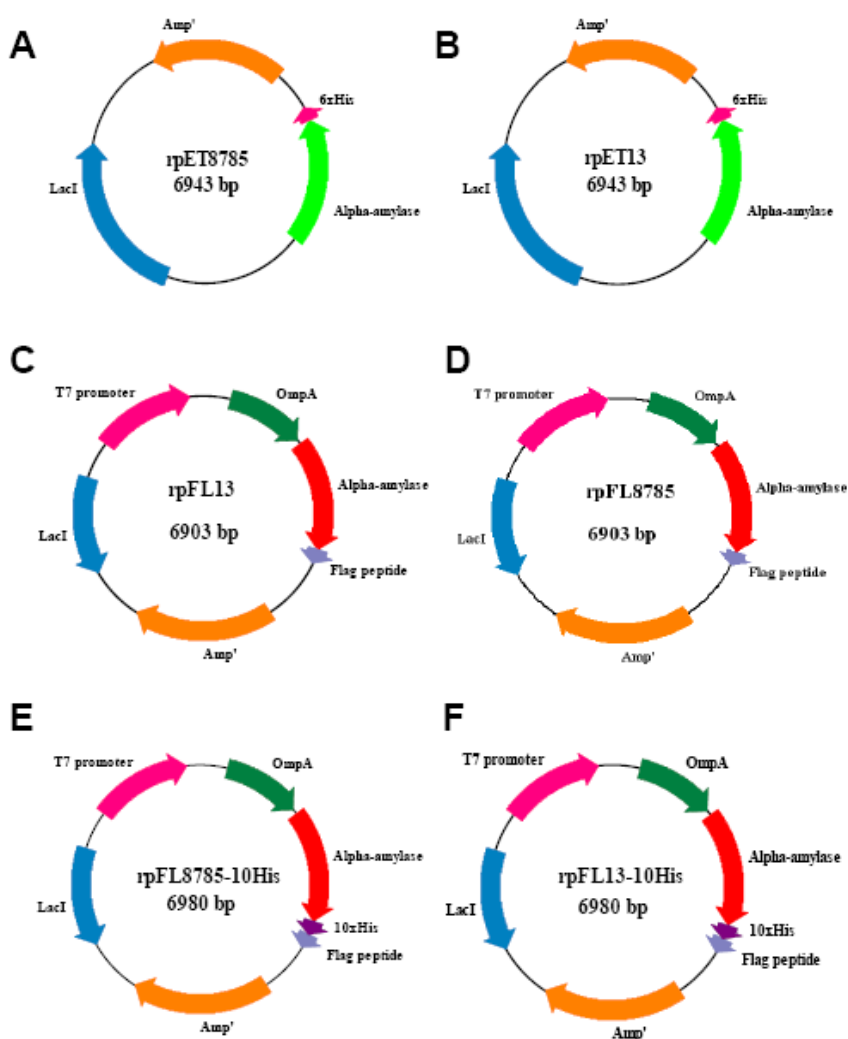
Figure 5. Maps of recombinant expression vectors of alpha-amylases.
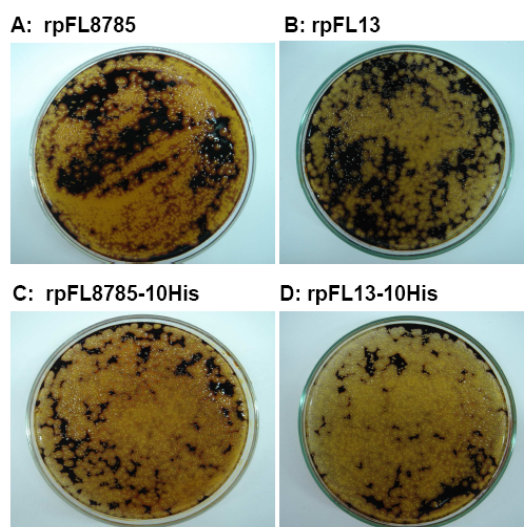
Figure 6. Expression of alpha-amylases from four recombinant vectors on soluble starch plate after transformation without IPTG induction.
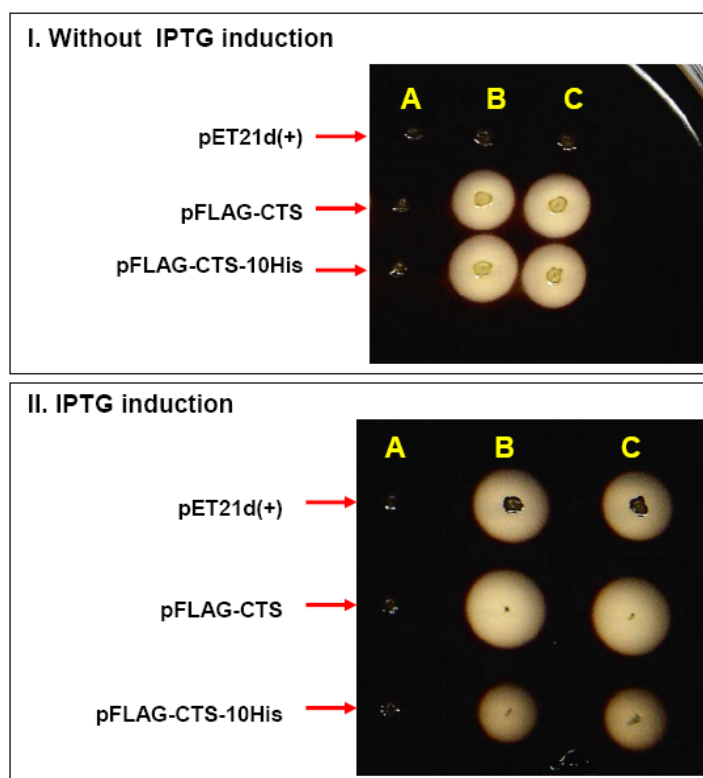
Figure 7. Expression of alpha-amylases from six recombinant vectors on soluble starch plate by tooth pick with or without IPTG induction.

A: empty vector; B: alpha-amylase DSM13; C: alpha-amylase DSM8785.

Multiple alignment of nucleotide sequences were done by using vector NTI software from invitrogen where as amino acid sequences analysis was done by ESpript 2.2 software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA). The analysis of secondary structure of alpha-amylase was based on well known secondary structure of alpha-amylase from *B. licheniformis* (pdb code; 1BLI.pdb) (Machius et al., 1998b). Nucleotide and amino acid sequences of recombinant alpha-amylase vectors from rpET13, rpET8785, rpFL13-10xHis, and rpFL8785-10xHis were analyzed and compared with native alpha-amylase sequence from *B. licheniformis* DSM 13 complete genome (Figure 8 and 9). The nucleotide sequences of recombinant alpha-amylase genes showed 100% and 99% of similarity to native alpha-amylase *B. licheniformis* DSM13 (Rey et al., 2004). Blastn analysis of both recombinant alpha-amylases showed similarity to *B. licheniformis* alpha-amylase strain ATCC 14580 and *B. licheniformis* alpha-amylase 584 (appendix). Alignment results showed that recombinants rpET13 and rpET8785 vector were fused with 6 histidine sequence at C-terminus as same as rpFL13-10xHis and rpFL8785-10xHis, which fuse with 10 histidine sequence. Histidine sequences were shown in green box. *Bacillus* signal peptide was also shown in blue box. The biggest part of sequence was mature enzyme for alpha-amylase. The amino acid sequence of both recombinant alpha-amylases rpET13 and rpET8785 contained 512 amino acids including *Bacillus* signal peptide of 29 amino acids whereas 483 of amino acids of all recombinant alpha-amylase are belong to mature alpha-amylase. The overall structures of all recombinant alpha-amylases appear to be similar to structure of thermostable *B. licheniformis* NH1 alpha-amylase (Hmidet et al., 2008) and well known structure of alpha-amylase (pdb code; 1BLI.pdb) (Machius et al., 1998b). The summarized in overall structure of both

recombinant alpha-amylase rpFL13 and rpFL8785 without additional *Bacillus* signal peptide was shown in Table 5. The structure contained three domains: domain A (amino acid 30-138 and 234-411), domain B (amino acid 139-233), and domain C (amino acid 412-512). Four highly conserved regions were also found in structure. The multiple alignment of amino acid suggested that these recombinant alpha-amylases rpET8785, rpFL8785 and rpFL8785-10xHis differ by 5 amino acids when compared to native sequence of alpha-amylase from *B. licheniformis* DSM13 or to three recombinant alpha-amylases rpET13, rpFL13 and rpFL13-10xHis (Figure 9). First different point of amino acid was in *Bacillus* signal peptide (amino acid 13). Four different points were in mature enzymes of alpha-amylase.

```
                              1                                                          60
        DSM13      (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTC
        rpET13     (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTC
        rpET8785   (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTC
        rpFL8785   (1)  ------------------------------------------------------------
 rpFL8785-10xHis   (1)  ------------------------------------------------------------
        rpFL13     (1)  ------------------------------------------------------------
  rpFL13-10xHis    (1)  ------------------------------------------------------------
                              61                                                         120
        DSM13     (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAAAGGGACGCTGATGCAGTATTTT
        rpET13    (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAAAGGGACGCTGATGCAGTATTTT
        rpET8785  (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
        rpFL8785   (1)  -----------------------GCAAATCTTAATGGGACGCTGATGCAGTATTTT
 rpFL8785-10xHis   (1)  -----------------------GCAAATCTTAATGGGACGCTGATGCAGTATTTT
        rpFL13     (1)  -----------------------GCAAATCTTAAAGGGACGCTGATGCAGTATTTT
  rpFL13-10xHis    (1)  -----------------------GCAAATCTTAAAGGGACGCTGATGCAGTATTTT
                              121                                                        180
        DSM13    (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
        rpET13   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
        rpET8785 (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
        rpFL8785  (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
 rpFL8785-10xHis  (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
        rpFL13    (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
  rpFL13-10xHis   (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
                              181                                                        240
        DSM13    (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
        rpET13   (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
        rpET8785 (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
        rpFL8785  (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
 rpFL8785-10xHis  (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
        rpFL13    (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
  rpFL13-10xHis   (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
                              241                                                        300
        DSM13    (241)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
        rpET13   (241)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
        rpET8785 (241)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
        rpFL8785 (154)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
 rpFL8785-10xHis (154)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
        rpFL13   (154)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
  rpFL13-10xHis  (154)  GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
                              301                                                        360
        DSM13    (301)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
        rpET13   (301)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
        rpET8785 (301)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
        rpFL8785 (214)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
 rpFL8785-10xHis (214)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
        rpFL13   (214)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
  rpFL13-10xHis  (214)  ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
```

Figure 8. Multiple alignment of nucleotide sequences of alpha-amylase.

DSM13; Native alpha-amylase from *B. licheniformis* DSM13, rpET and

rpET8785; Recombinant alpha-amylase from *B. licheniformis* DSM 13 and

DSM8785, repectively, by using pET21d (+) system, rpFL13 and

rpFL8785; Recombinant alpha-amylase from *B. licheniformis* DSM 13 and

DSM8785, repectively, by using pFLAG-CTS system, blue color; *Bacillus*

signal peptide, green color; histidine sequence, yellow color; different

nucleotide sequence.

```
                       361                                                    420
          DSM13  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
          rpET13  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
        rpET8785  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
        rpFL8785  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
   rpFL8785-10xHis  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
          rpFL13  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
     rpFL13-10xHis  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
                       421                                                    480
          DSM13  (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
          rpET13  (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
        rpET8785  (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
        rpFL8785  (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
   rpFL8785-10xHis  (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
          rpFL13  (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     rpFL13-10xHis  (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
                       481                                                    540
          DSM13  (481) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
          rpET13  (481) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
        rpET8785  (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
        rpFL8785  (394) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
   rpFL8785-10xHis  (394) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
          rpFL13  (394) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     rpFL13-10xHis  (394) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
                       541                                                    600
          DSM13  (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
          rpET13  (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
        rpET8785  (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
        rpFL8785  (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
   rpFL8785-10xHis  (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
          rpFL13  (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     rpFL13-10xHis  (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
                       601                                                    660
          DSM13  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
          rpET13  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
        rpET8785  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
        rpFL8785  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   rpFL8785-10xHis  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
          rpFL13  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     rpFL13-10xHis  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
                       661                                                    720
          DSM13  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
          rpET13  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
        rpET8785  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
        rpFL8785  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   rpFL8785-10xHis  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
          rpFL13  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     rpFL13-10xHis  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
                       721                                                    780
          DSM13  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
          rpET13  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
        rpET8785  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
        rpFL8785  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   rpFL8785-10xHis  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
          rpFL13  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     rpFL13-10xHis  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
                       781                                                    840
          DSM13  (781) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
          rpET13  (781) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
        rpET8785  (781) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
        rpFL8785  (694) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
   rpFL8785-10xHis  (694) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
          rpFL13  (694) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
     rpFL13-10xHis  (694) GCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
```

Figure 8. (Continued).

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                          841                                                   900
         DSM13    (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
         rpET13   (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
        rpET8785  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
        rpFL8785  (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
  rpFL8785-10xHis (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
         rpFL13   (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    rpFL13-10xHis (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
                          901                                                   960
         DSM13    (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
         rpET13   (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
        rpET8785  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
        rpFL8785  (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
  rpFL8785-10xHis (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
         rpFL13   (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    rpFL13-10xHis (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
                          961                                                  1020
         DSM13    (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
         rpET13   (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
        rpET8785  (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
        rpFL8785  (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  rpFL8785-10xHis (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
         rpFL13   (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
    rpFL13-10xHis (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
                         1021                                                  1080
         DSM13   (1021) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
         rpET13   (1021) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
        rpET8785  (1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
        rpFL8785  (934) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  rpFL8785-10xHis (934) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
         rpFL13   (934) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
    rpFL13-10xHis (934) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
                         1081                                                  1140
         DSM13   (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
         rpET13   (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
        rpET8785  (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
        rpFL8785  (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  rpFL8785-10xHis (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
         rpFL13   (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
    rpFL13-10xHis (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
                         1141                                                  1200
         DSM13   (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
         rpET13   (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
        rpET8785  (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
        rpFL8785  (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
  rpFL8785-10xHis (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
         rpFL13   (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
    rpFL13-10xHis (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
                         1201                                                  1260
         DSM13   (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
         rpET13   (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
        rpET8785  (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
        rpFL8785  (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
  rpFL8785-10xHis (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
         rpFL13   (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
    rpFL13-10xHis (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
                         1261                                                  1320
         DSM13   (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
         rpET13   (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
        rpET8785  (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
        rpFL8785  (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
  rpFL8785-10xHis (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
         rpFL13   (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
    rpFL13-10xHis (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
```

Figure 8. (Continued).

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                    1321                                                  1380
          DSM13 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
          rpET13 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
        rpET8785 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
        rpFL8785 (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  rpFL8785-10xHis (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
          rpFL13 (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    rpFL13-10xHis (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
                    1381                                                  1440
          DSM13 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
          rpET13 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
        rpET8785 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
        rpFL8785 (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  rpFL8785-10xHis (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
          rpFL13 (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    rpFL13-10xHis (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
                    1441                                                  1500
          DSM13 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
          rpET13 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
        rpET8785 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
        rpFL8785 (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  rpFL8785-10xHis (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
          rpFL13 (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    rpFL13-10xHis (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
                    1501                                                  1560
          DSM13 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGA------------------------
          rpET13 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA-
        rpET8785 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA-
        rpFL8785 (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC------------------
  rpFL8785-10xHis (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGACCACCATCACCATCACCAT
          rpFL13 (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC------------------
    rpFL13-10xHis (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGACCACCATCACCATCACCAT
                    1561    1572
          DSM13 (1537) ------------
          rpET13 (1560) ------------
        rpET8785 (1560) ------------
        rpFL8785 (1456) ------------
  rpFL8785-10xHis (1474) CACCATCATCAC
          rpFL13 (1456) ------------
    rpFL13-10xHis (1474) CACCATCATCAC
```

Figure 8. (Continued).

Figure 9. The secondary structure of alpha-amylases.

DSM13; Native alpha-amylase from *B. licheniformis* DSM13, rpET and

rpET8785; Recombinant alpha-amylase from *B. licheniformis* DSM 13 and

DSM8785, repectively, by using pET21d (+) system, rpFL13 and

rpFL8785; Recombinant alpha-amylase from *B. licheniformis* DSM 13 and

DSM8785, repectively, by using pFLAG-CTS system. Symbols α or ℓℓℓ;

Alpha-helix, β; Beta-sheet, TT; Turn helix, Red color; Same sequences,

Yellow color; Different sequences. The overall structure was shown in

Table 5.

β15 α9 α10 β16 β17
DSM13
380   390   400   410   420   430   440

DSM13	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpET13	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpET8785	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpFL13	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpFL8785	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpFL13_10His	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
rpFL8785_10His	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT
consensus>50	FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWT

β18 β19 η4 β20 β21 β22
DSM13
450   460   470   480   490   500

DSM13	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpET13	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpET8785	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpFL13	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpFL8785	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpFL13_10His	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
rpFL8785_10His	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG
consensus>50	REGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGG

β23
DSM13
510

DSM13	SVSIYVQR............
rpET13	SVSIYVQRLEHHHHHH....
rpET8785	SVSIYVQRLEHHHHHH....
rpFL13	SVSIYVQRLD..........
rpFL8785	SVSIYVQRLD..........
rpFL13_10His	SVSIYVQRLDHHHHHHHHHH
rpFL8785_10His	SVSIYVQRLDHHHHHHHHHH
consensus>50	SVSIYVQRldhhhhhh....

Figure 9. (Continued).

5.2 SDS-PAGE and zymogram analysis of recombinant alpha-amylases

The expression of alpha-amylases from two expression vectors; rpFL13-10xHis and rpFL8785-10xHis were optimaized under induction of 1mM IPTG as final concentration at 25^0C for 18 hr (Figure 10). The cell lystate of *E. coli* was used as crude enzyme. The crude enzyme showed big band of alpha-amylase on SDS-PAGE analysis. At 0 hr of induction, the small band of recombinant alpha-amylase rpFL13-10xHis and rpFL8785-10xHis (red arrow, Land 2 and 3) could be found. The biggest band of recombinant alpha-amylases amylase rpFL13-10xHis and rpFL8785-10xHis (red arrow, Land 4 and 5) were observed after 18 hr of induction. The effective purification by 10 histidine sequence at C-terminal of alpha-amylase on nickel column

showed 2 clear bands of purified alpha-amylase on gel (land 6 and 7). However, unexpected large complex of alpha-amylase was also found. Confirmation of activity of purified alpha-amylase was done by zymogram and two clear bands (white arrow) as results of starch hydrolysis on starch gel. However, the brighter-white bands of both recombinant alpha-amylases were observed. It was possible that recombinant alpha-amylase was bind to starch molecule on the gel and it was accumutated in that position, resulting in observation of starch hydrolysis as brighter-white clearing band. Therefore, the fusion of recombinant alpha-amylases with multiple 10 histidine sequences led to effective purification by nickel. Moreover, 10 histidine sequences did not inhibit the activity of alpha-amylase, which the observation of hydrolysis of starch could be found in polyacrylamide gel containing 0.2% (w/v) soluble starch. The efficience of purification by using 6 or 10 histidine sequences were observed in order to purify alpha-amylase and kinase, respectively (Belin et al., 2006; Hmidet et al., 2008). Molecular weight of recombinant alpha-amylases rpFL13-10xHis and rpFL8785-10xHis were estimated by *Rf* value on SDS-PAGE gel. The approximately MW was 56 kDa. The molecular weight was same as other alpha-amylase from thermophilic counterpart *B. licheniformis* (Khajeh et al., 2006). However, the MW of recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were calculated by using equation (http://www.scripps.edu/~cdputnam/protcalc.html). Approximately MW was about 58 kDa. This value was close to molecular weight, which was calculated by using relation between R_f value and molecular weight of protein marker. Moreover, thermostable alpha-amylase from newly isolated *B. licheniformis* NH1 was purified by Sephadex G-100 gel filtration and Q anion exchange chromatography. Results showed 58 kDa of purified alpha-amylase on SDS-

PAGE (Hmidet et al., 2008). However, the previous report showed the 65 kDa of alpha-amylase from other *B. licheniformis.* (I. C. Kim et al., 1992; Richardson T.H. et al., 2002). Moreover, molecular masses of 28, 22.5 amd 23.5 kDa, were also reported from *B. licheniformis* CUMU 305 (Krishnan and Chandra, 1983), *B. licheniformis* 584 (Saito, 1973), and *B. licheniformis* BLM 1777 (Chiang et al., 1979), respectively.
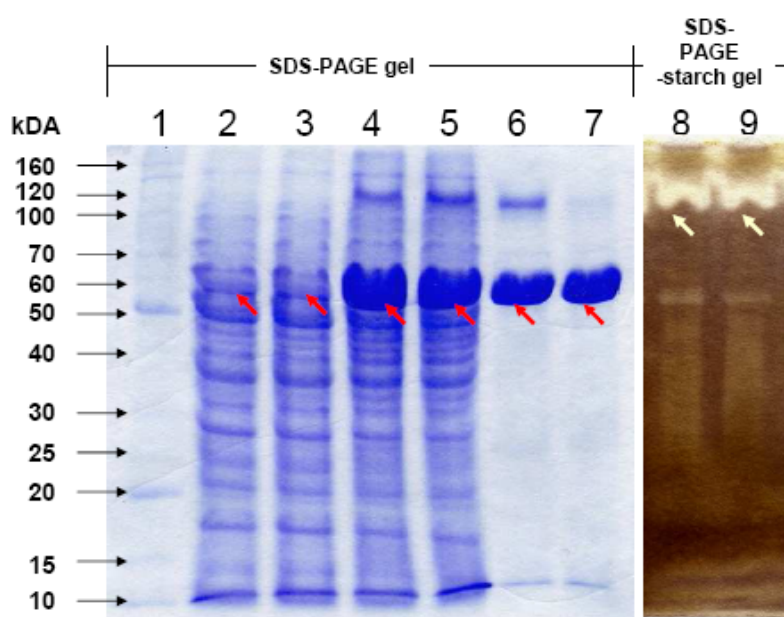
Figure 10. SDS-PAGE and zymogram analysis of purified alpha-amylase.

Land 1: molecular weight protein marker.

Land 2: rpFL13-10xHis at 0 hr of induction (9 μg).

Land 3: rpFL8785-10xHis at 0 hr of induction (9 μg).

Land 4: rpFL13-10xHis at 18 hr of induction (41 μg).

Land 5: rpFL8785-10xHis at 18 hr of induction (41 μg).

Land 6: purified alph-amylase from rpFL13-10xHis (28 μg).

Land 7: purified alpha-amylase from rpFL8785-10xHis (28 μg).

Land 8: activity of purified alpha-amylase from rpFL13-10xHis (6 μg).

Land 9: activity of purified alpha-amylase from rpFL8785-10xHis (6 μg).

5.3 Characterization of alpha-amylase and kinetic analysis

The effects of pH and temperature against activities and stabilization of purified alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were analyzed under presence or absence of 2mM $CaCl_2$ by using soluble starch as substrate. The pH-activity profile and temperature activity profile were shown in Figure 11 and 13, respectively. Moreover, the effects of pH and temperature on activity of both recombinant alpha-amylases were studied and the profiles were shown in Figure 12, 14 and 15.
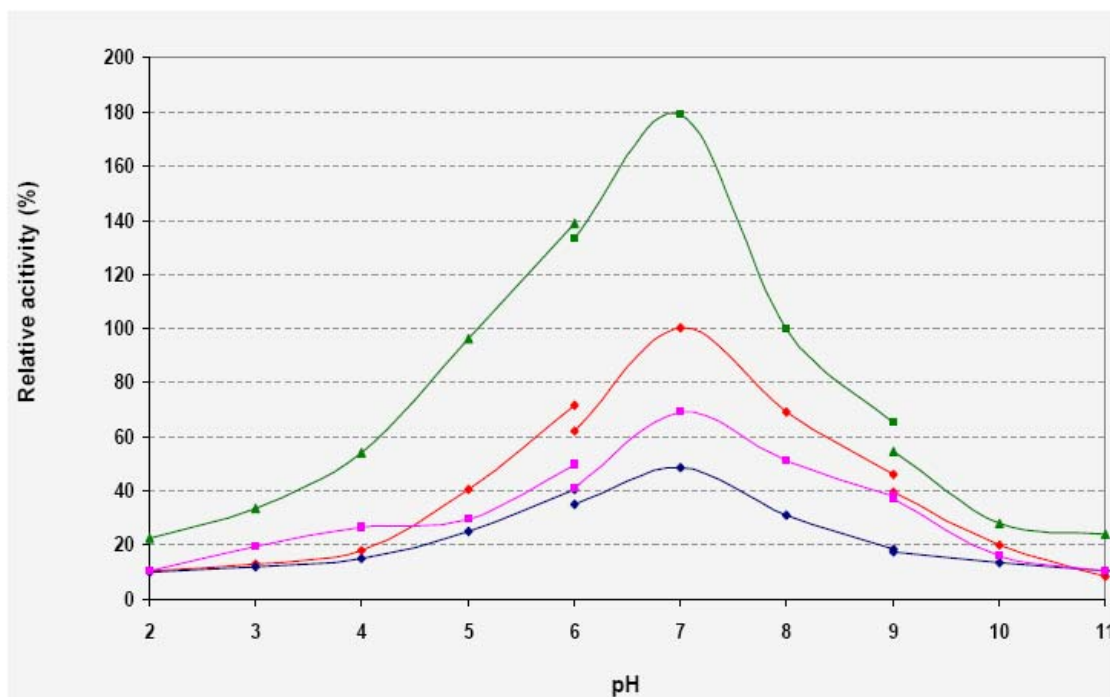
Figure 11. The pH profiles of two recombinant wild type alpha-amylases.

The Y axis was the (%) relative activity while X axis was pH values.

Symboles ▨ : relative activity of rpFL8785-10xHis plus 2mM $CaCl_2$, ▨ :

relative activity of rpFL8785, ◆ : relative activity of rpFL13-10xHis plus

2mM $CaCl_2$, and ◆ : relative activity of rpFL13-10xHis.

The optimal pH of both purified recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were determined with/without addition of 2 mM $CaCl_2$ (Figure 11). All percentages of relative activities were calculated by based on activity of purified recombinant alpha-amylase rpFL13-10xHis under non-addtion of 2mM $CaCl_2$. The purified alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were highly active under addition of calcium ions. However, the activity of both recombinant alpha-amylases were active without calcium ions in the assay. The results suggested that calcium ions enhanced the activity of both alpha-amylase rpFL13-10xHis and rpFL8785-10xHis at all conditions of pH assay. (Violet and Meunier, 1989). The optimal pH of both recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were at pH 7 as shown in Figure 11. The previous reports were studied the effect of pH on activity of *B. licheniformis* alpha-amylase. The optimal pH of this alpha-amylase was at pH 6 (Richardson T.H. et al., 2002) whereas the commercial alpha-amylase from *B. licheniformis* (Termamyl 300 L Type DX) had optimal pH at pH 7.5 in starch hydrolysis by using soluble starch as substrate (Bravo Rodriguez et al., 2006). The activities of two alpha-amylases rpFL13-10xHis and rpFL8785-10xHis increased when pH conditions of analysis were increased from pH 2 to 7. On another hand, the activity of both recombinant alpha-amylases rpFL13-10xHis and rpFL8785-10xHis were dramatically decreased when pH condition of assay were increased from pH 8 to 11. However, the activity was not detected when the pH condition of assay was lower or higher than pH 4 and pH10, respectively.

The effect of pH on stability of two recombinant alpha-amylase was studied at three different ranges of pH values by incubating enzymes at 37°C, for 30 min with or without calcium ions in the stability assay (Figure 12). The 85% and 75% of

relative activity of alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis were remained after incubation at pH 7 at 37°C for 30 min, without addition of $CaCl_2$. However, the remained activity of alpha-amylases rpFL13-10xHis and rpFL8785-10His were not detected under strong acidic or alkaline condition, even in the addition of calcium ions. The other research suggested that activity of thermostable *B. licheniformis* NH1 alpha-amylase was not stimulated by the presence of calcium ion (Hmidet et al., 2008). This alpha-amylase was highly active over a wide range of pH from 5.0 to 10.0. The relative activities at pH 5.0, 9.0 and 10.0 were about 89, 96.6 and 90%, of that at pH 6.5, respectively

Figure 12. The effect of pH on stability of two recombinanat alpha-amylases

rpFL13-10xHis and rpFL8785-10xHis at 37°C for 30 min.

The Y axis was the (%) relative activity while X axis was stability

condition at different pH values. Symboles ■ : relative activity of

rpFL8785-10xHis plus 2mM $CaCl_2$, ■ : relative activity of rpFL8785,

◆ : relative activity of rpFL13-10xHis plus 2mM $CaCl_2$, and

◆ : relative activity of rpFL13-10xHis.

Figure 13. Temperature profiles of recombinant wild type alpha-amylases**.**
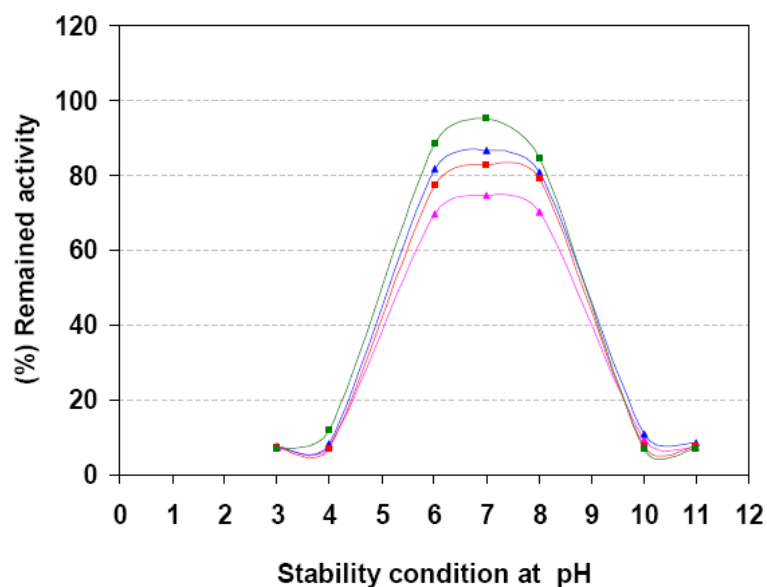
The Y axis was the (%) relative activity while X axis was temperature conditions. Symboles ■: relative activity of rpFL8785-10xHis plus 2mM CaCl$_2$, ■ : relative activity of rpFL8785, ◆: relative activity of rpFL13-10xHis plus 2mM CaCl$_2$, and ◆: relative activity of rpFL13-10xHis.
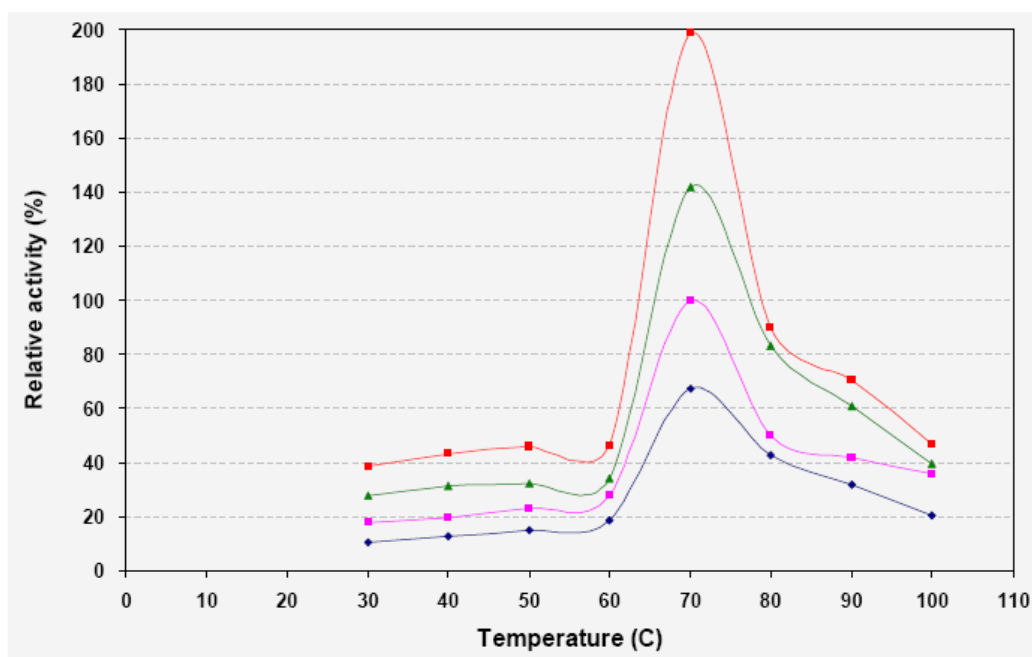
The efftects of temperature on activity and stabilization of alpha-amylases were observed as shown in Figure 13 and 14, respectively. The purified alpha-amylases rpFL13-10xHis and rpFL-10xHis were highly active at temperature of assay at 70°C (Figure 13), especially in the addition of 2 mM CaCl$_2$. The activity was dramatically increased. The temperature-activity profile of two recombinant alpha-amylases showed an optimum temperature at 70°C. The optimal condition was nearly to condition of themal alpha-amylase, which was 80°C (Violet and Meunier, 1989).

The newly isolated alpha-amylase from *B. licheniformis* had optimum temperature of the purified enzyme at 90°C (Hmidet et al., 2008). Moreover, optimal temperature of alpha-amylase from *B. licheniformis* CUMC305 was 91°C under alkaline condition (pH 9) (Kindle, 1983) The alpha-amylase from rpFL13-10xHis showed higher percentage of relative activity than alpha-amylase from rpFL8785-10xHis at all temperatures. The addition of CaCl₂ in activity assays showed the same relationship to effects of pH on activity of alpha-amylase. The enhancement of activity was occurred under presence of calcium ions.
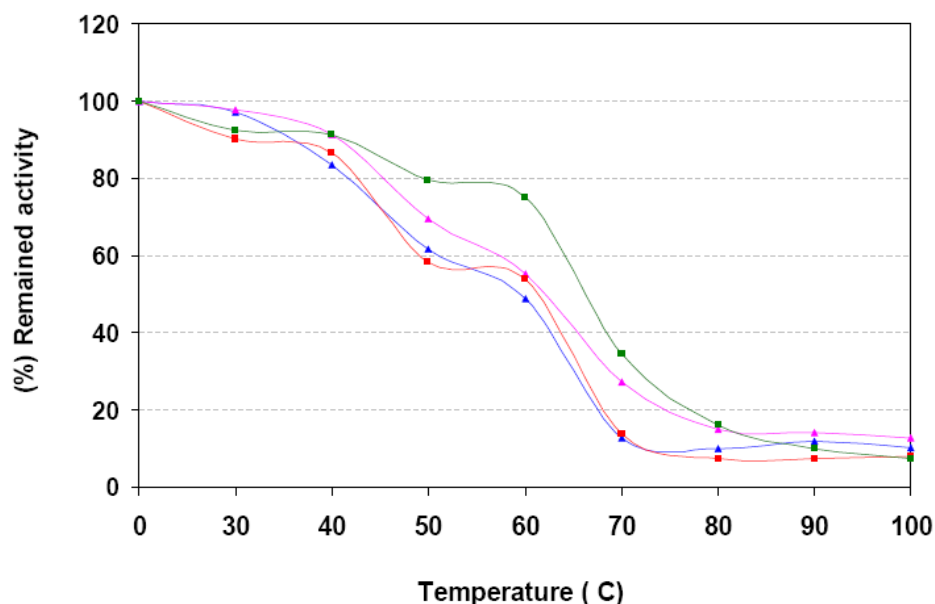
Figure 14. The effect of temperature on stability of two recombinanat alpha-amylases rpFL13-10xHis and rpFL8785-10xHis for 30 min.
The Y axis was the (%) relative activity while X axis was temperature conditions. Symboles ▪: relative activity of rpFL8785-10xHis plus 2mM CaCl₂, ▪ : relative activity of rpFL8785, ◆: relative activity of rpFL13-10xHis plus 2mM CaCl₂, and ◆: relative activity of rpFL13-10xHis.

The stability of wild type alpha-amylases were observed at temperature from 30°C to 100°C for 30 min with or without addition of $CaCl_2$ (Figure14). Stability analysis at temperature over 80°C showed (%) of remained activity less than 20% for all purified recombinant alpha-amylases. The fifty percentage of remained activity was detected at temperature 60°C. This point was shown as half life of reconmbinant alpha-amylases from rpFL13-10xHis and rpFl8785-10xHis. The inactivation of alpha-amylase by temperature was studied from *B. licheniformis* alpha-amylase (Kilic and Ozbek, 2004). The results showed that 50% of residual alpha-amylase activity on stability analysis were detected for 35 and 25 min at temperature 50°C and 60°C, respectively. It seemed that the relative activity decreased as the temperature increased due to inhibitory effects of temperature. The previous report showed that alpha-amylase from hyperthermostable *B. licheniformis* had half life at 85°C for 5 min.

The effects of temperature on thermal stability of recombinant alpha-amylsaes from rpFL13-10xHis and rpFL8785-10xHis were determined by incubating recombinant alpha-amylases under four different conditions for 2 hr. The result showed over 70% of remained activity after incubation at 37°C for 2 hr under presence or absence of calcium ions. The 40% of its orginal activity was remained under incucbation at 60°C for 2 hr. Nevertheless, the activities of two alpha-amylases from rpFL13-10xHis and rpFl8785-10xHis were dramatically decreased after incubation at 75°C and 100°C for 15 min. It seemed that two recombinant alpha-amylases were not stable under high temperature, even under addition of $CaCl_2$ (Figure 15).
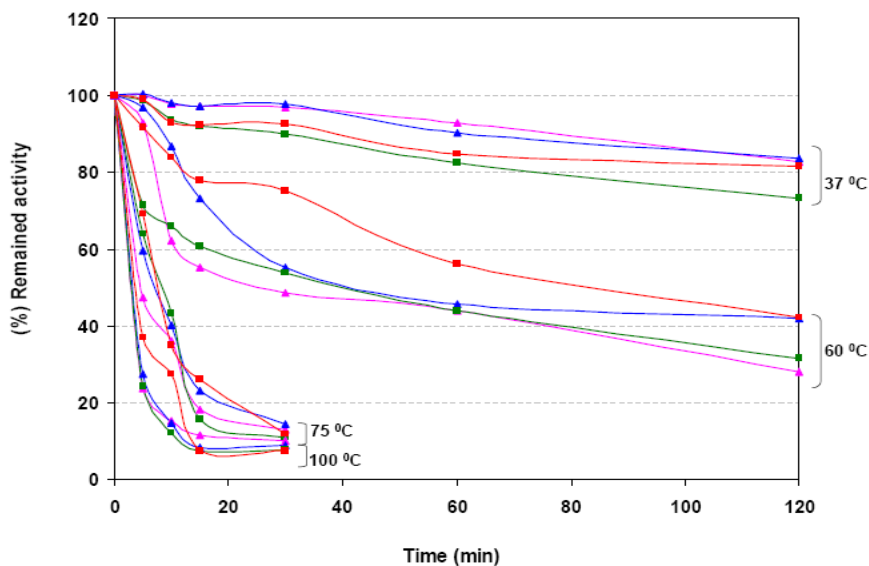
Figure 15. The effects of temperature on stability of two recombinanat alpha-

amylases rpFL13-10xHis and rpFL8785-10xHis for 120 min with

different sampling time.

The Y axis was the (%) relative activity while X axis was temperature

conditions. Symboles ▪: relative activity of rpFL8785-10xHis plus

2mM CaCl$_2$, ▪: relative activity of rpFL8785, ◆: relative activity of

rpFL13-10xHis plus 2mM CaCl$_2$, and◆: relative activity of rpFL13

10xHis.

The effect of calcium concentration on stability of alpha-amylase was

determined under high temperature. The results suggested that the presence of 5mM

calcium ions was the best concentration to stabilize the structure of alpha-amylase

under high temperature (90°C) (Hmidet et al., 2008). Moreover, the increase of

concentration of calcium ions over 5mM did not had effects on thermostability of

alpha-amylase (75 to 95°C) (Violet and Meunier, 1989). However, the properties of

alpha-amylases from *B. licheniformis* were various. Most alpha-amylase showed the

optimal temperature over 70°C whereas some alpha-amylases had optimal pH at 7 under presence of 4mM CaCl$_2$. The thermostability at 60°C and 70°C were analyzed. The results showed that enzyme had resistance at these conditions for 5 min. On the other hand, other alpha-amylase had 55% of remained activity after incubation at 80°C for 5 min (Kindle, 1983).

The kinetic parameters of purified alpha-amylases from both recombinants from rpFL13-10xHis and rpFL8785-10xHis were calculated by using Michaelis-menten, Lineweaver-burk and Edie-hofstee equations. GraphPad Prism program was used for drowing graph. However, Km values were calculated by Michaelis-menten and Lineweaver-burk equation resulting in variation in kinetic values (see appendix). Therefore, Eadie-Hofetee equation was used to evaluate all kineteic paremeters from two different substrates, which were commercial soluble starch and cassava starch from market. The parameters were summarized as shown in Table 4.

Table 4. Kinetic parameters of recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis.

Substrate	Kinetic parameters	Recombinant alpha-amylases	
		rpFL13-10xHis	**rpFL8785-10xHis**
Soluble starch	K_m (mg)	13.72	11.48
	V_{max} (μmol/min)	15.41	14.10
	k_{cat} (s^{-1})	96.99	89.78
	k_{cat}/K_m (s^{-1}/mg)	7.07	7.82
Cassava starch	K_m (mg)	9.25	8.85
	V_{max} (μmol/min)	12.79	12.88
	k_{cat} (s^{-1})	80.50	82.01
	k_{cat}/K_m (s^{-1}/mg)	8.70	9.27

The alpha-amylase rpFL13-10xHis showed higher reaction velocity than alpha-amylase rpF8785-10xHis by hydrolyzing soluble starch as substrate. This was inversely relation by hydrolyzing cassava starch. The better reaction velocity was found in hydrolization of soluble starch. They were 15.41 μmol.min^{-1} and 14.10 μmol.min^{-1} by recombinant rpFL13-10xHis and rpFL8785-10xHis, respectively. The comparison of Michaelis-menten constants suggested that alpha-amylase rpFL8785-10xHis was better specific binding to substrate than alpha-amylase rpFL13-10xHis same as the comparison of specific catalytic constants (k_{cat}/K$_m$) when using both cassava starch and soluble starch as substrate. The kinetic showed that both recombinant alpha-amylases were the best hydrolyzing enzyme by using cassava starch as substrate. The specific catalytic constants in the hydrolysis of cassava starch were higher than hydrolysis of soluble starch, which were 1.23 and 1.18 fold by using two recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis, respectively. However, these results were inversely related to component of starch. Natural starch consist of 2 main components in different ratios; amylose (10-20%) and amylopectic (80-90%) (Maarel et al., 2002). In case of cassava starch, 17% of amylose is found in the structure whereas 28% of amyloase is found in soluble starch (http://www.starch.dk/isi/starch/starch.htm). Because the main linkage in amylose is alpha-1,4-glycosidic bond (Maarel et al., 2002), it mean that the hydrolysis of soluble starch should be better than cassava starch by using alpha-amylase.

5.4 Library construction of shuffled alpha-amylases

The construction of two libraries of shuffled alpha-amylase genes for two expression vectors, pET21d (+) and pFLAG-CTS, were done under a similar condition. The DNA shuffling protocol was modified in order to optimize condition for alpha-amylases (Zhao and Arnold, 1997). Construction of shuffled alpha-amylases in pET21d (+) used rpET13 and rpET8785 as template, whereas rpFL13 and rpFL8785 were used as template for the 2nd library using pFLAG-CTS expression system. The secondary library of shuffled alpha-amylase was constructed after screening of the 1st library of shuffled alpha-amylase in pET21d (+) system was not successful. Since DNA sequence of *B. licheniformis* DSM13 and DSM8785 were highly similar. Thus, the diversity in nucleotide sequence was introduced by random mutagenesis of alpha-amylase gene by error prone PCR. (Figure 16-1 and 17-1). The addition of $MnCl_2$ into PCR amplification of alpha-amylase gene led to increase rate of mutation by using *Taq* DNA polymerase. The digestion of DNaseI (0.1-1.0 U/μg DNA) generated the poll of DNA fragment in randomly size (Figure 16-2 and 17-2). The previous research suggested that preparation of diversity of gene templates by plasmid digestion and addition of $MnCl_2$ in DNaseI digestion generated 95% of active clones (Zhao and Arnold, 1997). However, creation of diversity of genes was successful by error prone PCR under presence of $MgCl_2$ (Aubrey et al., 2008) or both $MgCl_2$ and $MnCl_2$ (Parikh and Matsumura, 2005). After DNaseI digestion, the fragments size in the range of 50-250 bp were purified by 15% (w/v) polyacrylamide gel and then, reassembled by PCR amplification without primers. The fragment size in rang from 50 to 300 bp was efficient to create full lengh of gene (Parikh and Matsumura, 2005) (Aubrey et al., 2008). On another hand, small fragment (< 50 bp)

was purified (Arnold and Moore, 1997). Recombination of short DNA fragments was occurred by crossover recombination. Moreover, shorter fragments size also acted as primers for PCR amplification by using longer fragments size as template. Reassembly reaction required *pfu* DNA polymerase to avoide additional A at 3'end, which could be generated by *Taq* DNA polymerase. The reassemble PCR by *pfu* DNA polymerase was better than reassemble reaction by using only *Taq* DNA polymerase (Arnold and Moore, 1997). The smear bands of reassembled products were observed on 1% (w/v) agarose gel (Figure 16-3 and 17-3). The addition of specific primers (3'-*BspH*I and 5'-*Xho*I or 3'-*EcoR*I and 5'-*Xho*I) into PCR amplification of shuffled alpha-amylase were performed to create corrected size of shuffled alpha-amylases. Approximately size of 1.5 kb of shuffled alpha-amylases that related to wild type alpha-amylase gene, was observed by 1% (w/v) agarose gel as showed in Figure 16-4 and 17-4. The shuffled products creating by primer set of 3'-*BspH*I and 5'-*Xho*I were cloned into pET21d (+) to construct the rpET-SH library same as the shuffled PCR products from amplification by primer set of 3'-*EcoR*I and 5'-*Xho*I, were inserted into pFLAG-CTS vector to create the rpFL-SH library for expression of shuffled alpha-amylases for further screening.
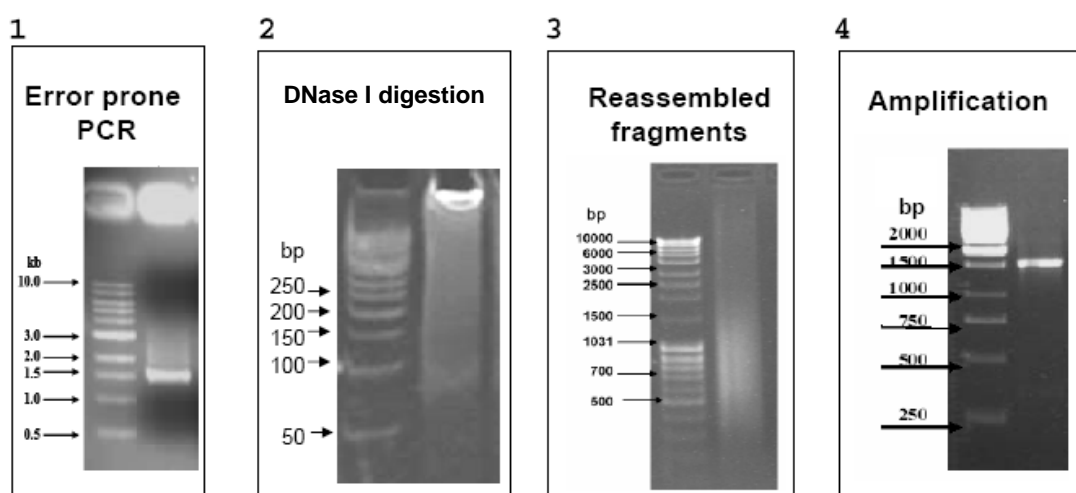
Figure 16. Construction of rpET-SH library of shuffled alpha-amylase.
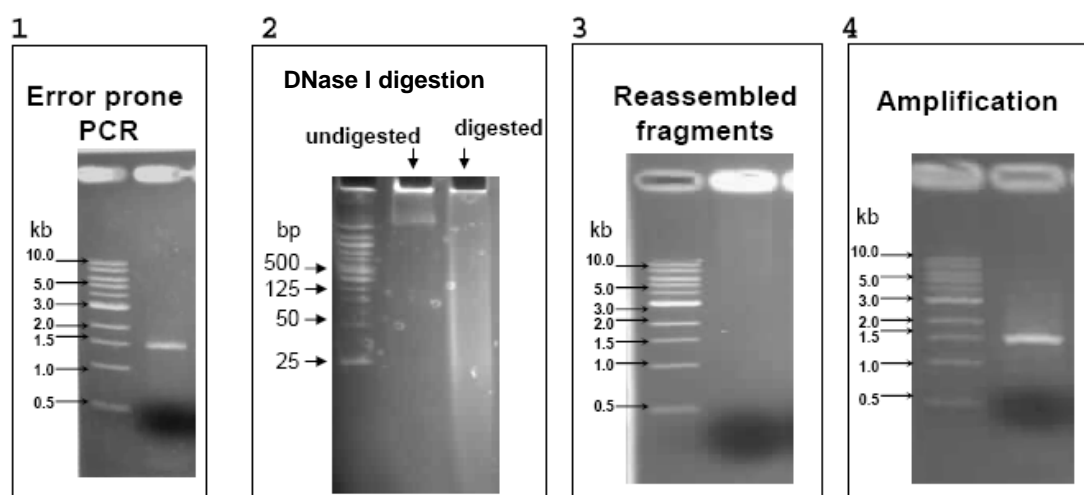
Figure 17. Construction of rpFL-SH library of shuffled alpha-amylase.

5.5 Library screening of shuffled alpha-amylases

5.5.1 Primary screening of library of shuffled alpha-amylase

5.5.1.1 Primary screening system of rpET-SH library

The expression of rpET-SH library of shuffled alpha-amylase gene in pET21d (+) vector system required the IPTG induction. The observation of starch hydrolysis on screening plate with KI was done for prescreening of other shuffled alpha-amylase. This methos was easy to do and kept the cost too low (Bessler et al., 2003). The colonies on transformation plate were replicated onto 2 plates. One plate was grown on LB agar and used as master plate, whereas another one was grown on LB agar containing 1% (w/v) of soluble starch and used as screening plate. Secretion of shuffled alpha-amylase outside the host cell using *Bacillus* signal peptide resulted in starch hydrolysis represented by clear zone around *E. coli* (Figure 18). The fusion of 6 histidine sequences to shuffled alpha-amylase gene was not inhibit the secretion system and activity of shuffled alpha-amylase (Khajeh et al., 2006). However, the bigger dimension of clearing zone on screening plate could not confirm the improvement of activity since the expression level of each host cell was not the same level. The 230 shuffled clones showing clear zone as the hydrolysis of soluble starch around *E. coli* host cells, were found from 5,000 transformants on screening plates. Thus, approximaltly 5% of positive clones were obtained from primary screening. Only shuffled alpha-amylase clones showing clear zone were selected for secondary screening.
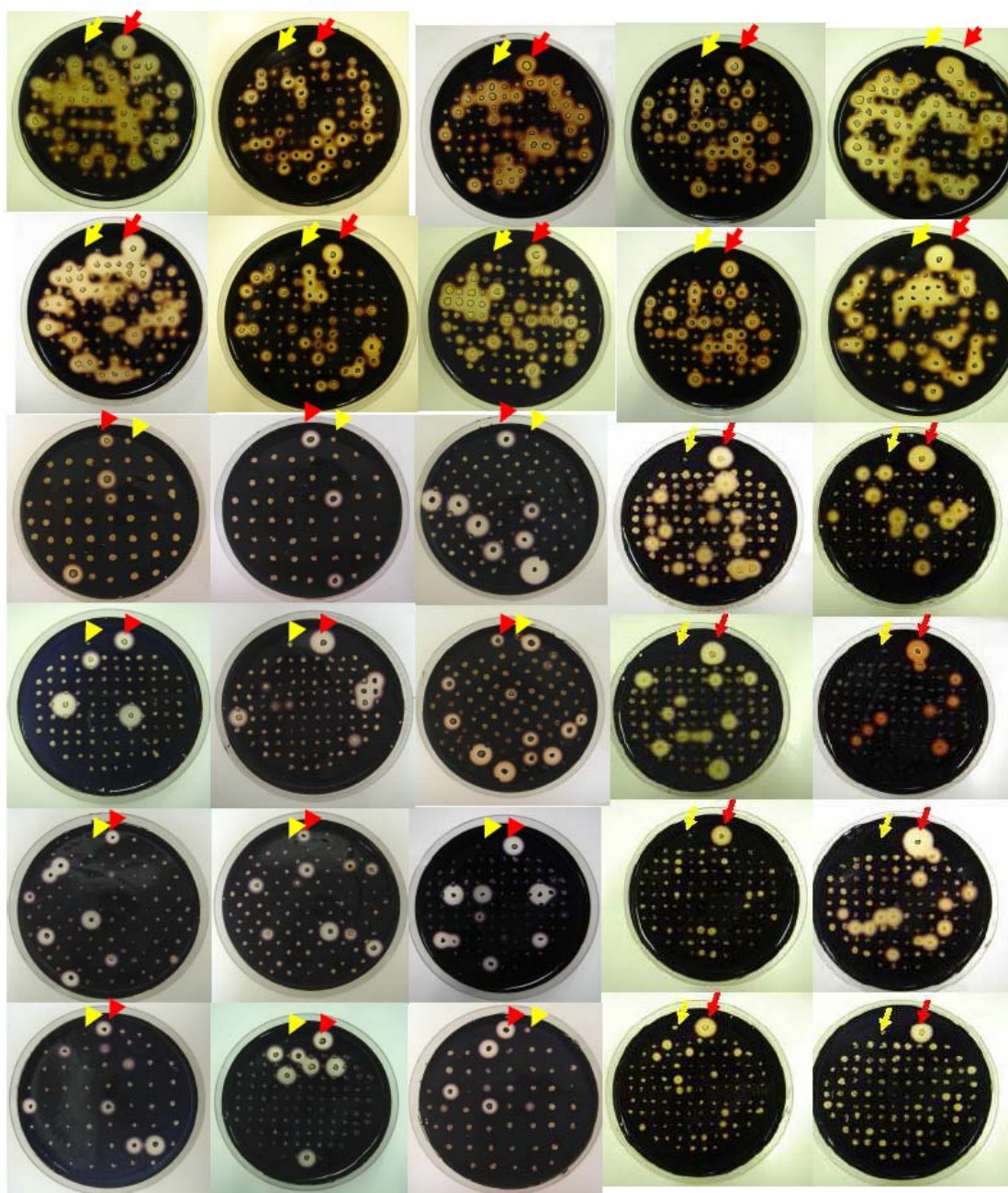
Figure 18. Primary screening of rpET-SH library of shuffled alpha-amylase on

screening plate under IPTG induction.

Red arrow head: rpET8785, yellow arrow head: empty pET21d (+).

5.5.1.2 Primary screening system of rpFL-SH library

Because pFLAG-CTS is a leakly vector, an expression of shuffled alpha-amylase was performed without IPTG induction after electroporation. This led to secretion of shuffled alpha-amylase by using *E. coli Omp*A signal peptide into its environment, resulting in an observation of digestion of soluble starch around *E. coli* host cells as clearing zone on screening plate after electroporation (Figure 19).
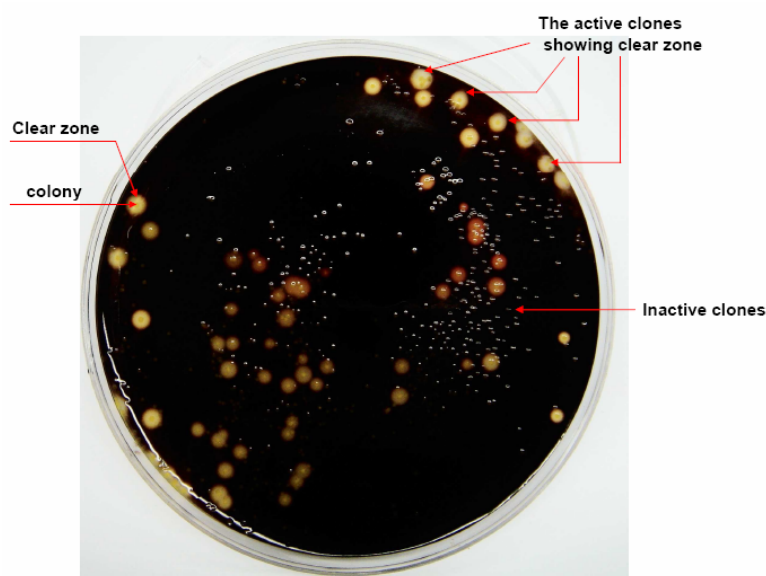
Figure 19. Primary screening of rpFL-SH library of shuffled alpha-amylase on

soluble starch plate without IPTG induction.

5.5.2 Secondary screening of library of shuffled alpha-amylase

Principle theory of secondary screening was based on colorimetric method by measuring absorbance of development color of reaction after hydrolysis of starch by alpha-amylase. The remaining starch in the reaction turned into black-blue color after addition of iodine solution whereas bright–yellow color showed complete conversion of starch to sugar monomers. Since the screening procedure is the most critical point in every directed evolution (Krammer et al., 2007), the screening of

E. coli mutant library by high throughput was undertaken for all secondary screening. The high throughput procedure was developed for many libraries such as library of beta-fucosidase (Parikh and Matsumura, 2005), alpha-amylase (Bessler et al., 2003), multogenic alpha-amylase (Aubrey et al., 2008), Hydroxynitrile lyases (Hnls) (Krammer et al., 2007), and etc.

5.5.2.1 Secondary screening system of rpET-SH library

The selected 230 clones from primary screening were obtained in secondary screening to select shuffled alpha-amylases, which showed higher activity than wild type alpha-amylase under high temperature at $90^{o}C$ for 10 min at 2 different pH values; pH 3.5 and pH 9.5. The screening condition at pH 6.5 was performed for control experiment. The reaction of starch hydrolysis was observed by developing color of reaction by iodine solution. The bright–yellow color of reaction showed better activity than brown color in hydrolysis. The results showed that some shuffled alpha-amylases had activity at pH 3.5 and 9.5 whereas the activity of rpET8785 was not detected under these conditions (Figure 20).
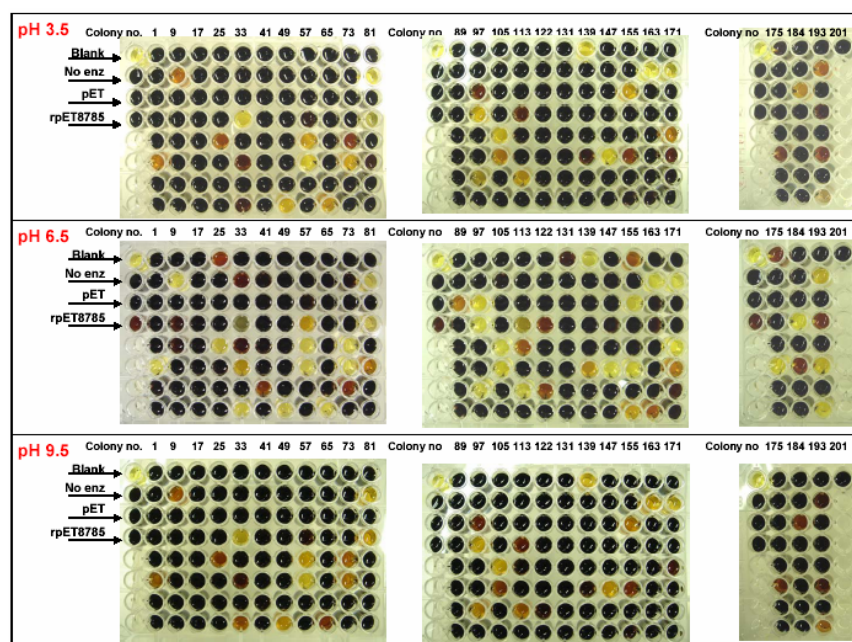
Figure 20. The starch hydrolysis under high temperature of shuffled alpha-amylase after development color by iodine.

The specific activity of all shuffled alpha-amylases and recombinant alpha-amylase rpET8785 were determined in all experiments. The base lines of activity of alpha-amylase rpET8785 were labled in green, blue and red color for activity at pH 3.5 and 9.5, respectively. Some of shuffled alpha-amylase showed the higher specific activity than specific activity of rpET8785 alpha-amylase under screening at pH 3.5 and 9.5. the results showed the improvement of actitity of shuffled alpha-amylase under acidic or alkaline condition. Although, the most of shuffled clones showed the lower specific activity than activity of recombinant rpET8785 at pH 6.5 (Figure 21).

To confirm these positive results, the second experiment was conducted in all conditions of screening (Figure 22). The subcultured of all shuffled alpha-amylasae

clones had performed by tooth picking from master plate. The subculture clones of shuffled alpha-amylases were grown on screening media and on normal LB agar for new master plate.
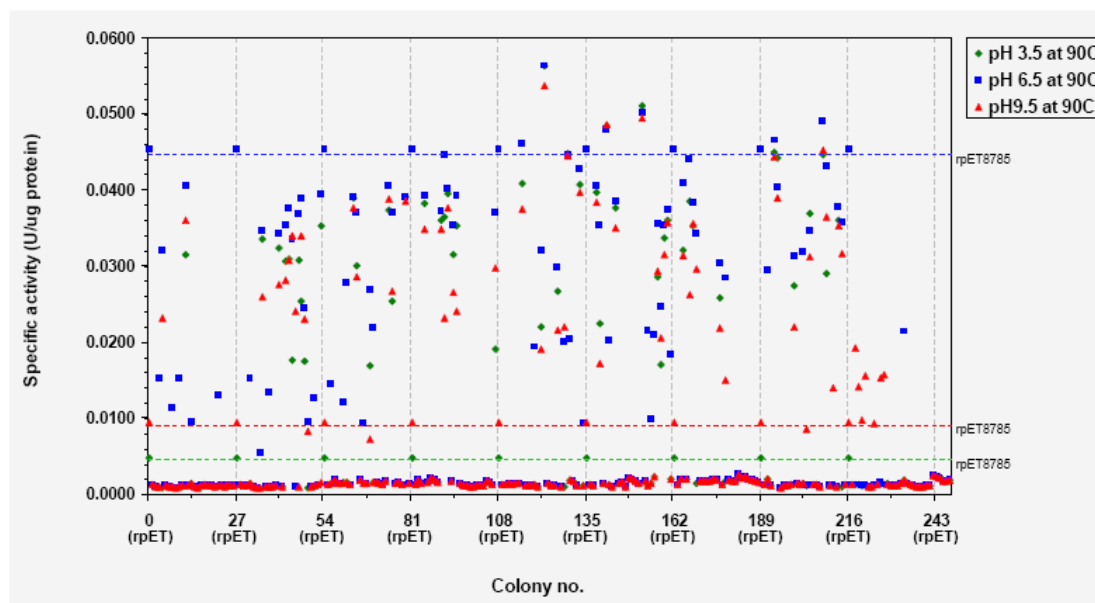
Figure 21. Specific activity of shuffled alpha-amylase in first experiment of

secondary screening.

Y axis was specific activity (U/µg protein) whereas X axis was colonly

no. Symbols . ◆ : specific activity at pH 3.5,■ : specific activity at pH

6.5, ▲ : specific activity at pH 6.5, ----, ----, ---- : activity level at pH 6.5,

3.5 and 9.5 of alpha-amylase from rpET8785, respectively.
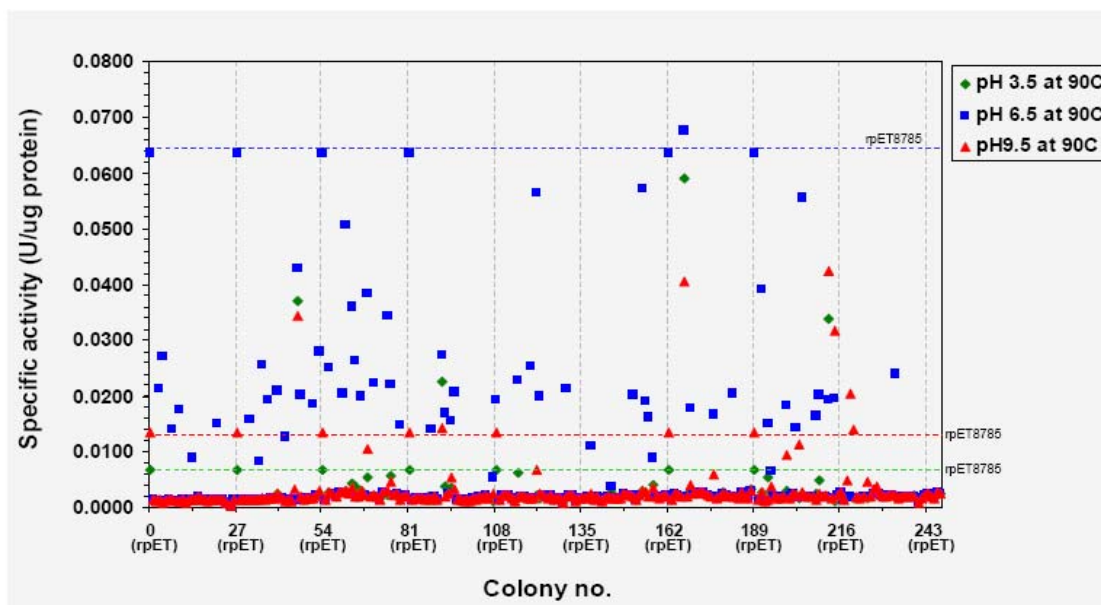
Figure 22. Specific activity of shuffled alpha-amylase in second experiment of

secondary screening.

Y axis was specific activity (U/μg protein) whereas X axis was colonly

no. Symbols . ◆ : specific activity at pH 3.5, ■ : specific activity at pH

6.5, ▲ : specific activity at pH 6.5, ----, ----, ---- : activity level at pH

6.5, 3.5 and 9.5 of alpha-amylase from rpET8785, respectively.

On the second experiment, the specific activity of shuffled alpha-amylase

(Figure 22) were decreased when compared values from 1st experiment of secondary

screening (Figure 21). Nevertheless, these values were lower than that of rpET8785

alpha-amylase. One possibility explaination was the expression level of host *E. coli*

BL21 (DE3). It seemed that expression level was decreased after re-cultivation on new

agar plate. Another reason was the reduction of plasmid stability that it was possible

gone if host cell was grown and kept on agar for a few weeks. However, activity of

some shuffled alpha-amylase clones could be observed at pH 3.5 and 9.5. These

clones were collected and named SHM43, SHM154, SHM197 and SHM199. The expression vectors of four shuffled clones were collected for analysis of nucleotide and amino acid sequences.

The third experiment was performed in test tube (10 ml). The observation of starch hydrolysis was deteced by using 3, 5-dinitrosalisylic acid. The reaction was done by using 1% (w/v) of cassava starch in 50mM potassium phosphate at pH 7. The analysis of four shuffled alpha-amylases (SHM43, SHM154, SHM197 and SHM199) were proformed at 90°C for 10 min. The experiment suggested that properties of shuffled alpha-amylase were not improved even at pH 6.5 (Figure 23).
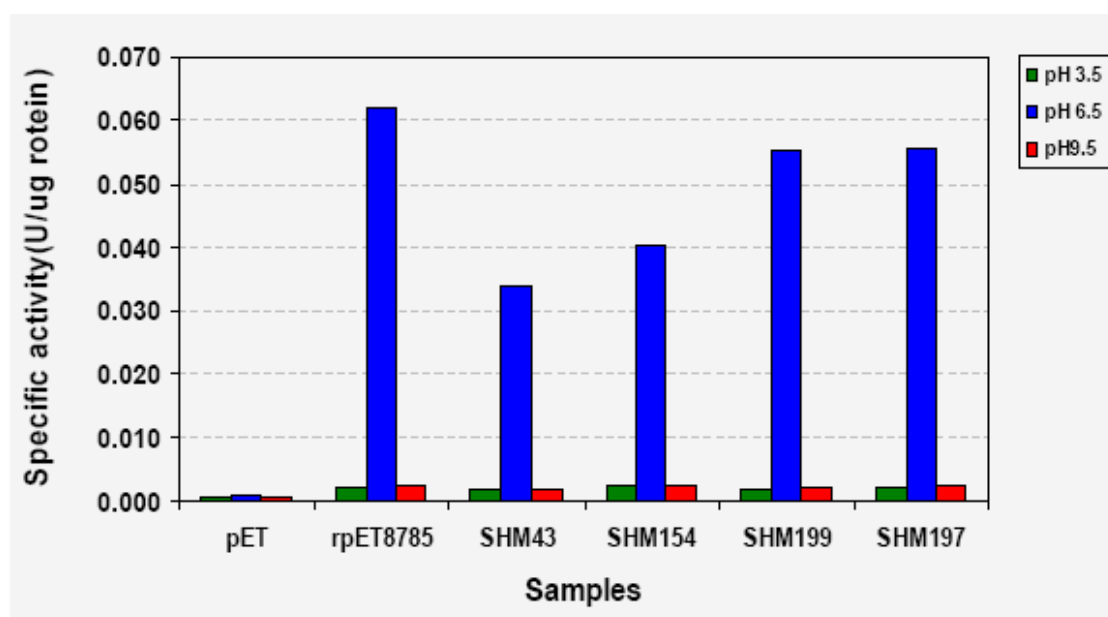
Figure 23. Specific activity of shuffled alpha-amylase in third experiment of

secondary screening**.**

Y axis was specific activity (U/µg protein) whereas X axis was colonly

no. Symbols . ◆ : specific activity at pH 3.5, ■ : specific activity at pH

6.5, ▲ : specific activity at pH 6.5.

Because the secondary screening of rpET-SH library was not successful, the new construction of shuffled alpha-amylase was done by using pFLAG-CTS as new expression vector. The pFLAG-CTS vector was more comfortable than rpET21d (+) vector because it could be used as cloning and expression vector.

5.5.2.2 Secondary screening system of rpFL-SH library

Primary experiments were analyzed to optimize conditions of secondary screening by using recombinant alpha-amylases from rpFL13 and rpFL8785 as positive control. The activity of clones carring original rpFLAG-CTS vector was used as negative control. Starch hydrolysis of recombinant alpha-amylases was determined under acidic conditions from pH 3 to 5. The thermostability analysis was done under high temperature ($75^{o}C$) at different sampling times (0, 30, 60, and 120 min) whereas the analysis of thermostability of recombinant alpha-amylases rpFL13 and rpFL8785 under presence of EDTA (0 mM, 0.25, 5 and 10 mM) was taken at $60^{o}C$ for 1 hr. The optimized conditions were summarized as shown in Figure 24. The acivities of both rpFL13 and rpFL8785 were not detected at under acidic condition (pH3), under thermostability at $75^{o}C$ for 2 hr, and under thermostability at $60^{o}C$ under presence of EDTA (10mM).
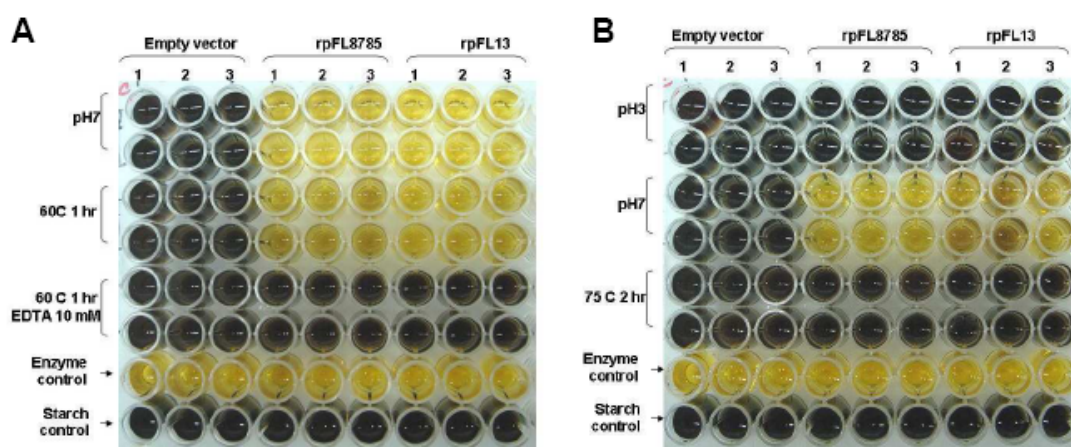
Figure 24. The starch hydrolysis of recombinant alpha-amylases under different optimized conditions for primary experiment.

Shuffled clones (1,100 clones) showing clear zone around colony on screening medium from primary screening, were conducted for secondary screening under 6 different conditions on 96 wells microtiter plate (Figure 25). All shuffled clones did not show the improve activity or thermostability from all screening assays. Only control experiment of screening (activity assay at pH 7, 37°C, for 30 min by using 0.025% (w/v) cassava starch as substrate), the activities of most shuffled clones were detected (Figure 26). Some shuffled clones showed the lower activity than wild type recombinant alpha-amylases from rpFL13 and rpFL8785, whereas the activity of most shuffled alpha-amylase clones were detected at the same level as activity of two wild types rpFL13 and rpFL8785 alpha-amylase.
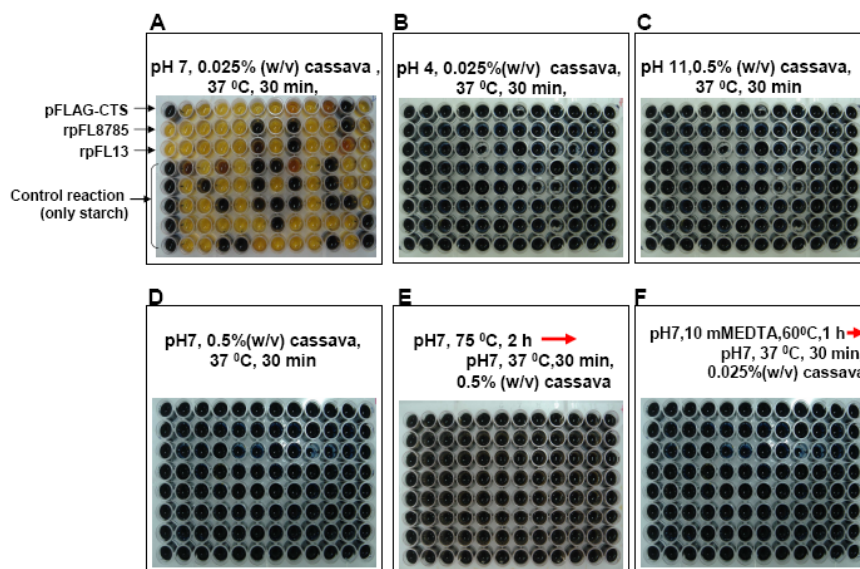
Figure 25. The starch hydrolysis of shuffled alpha-amylase under 6 different

condtions after development color with iodine.
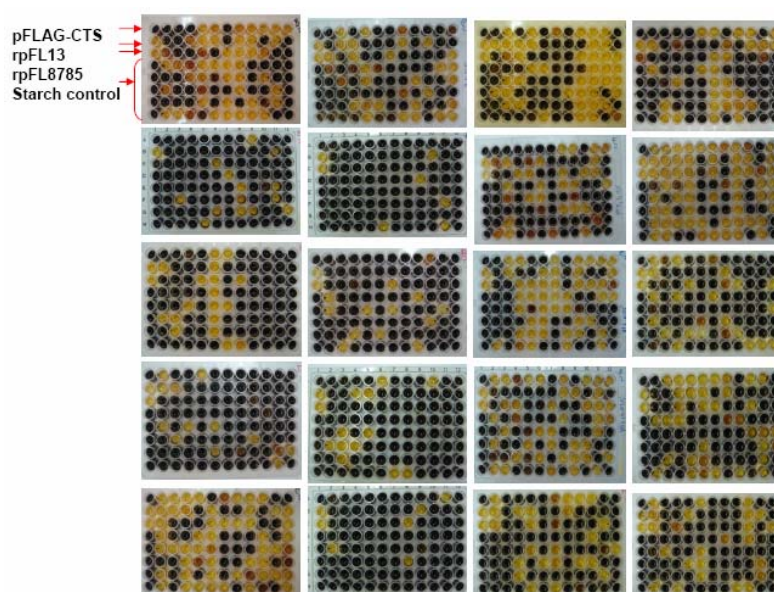
Figure 26. The starch hydrolysis of shiuffled alpha-amylase under control

expereiment at pH 7, 37 $^{\circ}$C for 30 min by using 0.025% (w/v) cassava

starch as substrate.

The two-dimensional plot according to enzyme activity was used in order to observe improvement of properties (Aubrey et al., 2008; Bessler et al., 2003). The activity (U/ml) of wild type clones and clones carring orginal vector were plotted and compared to activities of shuffled alpha-amylases. Mostly, activities of both wild type clones were separated distinctly from original vector. The activities of shuffled alpha-amylases were plotted and shown in blue color whereas activities of recombinant rpFL13 and rpFL8785 were shown in red color. The green color was the activity of original vector (pFLAG-CTS). The wild type-like clones or inactive shuffled clones were lied in the same position of wild types. The shuffled clone showing the better properties than wild types were lied separately from groups. The activity of shuffled alpha-amylases were determined and compared with activities of both recombinant alpha-amylases rpFL13 and rpFL8785 under all screening conditions. Two dimension plot of activity between 2 different conditions: activity at pH 4 and at pH 7. The activities of shuffled clons at neutral condition (at pH 7) were separated into 2 groups. The first group had activities in range of 0.012 to 0.018 U/ml, approximately. These activity values were near to activity of both wild type rpFL13 and rpFL8785 as shown in red color. Most the activities of shuffled enzymes were accumulated in second group, which activities were lower than 0.010 U/ml. The improvement of activity of shuffled alpha-amylase under acidic condition was not detected from all shuffled enzymes (Figure 27). Nevertheless, the recombinant alpha-amylases from rpFL13 and rpFl8785 had higher activity than the activity of shuffled alpha-amylases under acidic condition. However, it was hard to concern that activities of shuffled alpha-amylases were better or lower activity than recombinants rpFL13 and rpFL8785 since specific

activity was not reported. By plotting the activity values under alkaline and neutral condition (Figure 28), it was shown the different pattern from Figure 27. All activity patterns were not accumulated and distinctly separated between the active shuffled alpha-amylase clones and the wild type-like clones, even the pattern of activity of rpFL13 and rpFL8785 alpha-amylases. However, the separately group between acitivity of wild type clones and clones carrying empty vector was cleary. The activities of shuffled alpha-amylases were higher than activity of recombinant rpFL13 and rpFL8785 alpha-amylase from both conditions. However, it could be not confirm the improvement of activity because specific acitivity was not reported. Because the expression level of host cell was not the same expression level in each host cell, resulting in the non equal amount of enzyme in the reaction. However, the condition of screening under alkaline condition for shuffled alpha-amylases was also compared to screening condition from previous report (Bessler et al., 2003). It was slightly higher than previous study. The previous report suggested that the screening under alkaline condition (pH 10) was performed to screen mutant alpha-amylases creating by error prone PCR and DNA shuffling. The shuffled alpha-amylase showed 5 folds higher activity than its wild type at pH 10, even optimal pH was at pH 6. The many reasons should be recommended for screening under different pH conditions. The low buffer capacity of enzyme should be recommened for screening at different pH values. The host *E. coli* should not have mechanisms for the active secreation of other expressed proteins into media. These reasons avoid the alteration of pH values and interference of heterologous proteins during the secreening (Bessler et al., 2003).
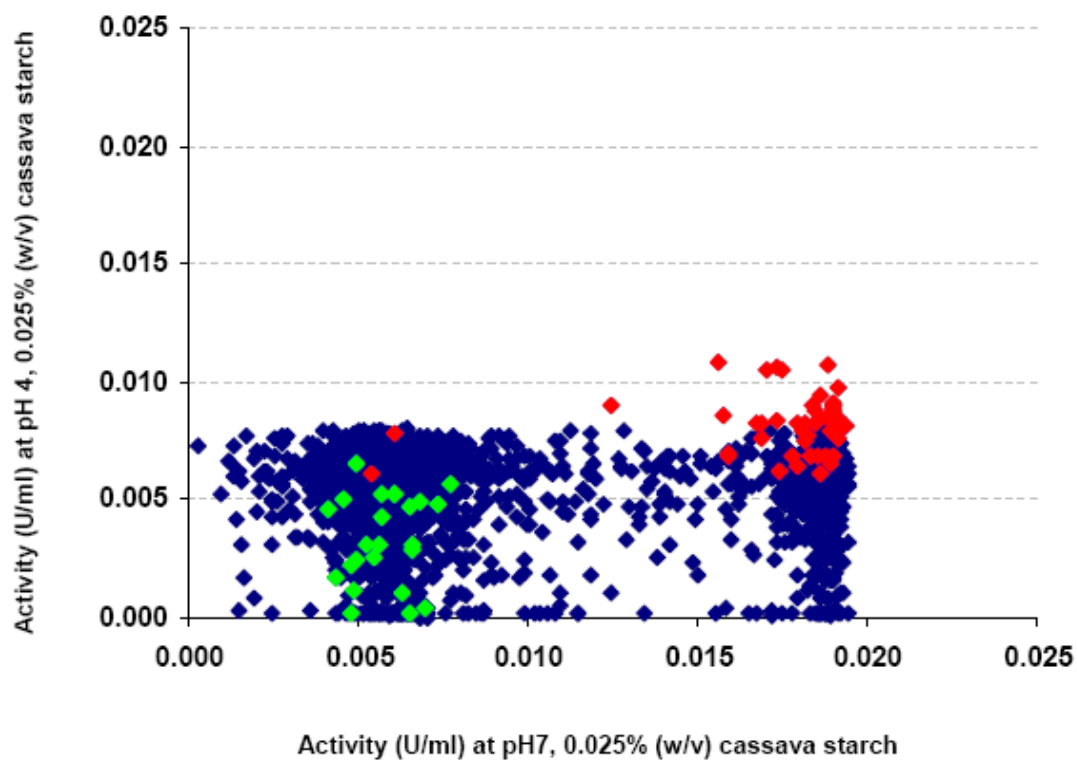
Figure 27. Relationship of shuffled alpha-amylase activity between condition at pH 3

and pH 7.

Y axis was activity (U/ml) by using 0.025% (w/v) of cassava starch as

substrate at pH 3, 37 $^{\circ}$C for 30 min.

X axis was activity (U/ml by using 0.025% (w/v) of cassava starch as

substrate at pH 7, 37 $^{\circ}$C for 30 min.

Symbols . ◆ : activities of shuffled alpha-amylases.

◆ : activities of both wild types and empty pFLAG-CTS.

◆ : activities of both recombinant alpha-amylase rpEF13 and

rpFL8785.
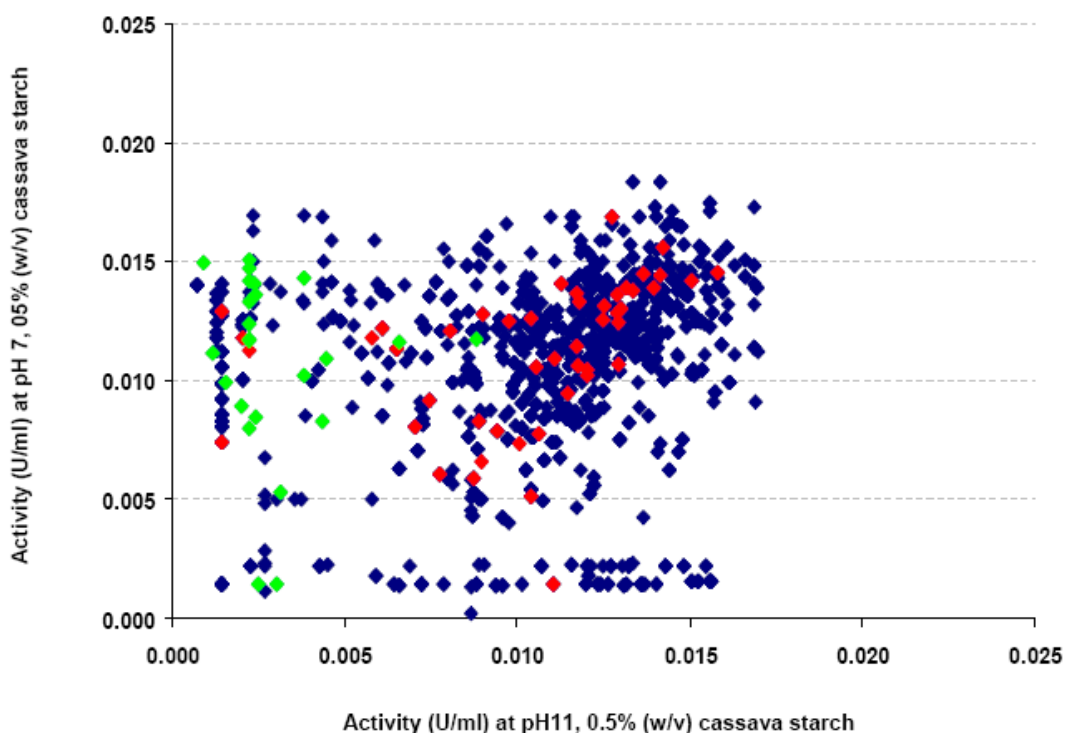
Figure 28. Relationship of shuffled alpha-amylase activity between condition at pH 7

and pH 11.

Y axis was activity (U/ml) by using 0.5% (w/v) of cassava starch as

substrate at pH 7, 37 $^{\circ}$C for 30 min.

X axis was activity (U/ml) by using 0.5% (w/v) of cassava starch as

substrate at pH 11, 37 $^{\circ}$C for 30 min.

Symbols ◆ : activities of shuffled alpha-amylases.

◆ : activities of both wild types and empty pFLAG-CTS.

◆ : activities of both recombinant alpha-amylase rpEF13 and

rpFL8785.
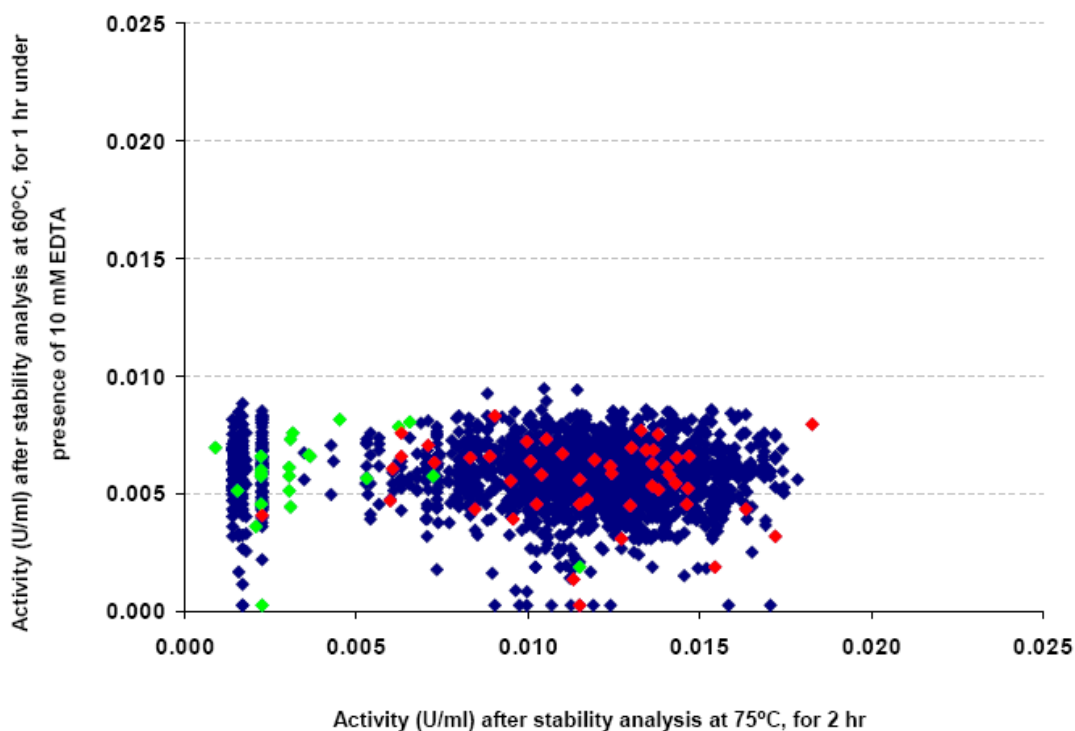
Figure 29. Relationship of shuffled alpha-amylase between stability condition under

high temperature or under presence of EDTA.

Y axis was activity (U/ml) after stability analysis at 60°C, for 1 hr under

presence of 10 mM EDTA. The activity was determined by using

0.025% (w/v) cassava starch as substrate at pH7, 37 °C for 30 min.

X axis was activity (U/ml) after stability analysis at 75°C, for 2 hr. The

activity was determined by using 0.5% (w/v) cassava starch as substrate

at pH 7, 37 °C for 30 min.

Symbols   ◆ : activities of shuffled alpha-amylases.

◆ : activities of both wild types and empty pFLAG-CTS.

◆ : activities of both recombinant alpha-amylase rpEF13 and

rpFL8785.

The analysis of thermostability was done to screen expected property of shuffled alpha-amylase under high temperature ($75^{o}C$) or under presence of 10 mM EDTA (Figure 29). The screening was done without addition of calcium ions into the reaction. To prevent the inhibition of activity by thermal denaturation, all enzymes were kept on ice immediately to cool down temperature for 15 min. The cooling on ice allowed the reversibly unfolded form to fold into native form of enzyme (Violet and Meunier, 1989). Two-dimension plot between both stability assay was not shown better property of shuffle alpha-amylases than property of wild types. Some previous reports suggested that inhibition of activity was detected by incubating alpha-amylase under different conditions. The incubation of alpha-amylase from *B. licheniformis* at $45^{o}C$, for 1 hr under presence of 1 mM EDTA resulting in the decrease of remained activity to 50% of original activity (Hagihara et al., 2001). The result was same as the incubation of alpha-amylase with 10 mM EDTA, resulting in completely inhibition of activity (H.-F. Lo et al., 2001). Moreover, the stability of enzyme under presence of EDTA upto 15 mM at $20^{o}C$, for 30 min was studied. The 15% of remained activity was determined (Hmidet et al., 2008). The activity of alpha-amylase was completely inhibited by EDTA, indicating the involvement of calcium ions in the enzyme function as shown in Figure 24 and 25. Other reports showed the success of thermostable screening. The thermostable screening of *Bacillus* library variants were done to screen thermostable of alpha-amylase by incubating at $80^{o}C$ of 25 min with addition of calcium into media. The 34 of variant clones were collected from 50,000 transformant clones in first round of DNA shuffling (Aubrey et al., 2008). The possibly reason was the absence of calcium in reaction, resulting in the unstable structure of alpha-amylase, which led to decrease in activity. Moreover, the presence

of calcium ion caused in decrease in activation entropy during thermal denaturation of thermophilic alpha-amylase, led to enhance stability (H.-F. Lo et al., 2001).

The improvement of properties of shuffled alpha-amylase was not successful with some possible reasons. The first was that the condition was probably too strong led to non-detection of small improved activity. Second reason was position of mutation in gene regions. If mutation was in conserved regions, it was possible to decrease or increase in activity and stability. However, some shuffled alpha-amylase genes were collected and analyzed the nucleotide and amino acid sequences. The collection of shuffled gene was based on activity under control experiment at pH 7, 37°C, for 30 min. The shuffled clones showing non activity or lower activity than their recombinant wild type enzyme were collected.

5.6 Multiple sequence alignment of shuffled alpha-amylase

Multiple alignment and analysis of secondary structure of shuffled alpha-amylase from two libraries, rpET-SH and rpFL-SH, were analyzed by Espript 2.2 program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA) and vector NTI software, respectively. The similarity of amino acid sequences was determined by ClustalW 2 from EMBL-EBI software on-line. The multiple alignment of complete and non-complete nucleotide sequences were analyzed as shown in Figure 30 and 31. The 4 and 10 of nucleotide sequences of shuffled alpha-amylase from rpET-SH and rpFL-SH library, respectively, were analyzed by based on sequences of recombinant alpha-amylases from rpET13, rpET8785, rpFL13 and rpFL8785. The 4 shuffled alpha-amylases from rpET-SH library appeared to be 100% and 99% of similarity to alpha-amylases from rpET8785 and rpET13, respectively. These were not precisely results to confirm that the construction of rpET-SH library was successful since the four

sequences of rpET-SH library were small population of samples. Shuffled no. 2b8b, 2a11h, and 4d2d from library of shuffled alpha-amylase (rpFL-SH) showed successfully nucleotide base shuffling between recombinant alpha-amylase genes from *B. licheniformis* DSM13 and DSM8785. The positions of shuffled nucleotide sequence were at 154, 488, and 1045 whereas positions of amino acid were at 6, 136, 240 and 322 of amino acid sequence. Moreover, the point mutations of nucleotide base by error prone PCR were found on ten nucleotide sequences of shuffled alpha-amylase from rpFL-SH library. These results suggested the creation of gene diversity by error prone PCR was successful in early step of DNA shuffling. The fidelity of error prone PCR could be controlled by altering the concentration of $MnCl_2$ coupled with an unbalanced mixture of nucleotides (Aubrey et al., 2008). Moreover, the shuffled points in nucleotides sequneces (Figure 30, 31) and amino acid sequences (Figure 32, and 33) could be confirmed the successful of library construction by DNA shuffling. However, the rate of mutation was not high because the percentage of similarity was 98 to 99% of similarity to all recombinant alpha-amylases rpET13, rpFT8785, rpFL13 and rpFL8785. DNA shuffling technique created one to six points of amino acid mutation of shuffled alpha-amylases. One point mutation of rpFL-SH library was found on shuffled no. 1b8b, 2d11h, 7c12h, and 7c4h. Five mutation points were occurred on 2e2a and 9g7d. The shuffled no. 2a11h had the highest mutation points on nucleotide sequence. Moreover, the termination codon (TAA) was occurred. Alteration of nucleotide sequences were occurred by changing between each purine or purimidine base, or by replacing of pyrimidine into purine base or *vice versa*.

```
                    1                                                        60
     1b8b     (1)  ------------------------------------------------------------
     rpET13   (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTC
     rpFL13   (1)  ------------------------------------------------------------
     2b8b     (1)  ------------------------------------------------------------
     rpET8785 (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTC
     SHM154   (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTC
     SHM199   (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTTATTTGCGCTCATCTTC
     SHM43    (1)  ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTC
     SHM197   (1)  ATGAAACAACAAAAACGGCTTTACGTCCGATTGCTGACGCTGTTATTTGCGCTCATCTTC
     rpFL8785 (1)  ------------------------------------------------------------
     1b9a     (1)  ------------------------------------------------------------
     1c5b     (1)  ------------------------------------------------------------
     2b7g     (1)  ------------------------------------------------------------
     2e2a     (1)  ------------------------------------------------------------
     2d11h    (1)  ------------------------------------------------------------
     7b3a     (1)  ------------------------------------------------------------
     7c12c    (1)  ------------------------------------------------------------
     7c4h     (1)  ------------------------------------------------------------
     9G7D     (1)  ------------------------------------------------------------
                    61                                                       120
     1b8b     (1)  -------------------GAATTCGCAAATCTTAAAGGGACGCTGATGCAGTATTTT
     rpET13  (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAAAGGGACGCTGATGCAGTATTTT
     rpFL13   (1)  ------------------------GCAAATCTTAAAGGGACGCTGATGCAGTATTTT
     2b8b     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     rpET8785(61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     SHM154  (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     SHM199  (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     SHM43   (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     SHM197  (61)  TTGCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     rpFL8785 (1)  ------------------------GCAAATCTTAATGGGACGCTGATGCAGTATTTT
     1b9a     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     1c5b     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     2b7g     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     2e2a     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     2d11h    (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     7b3a     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     7c12c    (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     7c4h     (1)  -------------------GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTT
     9G7D     (1)  -------------------GAATTCGAAAATATTAATGGGACGCTGATGCAGTATTTT
```

Figure 30. Multiple alignment of complete nucleotide sequences of shuffled alpha-amylase from rpFL-SH and rpET-SH library.

Sample SHM43, SHM154, SHM197, and SHM199 was shuffled alpha-amylase from rpET-SH library. The rest of samples were shuffled alpha-amylase from rpFL-SH library, rpFL13 and rpET13; recombinant *B. licheniformis* alpha-amylase DSM13, rpET8785 and rpFL8785; recombinant *B. licheniformis* alpha-amylase DSM8785. Blue color; conservative sequence, yellow color; different sequences.

```
              121                                                          180
1b8b      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
rpET13   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
rpFL13    (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
2b8b      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
rpET8785 (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM154   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM199   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM43    (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM197   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
rpFL8785  (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
1b9a      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
1c5b      (40)  GAATGGTACATGCCCAATGACGGCCGACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2b7g      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2e2a      (40)  GAATGGTATATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2d11h     (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7b3a      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7c12c     (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7c4h      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
9G7D      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
              121                                                          180
1b8b      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
rpET13   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
rpFL13    (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATAT
2b8b      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
rpET8785 (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM154   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM199   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM43    (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
SHM197   (121)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
rpFL8785  (34)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
1b9a      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
1c5b      (40)  GAATGGTACATGCCCAATGACGGCCGACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2b7g      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2e2a      (40)  GAATGGTATATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
2d11h     (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7b3a      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7c12c     (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
7c4h      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
9G7D      (40)  GAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATAT
              181                                                          240
1b8b     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
rpET13   (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
rpFL13    (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
2b8b     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
rpET8785 (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
SHM154   (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
SHM199   (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
SHM43    (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
SHM197   (181)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
rpFL8785  (94)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
1b9a     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
1c5b     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
2b7g     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
2e2a     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
2d11h    (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
7b3a     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
7c12c    (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
7c4h     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
9G7D     (100)  TTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAA
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Figure 30. (Continued).

```
             241                                                        300
      1b8b  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
     rpET13  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
     rpFL13  (154) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      2b8b  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
   rpET8785  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
    SHM154  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
    SHM199  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
     SHM43  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
    SHM197  (241) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
   rpFL8785  (154) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      1b9a  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      1c5b  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      2b7g  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCGTCAAAAAGGG
      2e2a  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAACAAGGG
     2d11h  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      7b3a  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      7c12c  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      7c4h  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
      9G7D  (160) GCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGG
             301                                                        360
      1b8b  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
     rpET13  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
     rpFL13  (214) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      2b8b  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
   rpET8785  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
    SHM154  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
    SHM199  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
     SHM43  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
    SHM197  (301) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
   rpFL8785  (214) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      1b9a  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      1c5b  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      2b7g  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      2e2a  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
     2d11h  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      7b3a  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      7c12c  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      7c4h  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
      9G7D  (220) ACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCAT
             361                                                        420
      1b8b  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
     rpET13  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
     rpFL13  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      2b8b  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
   rpET8785  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
    SHM154  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
    SHM199  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
     SHM43  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
    SHM197  (361) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
   rpFL8785  (274) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      1b9a  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      1c5b  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      2b7g  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      2e2a  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
     2d11h  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      7b3a  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      7c12c  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      7c4h  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
      9G7D  (280) TCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCG
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Figure 30. (Continued).

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                      421                                                          480
     1b8b    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
    rpET13    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
    rpFL13    (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     2b8b    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
  rpET8785    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
   SHM154    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
   SHM199    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
    SHM43    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
   SHM197    (421) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
  rpFL8785    (334) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     1b9a    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     1c5b    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     2b7g    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     2e2a    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
    2d11h    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     7b3a    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     7c12c    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     7c4h    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
     9G7D    (340) ACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGA
                      481                                                          540
     1b8b    (400) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
    rpET13    (481) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
    rpFL13    (394) GAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     2b8b    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAACACATACAGC
  rpET8785    (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
   SHM154    (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
   SHM199    (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
    SHM43    (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
   SHM197    (481) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
  rpFL8785    (394) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     1b9a    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     1c5b    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     2b7g    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     2e2a    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
    2d11h    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     7b3a    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     7c12c    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     7c4h    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
     9G7D    (400) GAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGC
                      541                                                          600
     1b8b    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
    rpET13    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
    rpFL13    (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     2b8b    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
  rpET8785    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
   SHM154    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
   SHM199    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
    SHM43    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
   SHM197    (541) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
  rpFL8785    (454) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     1b9a    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     1c5b    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     2b7g    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     2e2a    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGGCGAGTCCCGAAAGCTG
    2d11h    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     7b3a    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     7c12c    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     7c4h    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
     9G7D    (460) GATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTG
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Figure 30. (Continued).

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              601                                                    660
     1b8b  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   rpET13  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   rpFL13  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     2b8b  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
 rpET8785  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   SHM154  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   SHM199  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
    SHM43  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
   SHM197  (601) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
 rpFL8785  (514) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     1b9a  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     1c5b  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCATTGAAAACGGC
     2b7g  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     2e2a  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
    2d11h  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     7b3a  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     7c12c (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     7c4h  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
     9G7D  (520) AACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGC
              661                                                    720
     1b8b  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   rpET13  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   rpFL13  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     2b8b  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
 rpET8785  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   SHM154  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   SHM199  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
    SHM43  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
   SHM197  (661) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
 rpFL8785  (574) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     1b9a  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     1c5b  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     2b7g  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     2e2a  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
    2d11h  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     7b3a  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     7c12c (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     7c4h  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
     9G7D  (580) AACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAA
              721                                                    780
     1b8b  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   rpET13  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   rpFL13  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     2b8b  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
 rpET8785  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   SHM154  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   SHM199  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
    SHM43  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
   SHM197  (721) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
 rpFL8785  (634) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     1b9a  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     1c5b  (640) ATTAAAAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     2b7g  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     2e2a  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
    2d11h  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     7b3a  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     7c12c (640) ATTAAGAAATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     7c4h  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
     9G7D  (640) ATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGAT
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Figure 30. (Continued).

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                        781                                                                     840
      1b8b  (700) GCTGTCAAACACATTAAATTGTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
    rpET13  (781) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
    rpFL13  (694) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      2b8b  (700) GCTGTCAAACACATTAAATTGTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
  rpET8785  (781) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
    SHM154  (781) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
    SHM199  (781) GCTGTCAAACACATTAAATTTCTTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
     SHM43  (781) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
    SHM197  (781) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
  rpFL8785  (694) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      1b9a  (700) GCTGTCAAACCCATTAAATTTTCTTTTTTTGCGGGATTGGGTTAAACATGTCAGGGAAAAA
      1c5b  (700) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      2b7g  (700) GCTGTCAAACACATTAAATTTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      2e2a  (700) GCTGTCAAACACATTAAATTTTTTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
     2d11h  (700) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      7b3a  (700) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      7c12c (700) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      7c4h  (700) GCTGTCAAACACATTAAATCTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAA
      9G7D  (700) GCTGTCAAACACATTAAATTTTCTTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAGAA
                        841                                                                     900
      1b8b  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    rpET13  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    rpFL13  (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      2b8b  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
  rpET8785  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    SHM154  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    SHM199  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
     SHM43  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
    SHM197  (841) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
  rpFL8785  (754) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      1b9a  (760) ACGGGGAAGGAAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      1c5b  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      2b7g  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      2e2a  (760) ATGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
     2d11h  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      7b3a  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      7c12c (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      7c4h  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
      9G7D  (760) ACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAA
                        901                                                                     960
      1b8b  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    rpET13  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    rpFL13  (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      2b8b  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
  rpET8785  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    SHM154  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    SHM199  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
     SHM43  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
    SHM197  (901) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
  rpFL8785  (814) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      1b9a  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      1c5b  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      2b7g  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      2e2a  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
     2d11h  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      7b3a  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      7c12c (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      7c4h  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
      9G7D  (820) AACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAG
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Figure 30. (Continued).

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                961                                                           1020
   1b8b   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  rpET13  (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  rpFL13  (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   2b8b   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
 rpET8785 (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  SHM154  (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  SHM199  (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  SHM43   (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  SHM197  (961) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
 rpFL8785 (874) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   1b9a   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   1c5b   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   2b7g   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   2e2a   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
  2d11h   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   7b3a   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   7c12c  (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   7c4h   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
   9G7D   (880) TTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACG
                1021                                                          1080
   1b8b   (940) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
  rpET13 (1021) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
  rpFL13  (934) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
   2b8b   (940) GTCGTTTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCG
 rpET8785(1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  SHM154 (1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  SHM199 (1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  SHM43  (1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  SHM197 (1021) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
 rpFL8785 (934) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   1b9a   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   1c5b   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   2b7g   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   2e2a   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
  2d11h   (940) GTCGTTTCCAAGCATCCGTTGAAGTCGGTTACATTTGTCGATAACCATGATACACAGCCG
   7b3a   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   7c12c  (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   7c4h   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
   9G7D   (940) GTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCG
                1081                                                          1140
   1b8b  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  rpET13 (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  rpFL13  (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   2b8b  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
 rpET8785(1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  SHM154 (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  SHM199 (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  SHM43  (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  SHM197 (1081) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
 rpFL8785 (994) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   1b9a  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   1c5b  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   2b7g  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   2e2a  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
  2d11h  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   7b3a  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   7c12c (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   7c4h  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
   9G7D  (1000) GGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATT
```

Figure 30. (Continued).

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                 1141                                                    1200
     1b8b (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
   rpET13 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
   rpFL13 (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     2b8b (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
 rpET8785 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
   SHM154 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
   SHM199 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
    SHM43 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
   SHM197 (1141) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
 rpFL8785 (1054) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     1b9a (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     1c5b (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     2b7g (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     2e2a (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
    2d11h (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     7b3a (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     7c12c (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     7c4h (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
     9G7D (1060) CTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGA
                 1201                                                    1260
     1b8b (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
   rpET13 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
   rpFL13 (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     2b8b (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
 rpET8785 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
   SHM154 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
   SHM199 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
    SHM43 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
   SHM197 (1201) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
 rpFL8785 (1114) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     1b9a (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     1c5b (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     2b7g (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     2e2a (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
    2d11h (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     7b3a (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     7c12c (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     7c4h (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
     9G7D (1120) GACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGA
                 1261                                                    1320
     1b8b (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
   rpET13 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
   rpFL13 (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     2b8b (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
 rpET8785 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
   SHM154 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
   SHM199 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
    SHM43 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
   SHM197 (1261) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
 rpFL8785 (1174) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     1b9a (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     1c5b (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     2b7g (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     2e2a (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
    2d11h (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     7b3a (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     7c12c (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     7c4h (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
     9G7D (1180) AAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGG
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Figure 30. (Continued).

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                1321                                                        1380
    1b8b (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  rpET13 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  rpFL13 (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    2b8b (1240) ACAAGGGAAGGCGACAGCTCAGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
rpET8785 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  SHM154 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  SHM199 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
   SHM43 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
  SHM197 (1321) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
rpFL8785 (1234) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    1b9a (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    1c5b (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    2b7g (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    2e2a (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
   2d11h (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    7b3a (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
   7c12c (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    7c4h (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA
    9G7D (1240) ACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGA

                1381                                                        1440
    1b8b (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  rpET13 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  rpFL13 (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    2b8b (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
rpET8785 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  SHM154 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  SHM199 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
   SHM43 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
  SHM197 (1381) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
rpFL8785 (1294) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    1b9a (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    1c5b (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    2b7g (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    2e2a (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
   2d11h (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    7b3a (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
   7c12c (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    7c4h (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC
    9G7D (1300) CCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGAC

                1441                                                        1500
    1b8b (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  rpET13 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  rpFL13 (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    2b8b (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
rpET8785 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  SHM154 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  SHM199 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
   SHM43 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
  SHM197 (1441) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
rpFL8785 (1354) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    1b9a (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    1c5b (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    2b7g (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCGC
    2e2a (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
   2d11h (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    7b3a (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
   7c12c (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    7c4h (1360) ATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC
    9G7D (1360) ATTACCGGAATCCGTTCGGAGCCGGTCGTCATCAATTCGGAAGGCTGGGGAGAGTTTCAC

                1551                                          1559
    1b8b (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
  rpET13 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
  rpFL13 (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    2b8b (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
rpET8785 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
  SHM154 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
  SHM199 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
   SHM43 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
  SHM197 (1501) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAGCACCACCACCACCACCA
rpFL8785 (1414) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    1b9a (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    1c5b (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    2b7g (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    2e2a (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
   2d11h (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAGAGACTCGAC-----------------
    7b3a (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
   7c12c (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    7c4h (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
    9G7D (1420) GTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGACTCGAC-----------------
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Figure 30. (Continued).

```
              1                                                          60
1c12b      (1) GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
2a11h      (1) GAATTCGCAAATCTTAAAGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
2e5e       (1) GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
2a2b-full  (1) GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
2d11d      (1) GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
4b2d       (1) GAATTCGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
rpFL13     (1) ------GCAAATCTTAAAGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
rpFL8785   (1) ------GCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGAC
              61                                                        120
1c12b     (61) GGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
2a11h     (61) GGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
2e5e      (61) GGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
2a2b-full (61) GGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
2d11d     (61) GGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
4b2d      (61) GGCCAACATTGGAAGCGCTTGCAAAACGACGCGGCATATTTGGCTGAACACGGTATTACT
rpFL13    (55) GGCCAACATTGGAAGCGCTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
rpFL8785  (55) GGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACT
              121                                                       180
1c12b    (121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
2a11h    (121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
2e5e     (121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
2a2b-full(121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
2d11d    (121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
4b2d     (121) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
rpFL13   (115) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
rpFL8785 (115) GCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCT
              181                                                       240
1c12b    (181) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
2a11h    (181) TACGACCTTTATGATTTAGGGGAGTCTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
2e5e     (181) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
2a2b-full(181) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
2d11d    (181) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
4b2d     (181) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
rpFL13   (175) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
rpFL8785 (175) TACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGC
              241                                                       300
1c12b    (241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
2a11h    (241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
2e5e     (241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
2a2b-full(241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
2d11d    (241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
4b2d     (241) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
rpFL13   (235) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
rpFL8785 (235) ACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTAC
              301                                                       360
1c12b    (301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
2a11h    (301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
2e5e     (301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
2a2b-full(301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
2d11d    (301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
4b2d     (301) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
rpFL13   (295) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
rpFL8785 (295) GGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTT
```

Figure 31. Multiple alignment of non-complete nucleotide sequences of shuffled

alpha-amylase from rpFL-SH library.

The rpFL13 and rpET13; recombinant *B. licheniformis* alpha-amylase DSM13 in

pFLAG-CTS system and pET21d (+) system, the rpET8785 and rpFL8785;

recombinant *B. licheniformis* alpha-amylase DSM8785 in pFLAG-CTS system

and pET21d (+) system. Blue color; conservative sequences, green color;

block of similar sequences, yellow color; non-similar sequences.

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               361                                                          420
1c12b    (361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGG
2a11h    (361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCCAATTAAAGCCTGG
2e5e     (361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGG
2a2b-full(361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGG
2d11d    (361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGG
4b2d     (361) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCGAATTAAAGCCTGG
rpFL13   (355) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCGAATTAAAGCCTGG
rpFL8785 (355) GAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGG
               421                                                          480
1c12b    (421) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
2a11h    (421) ACACATTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2e5e     (421) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
2a2b-full(421) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
2d11d    (421) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
4b2d     (421) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
rpFL13   (415) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
rpFL8785 (415) ACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTGGTAC
               481                                                          540
1c12b    (481) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAA
2a11h    (481) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2e5e     (481) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTTCA
2a2b-full(481) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAA
2d11d    (481) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCCATAAGTTTCAA
4b2d     (481) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAA
rpFL13   (475) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAA
rpFL8785 (475) CATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAA
               541                                                          600
1c12b    (541) GGAAAGGCTTGGGATTGGGAAGTTTCCCATGAAAACCGCAACTATGATTATTTGATGTAT
2a11h    (541) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2e5e     (541) GGAAAGGCTTGGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2a2b-full(541) GGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
2d11d    (541) GGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
4b2d     (541) GGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
rpFL13   (535) GGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
rpFL8785 (535) GGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTAT
               601                                                          660
1c12b    (601) GCCGACATCGATTANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2a11h    (601) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2e5e     (601) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2a2b-full(601) GCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGG
2d11d    (601) GCCGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
4b2d     (601) GCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGANNNNNNNNNNNNNN
rpFL13   (595) GCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGG
rpFL8785 (595) GCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGG
               661                                                          720
1c12b    (661) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAATTT
2a11h    (661) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2e5e     (661) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2a2b-full(661) TATGCCAATGAACTGCAATTGGACGGTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
2d11d    (661) TATGCCAATGAACTGCAATTGGGCGGTTTCCGTCTTAATGCTGTCAAACACATTAAATTT
4b2d     (661) TATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTT
rpFL13   (655) TATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTT
rpFL8785 (655) TATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTT
               721                                                          780
1c12b    (721) TCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
2a11h    (721) NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAAAAAACGGGGAAGGAAATGTTTACG
2e5e     (721) NNNNNNNNNNNNNNNNNGGGTTAATCATGTCAGGGAAAAAACGGGGAAAGAAATGTTTACG
2a2b-full(721) NNNNNNNNNNGGGATTGGGGTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
2d11d    (721) TCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
4b2d     (721) TCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
rpFL13   (715) TCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
rpFL8785 (715) TCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACG
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Figure 31. (Continued).

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                    781                                                    840
      1c12b    (781) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAACCTATTTGAACAAAACAAAT
      2a11h    (781) GTAGCTGAATATGGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
      2e5e     (781) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
   2a2b-full   (781) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
      2d11d    (781) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
      4b2d     (781) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
     rpFL13    (775) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
    rpFL8785   (775) GTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAAT
                    841                                                    900
      1c12b    (841) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
      2a11h    (841) TTTAATCTTTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGTCACAG
      2e5e     (841) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
   2a2b-full   (841) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
      2d11d    (841) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
      4b2d     (841) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
     rpFL13    (835) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
    rpFL8785   (835) TTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAG
                    901                                                    960
      1c12b    (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
      2a11h    (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
      2e5e     (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
   2a2b-full   (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
      2d11d    (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
      4b2d     (901) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGATTCCAAGCATCCGTTG
     rpFL13    (895) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
    rpFL8785   (895) GGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTG
                    961                                                   1020
      1c12b    (961) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
      2a11h    (961) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
      2e5e     (961) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
   2a2b-full   (961) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
      2d11d    (961) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
      4b2d     (961) AAAGCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
     rpFL13    (955) AAAGCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
    rpFL8785   (955) AAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
                   1021                                                   1080
      1c12b   (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
      2a11h   (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
      2e5e    (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
   2a2b-full  (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGGTAC
      2d11d   (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
      4b2d    (1021) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCGGGATAC
     rpFL13   (1015) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
    rpFL8785  (1015) GTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATAC
                   1081                                                   1140
      1c12b   (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
      2a11h   (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
      2e5e    (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
   2a2b-full  (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
      2d11d   (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
      4b2d    (1081) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
     rpFL13   (1075) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
    rpFL8785  (1075) CCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCT
                   1141                                                   1200
      1c12b   (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
      2a11h   (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
      2e5e    (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
   2a2b-full  (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
      2d11d   (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
      4b2d    (1141) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
     rpFL13   (1135) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
    rpFL8785  (1135) GCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCA
```

Figure 31. (Continued).

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                    1201                                                    1260
    1c12b    (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
    2a11h    (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGTAAGGCGACAGCTCG
     2e5e    (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
 2a2b-full   (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
    2d11d    (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
     4b2d    (1201) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
    rpFL13   (1195) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
   rpFL8785  (1195) CAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCG
                    1261                                                    1320
    1c12b    (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
    2a11h    (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
     2e5e    (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
 2a2b-full   (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
    2d11d    (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
     4b2d    (1261) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
    rpFL13   (1255) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
   rpFL8785  (1255) GTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATG
                    1321                                                    1380
    1c12b    (1321) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
    2a11h    (1321) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
     2e5e    (1321) TATGTCGGCCGGCAAAACGCCGGTGAGAAATGGCATGACATTACCGGAAACCGTTCGGAG
 2a2b-full   (1321) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
    2d11d    (1321) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
     4b2d    (1321) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
    rpFL13   (1315) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
   rpFL8785  (1315) TATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAG
                    1381                                                    1440
    1c12b    (1381) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCANNNNNNNNNNNNNNNNNNNNNNN
    2a11h    (1381) CCGGTTGTCATCAATTCGGAAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
     2e5e    (1381) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCA
 2a2b-full   (1381) CCGGTTGTCATCAATTCGGAAGGTTGGGGAGAGTTTCACNNNNNNNNNNNNNNNNNNNNNN
    2d11d    (1381) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCA
     4b2d    (1381) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCA
    rpFL13   (1375) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCA
   rpFL8785  (1375) CCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCA
                    1441              1462
    1c12b    (1441) NNNNNNNNNNNNNNNNNNNNNNNN-
    2a11h    (1441) NNNNNNNNNNNNNNNNNNNNNNNNC
     2e5e    (1441) ATTTATGTTCAAAGACNNNNN-
 2a2b-full   (1441) NNNNNNNNNNNNNNNNNNNNNN
    2d11d    (1441) ATTTATGTTCAAAGACTCGAC-
     4b2d    (1441) ATTTATGTTCAAAGACTCGAC-
    rpFL13   (1435) ATTTATGTTCAAAGACTCGAC-
   rpFL8785  (1435) ATTTATGTTCAAAGACTCGAC-
```

Figure 31. (Continued).

The analysis of secondary structure and multiple alignment of amino acid sequences of shuffled alpha-amylases and sequences of recomnbinant alpha-amylases rpFL13 and rpFL8785 were analyzed by based on well known alpha-amylase structure (Machius et al., 1998b). The overall structure of recombinant alpha-amylase was summarized as shown in Table 5. The order sequence was based on sequences from Figure 32. The structure comprised of 3 domains (A, B and C), which belong to

catalytic domain of enzyme (amino acid 9-379). The domain A was amino acid sequence in range of 3-111 and 207-384. The positions of amino acid at 112-206 and 385-482 were in domain B and C, respectively. Four highly conserved regions among all alpha-amylases were homology to other *B. licheniformis* alpha-amylase and they were designated as I, II, III, and IV (Aubrey et al., 2008; Bessler et al., 2003; Hmidet et al., 2008; Kuriki and Imanaka, 1999; Nielsen et al., 1999). Three conserved amino acid of active sites were in region II, III and IV, respectively. The residual sequences involved in the active sites (Asp233, Glu257, and Asp330), the 1^{st} calcium binding sites (Asn106, Asp196, Asp202, and His237), the 2^{nd} calcium binding sites (Asp163, Ala183, Asp185, Asp294, and Asp206), the 3^{rd} calcium binding sites (Gly302, Try304, His408, Asp409, and Arp423), and sodium binding sites (Asp163, Asp185, Asp196, Asp202, and Ile203) were conserved sequences for most shuffled alpha-amylases, except shuffled 1b9a, which mutations were occured in conserved regions (H237P, M258N, and F259V) and at 1^{st} calcium binding site (H237P) (Table 5 and 6, Figure 34). These conserved regions were same as other *B. licheniformis* alpha-amylase (Machius et al., 1998b). The calcium ions is important for stability of alpha-amylase, thus mutation in calcium binding site of shuffled alpha-amylase no. 1b9a may be decreased or increased the stability. The alteration of amino acids (shuffled no. 1b9a and no. 2a11h) in conserved regions could be changed in catalytic and substrate binding site of alpha-amylase (Kuriki and Imanaka, 1999). The nearly mutation points to conserved regions could be reduced the activity of alpha-amylase by changing in hydrogen bond network of amino acid complex at active site (Nielsen et al., 1999). These reasons confirmed the reduction of activity in shuffled alpha-amylase no. 9d7g (K251E) and no. 2e2a (T252M), which were 30% and 67% relative activity,

respectively. Moreover, the previous report suggested that the mutation in domain B made substrate specificity and stability changed in among the alpha-amylase (Svensson, 1994). The mutation in this region was found in shuffled alpha-amylase no. 2a11h, 2e2a, 2d11d, and 2e5e (Table 6).

Phylogenic tree of complete amino acid sequences of shuffled alpha-amylases was analyzed by vector NTI software as shown in Figure 32. The analysis was based on mature amino acid sequence of alpha-amylases. The *Bacillus* signal peptide and multiple histidine sequences were deleted to avoid the mistake of relation on phylogenic tree analysis. The results showed that the sequences of shuffled alpha-amylase no. 1b8b was similar to rpFL13 or rpFL13. The 4 shuffled alpha-amylases from rpET-SH library (SHM43, SHM154, SHM197, and SHM197) had sequences same as rpFL8785 or rpET8785 since the diversity was not detected among these shuffled alpha-amylases. This was one possible reason that made unsuccessful screening of rpET-SH library. The sequences of shuffled alpha-amylases (no. 1b9a, 2e2a, 9g7d, 2b7g, 1c5b, and 7c12h) were not closely related to rpFL13 or rpFL8785 recombinant alpha-amylase, especially shuffled no. 1b9a, 2e2a, 9g7d and 2b7g. They had high evolution of amino acid seqences from others. Sequences no. 2d11h, 7b3a had closely related to rpFL8785.

Table 5. The overall structure of recombinant alpha-amylases by based on well known alpha-amylase**.**

Characterization	Position of amino acid	Details
Alpha-amylase	4-485	
Catalytic domain	9-379	
Domain A	3-111, 207-384	
Domain B	112-206	
Domain C	385-482	
1st conserved region	102-107	(102-DVVINH-107)
2nd conserved region	226-239	(226-QLDGFRL**D**AVKHIK-239)
3rd conserved region	256-267	(256-K**E**MFTVAEYWQN-267)
4th conserved region	325-333	(325-FVDNH**D**TQP-333)
1st active site	233	D, in 2nd conserved region
2nd active site	257	E, in 3rd conserved region
3rd active site	330	D,in 4th conserved region
Domain A — Beta sheet 1	9-11	9-LMQ-11
Alpha helix 1	23-35	23-HWKRLQNDSAYLA-35
Beta sheet 2	41-43	41-AVW-43
Alpha helix 2	83-95	83-GELQSAIKSLHSR-95
Beta sheet 3	98-103	98-NVYGDV-103
Alpha helix 3	208-225	208-PDVAAEIKRWGTWYANEL-225
Beta sheet 4	229-232	229-GFRL-232
Alpha helix 4	240-252	240-SFLRDWVNHVRE-252
Beta sheet 5	259-262	259-FTVAEYWQN-262
Alpha helix 5	269-279	269-LGALENYLNKT-279
Beta sheet 6	284-286	284-SVF-286
Alpha helix 6	288-299	288-VPLHYQFHAAST-299
Beta sheet 7	322-325	322-AVT-325
Alpha helix 7	346-357	346-KLPAYAFILTRE-357
Beta sheet 8	360-364	360-YPQVF-364
Domian B — Alpha helix 8	385-395	385-KIEPILKARKQ-395
1st calcium binding site	106, 196, 202, 237	N, D, D, H,
2nd calcium binding site	163, 183, 185, 294, 206	D, A, D, D, D,
3rd calcium binding site	302, 304, 408, 409, 432	G, Y, H, D, D
sodium binding site	163, 185, 196, 202, 203,	D, D, D, D, I,
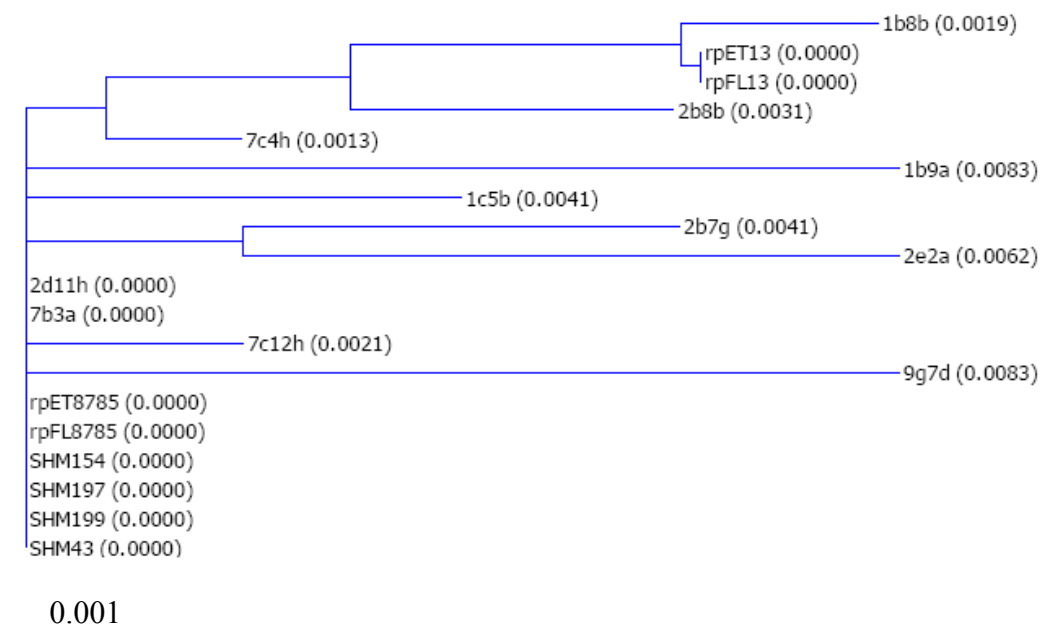
Figure 32. Phylogenic tree of shuffled alpha-amylases.

The evolution of amino acid sequences were created by DNA shuffling and error prone PCR in gene level. Alteration of new amino acid sequences were occurred by replacing with nonpolar to polar, neutral to acidic, basic to strong basic, strong basic to weaker basic, acidic to neutral, or by changing from basic to acidic amino acid. One possibility of evolution level was very high in shuffled alpha-amylase no. 1b9a, 2e2a, and 9g7d because of mutation in conserved region. Alpha-amylase family, mostly, has four conserved regions that spontaneous mutation is not often occurred by natural evolution. These regions have similar amino acid sequence among each species of microorganism sourece. Three conserved regions contain conserved amino acid involved in the active site of alpha-amylase that possibly changed in properties if mutation occurred. Shuffled no.1b9a showed three mutation points in two conserved

regions at position 237, 258, and 259 (Figure 33). Moreover, the position at 237 was the binding site of 1st calcium. The replacement of histidine with proline likely changed the bonding between protein and ions. Shuffled alpha-amylase no.2e2a and no. 9d7g showed one mutation point in conserved region at postion 252 and 251, respectively. The basic amino acid (K) was replaced by acidic amino acid (E) in shuffled alpha-amylase no. 9g7d, showing high evolution in genetic level as same as replacement of theronin (polar and neutral amino acid) with metionine (non polar and neutral amino acid). Shuffled no. 2a11h had 6 mutation points in amino acid sequence. The mutation at site 263 was occurred in conserved region. Moreover, DNA shuffling created terminated codon that made this shuffled enzyme was not active (Figure 34). Shuffled alpha-amylase no. 2a11h, 2b8b, and 4d2d showed successfully evolution by DNA shuffling. These enzymes contained shuffled amino acids between alpha-amylase rpFL13 and rpFL8785. Shuffled alpha-amylase no. 2b8b showed two amino acids (Asparagine (N) and leucine (L)) from recombinat alpha-amylase DSM 8785 at position 6 and 136 whereas two amino aicds at position 240 (leucine (L)) and 320 (alanine (A)) showed sequences from recombinant alpha-amylase DSM13. The mutation outside the acitive site was studied by previous report. The mutation at N106D, L272D, L272H, and Y292K changed in stability. It was difficult to know whether the changes were due to increase or decrease stability (Nielsen et al., 1999). The summarized informations of evolution of amino acid sequence by DNA shuffling technology was showen in Table 5 and 6.

The directed evolution of recombinant alpha-amylases from *B. licheniformis* DSM13 and DSM8785 created diversity in nucleotide and amino acid sequences by error prone PCR and by DNA shuffling technique. However, the high throughput

sceening could not detected the expected property of shuffled enzymes. The effects of each mutation point on sequences should take more attention on the alteration of property of shuffled alpha-amylase in further. This study will be the principle knowledges for development of alpha-amylase property by other methods or by DNA shuffling. Moreover, the method of DNA shuffling should be developed or modified to create the large diveristity of gene sequnences, resulting in the higher chance to observe improvement of properties. Many reports required the screening of variants enzymes, which were created by error prone PCR. Then, the selected gene was act as gene template for second delelopment of gene by DNA shuffling (Bessler et al., 2003; Parikh and Matsumura, 2005; Verhaert et al., 2002).

The disadvantage of whole gene mutagenesis is the requirement of screening method, which are more sensitive, precise, broder in range, and more round of evolution (Parikh and Matsumura, 2005). The development of enzymes properties is not required only one method but, some time, the recombination of various methods should be taken. Moreover, the desired activity should be able to screen by high throughput. The enzyme is over-expressed in *E. coli*, and substrates are available (Parikh and Matsumura, 2005). Therefore, the development of mutagenesis methods and high throughput screening are recommended to create new property of proteins.

Table 6. Summary of amino acid sequences from two libraries.

Mutants	Summarized details	Mutation or shuffled amino acid	%Relative activity
rpET-SH library	All alpha-amylases in this library had *Bacillus* signal peptide at N-terminal and 6-histidine sequence at C-terminus. The expression for screening was done by using *E.coli* BL21 (DE3) as host cell under IPTG induction		
SHM43	Completely sequences were same as rpET8785.	None	100% relative activity to rpET8785 activity
SHM154		None	
SHM199		None	
SHM197	One mutation was in *Bacillus* signal peptide. Completely sequences were same as rpET8785	A--> V	
rpFL-SH library	All alpha-amylases were fuse with *E.coli* signal peptid at N-terminus. Sequence did not have histidine sequence. The expression for screening was done by using *E.coli* TOP10 as host cell without IPTG induction.		
1b8b (complete sequence)	Sequence was same as rpFL13	None	100% to rpFL13
1b9a (complete sequence)	4 mutation points 4 shuffled amino acids from rpFL8785	N6 (rpFL8785) L136 (rpFL8785) H237P F240 (rpFL8785) N248K M258N F254V S322 (rpFL8785)	70% to rpFL8785

Table 6. (Continued).

Mutants	Summarized details	Mutation or shuffled amino acid	%Relative activity
1c5b (complete sequence)	2 mutation points Sequence was same as rpFL8785	N6 (rpFL8785) Q20R L136 (rpFL8785) N190I F240 (rpFL8785) S322 (rpFL8785)	65 % to rpFL8785
2b7g (complete sequence)	3 mutation points Sequence was same as rpFL8785	N6 (rpFL8785) H70R L136 (rpFL8785) F240 (rpFL8785) S241F S322 (rpFL8785) H473R	100 % to 8785
2b8b (complete sequence)	1 mutation point Sequence had shuffled amino acids between rpFL13 (2 points) and rpFL8785 (2 points)	N6 (rpFL8785) L136 (rpFL8785) S150N L240 (rpFL3) A322 (rpFL13)	95 % to 8785
2d11h (complete sequence)	Sequences was same as rpFL8785	None	100% to rpFL8785
2e2a (complete sequence)	3 mutation points Sequence was same as rpFL8785	N6 (rpFL8785) L136 (rpFL8785) D168G F240 (rpFL8785) S241F T254M S322 (rpFL8785)	67 % to rpFL8785

Table 6. (Continued).

Mutants	Summarized details	Mutation or shuffled amino acid	%Relative activity
7b3a (complete sequence)	Sequences was same as rpFL8785	None	100% to rpFL8785
7c4h (complete sequence)	Sequences was same as rpFL8785 1 mutation point	N6 (rpFL8785) L136 (rpFL8785) F or L240S S322 (rpFL8785)	90% to rpFL8785
7c12h (complete sequence)	1 mutation point Sequence was same as rpFL8785	N6 (rpFL8785) L136 (rpFL8785) R216K F240 (rpFL8785) S(322) (rpFL8785)	75% to rpFL8785
9g7d (complete sequence)	4 mutation points Sequence was same as rpFL8785	A3E L5I N6 (rpFL8785) L136 (rpFL8785) F240 (rpFL8785) K253E S322 (rpFL8785) N457I	30% activtity
2a11h (none complete sequence)	Partial sequence had shuffled amino acids between rpFL13 (one point) and rpFL8785 (one point) 6 mutation points	K6 (rpFL13) S31A F69S Lor R136P W265G H283L T299S S322 (rpFL8785) Stop codon (416)	0% activity

Table 6. (Continued).

Mutants	Summarized details	Mutation or shuffled amino acid	%Relative activity
4d2d (none complete sequence)	Partial sequence had shuffled amino acids between rpFL13 (two points) and rpFL8785 (two points)	N6 (rpFL8785) R136 (rpFL13) F240 (rpFL8785) A322 (rpFL13)	80% to rpFL8785
2e5e (none complete sequence)	Partial sequence was same as rpFL8785	N6 (rpFL8785) L136 (rpFL8785)	89% to rpFL8785
2d11d (none complete sequence)	Sequence was same as rpFL8785. Two mutation points	N6 (rpFL8785) L136 (rpFL8785) Y177H D228G F240 (rpFL8785) S322 (rpFL8785)	75% to rpFL8785
2a2b (none complete sequence)	Partial sequence was same as rpFL8785 One mutation point	N6 (rpFL8785) L136 (rpFL8785) V247G S322 (rpFL8785)	55% to rpFL8785
1c12b (none complete sequence)	Partial sequence was same as rpFL8785 Two mutation points	N6 (rpFL8785) L136 (rpFL8785) N190H G192F F240 (rpFL8785) S322 (rpFL8785)	66% to rpFL8785

Table 7. Summary of alteration of amino acids creating by DNA shuffling.

| Site	Amino acid alteration	Evolution details	Shuffled no.	% Relative activity
3	A1E	Mutation was in domain A. Replaced by polar and acidic amino acid (E)	9g7d	30
5	L3I	Mutation was in domain A. Replaced by same property of amino acid	9g7d	30
6	K (DSM13) N (DSM8785)	Shuffled amino acid between recombinant alpha-amylase *B. licheniformis* DSM13 and DSM 8785. This shuffled site was in domainA	rpFL13 1b8b 2a11h rpET13	100 100 0 100
22	Q22R	Mutation was near 1st alpha sheet in domain A Replaced by polar and strong amino acid	1c5b	65
31	S31A	Mutation was in 1st alpha sheet in damain A. Replaced by nonpolar and neutral amino acid	4d2d	80
69	F69S	Mutation was in domain A. Replaced by polar and neutral amino acid	2a11h	0
70	H70R	Mutation was in domain A. Replaced by polar and stronger basic amino acid (R)	2b7g	100
72	K72Q	Mutation was in domain A. Replaced by polar and neutral amino acid	2e2a	67
136	R (DSM13) L (DSM 8785) L or R 134P	Shuffled amino acid between recombinant alpha-amylase *B. licheniformis* DSM13 and DSM 8785. This shuffled site was in domain B. Replaced by nonpolar and neutral amino acid (P)	rpFL13, 1b8b, 4d2d, rpET13 2a11h (134P)	100 100 80 100 0

Table 7. (Continued).

Site	Amino acid alteration	Evolution details	Shuffled no.	% Relative activity
150	S150N	Mutation was in domain B Replaced by bigger size of amino acid	2b8b	95
168	D168G	Mutation was in domain B Replaced by nonpolar and neutral amino acid	2e2a	67
177	Y177H	Mutaiton was in domain B Replaced by polar and weakly basic amino acid	2d11d	100
180	Q180S	Mutation was in domain B Replaced by polar and neutral amino acid	2e5e	89
190	N190I	Mutation was in domain B Replaced by nonpolar and neutral amino acid	1c5b	65
216	R216K	Mutation was in 3rd alpha sheet of domain A. Replaced by polar and weaker basic amino acid	7c12h	75
237	H237P	Mutation was occurred in conserved region (226-QLDFRVDAVKHIK-239). This conserved region is in between 3rd and 4th alpha sheet and it contain 1st amino acid (D; aspartic acid) of active site (at site 232). This site involved in 1st Ca binding site. Replaced by nonpolar amino acid	1b9a	70

Table 7. (Continued).

Site	Amino acid alteration	Evolution details	Shuffled no.	% Relative activity
240	L (DSM13) F (DSM 8785) L or F(240)S	Shuffled amino acid between recombinant alpha-amylase *B. licheniformis* DSM13 and DSM 8785. This shuffled site was in 4[th] alpha sheet.One mutation was in this site (7c4h)	L (rpFL13, 1b8b, 2b8b, rpET13) 7c4h	100 100 95 100 90
241	S239F	Mutaion was in 4[th] alpha sheet in domain A. Replaced by nonpolar, neutral and bigger size of amino acid	2b7g 2e2a	100
247	V245G	Mutaion was in 4[th] alpha sheet in domain A. Replaced by nonpolar and neutral amino acid	2a2b	55
248	N246K	Mutation was in 4[th] alpha sheet in domain A. Replaced by polar and basic amino acid	1b9a	70
253-255	K253E T255M	Mutations were in 4[th] alpha sheet of domain A, and sites were nearly to conserved region. Basicamino acid (K) was replaced by acidic amino acid (E). Replaced by neutral and non polar amino acid (M) at site 254	9d7g (K251E) 2e2a (T252M)	30 67

Table 7. (Continued).

Site	Amino acid alteration	Evolution details	Shuffled no.	% Relative activity
258 259	M258N F259V	These points were in conserved region (256-KEMFTVAEY-267). This conserved region was closely to 3rd alpha sheet containing 2nd amino acid(E; glutamic acid) of active site (at site 257). Replaced by polar and neutral amino acid (N) and replaced with smaller size of amino acid (V)	1b9a	70
265	W265G	Mutation was in conserved region (256-KEMFTVAE Y-267). Replaced by nonpolar and neutral amino acid	2a11h	0
274	N274T	Mutation was in domain A. Replaced by polar and neutral amino acid	2a11h	0
299	T299S	Mutation was in 6th of alpha sheet of domain A. Replaced by polar and neutral amino acid	2a11h	0
315	V315D	Mutation was in 7th of alpha sheet of domain A. Replaced by polar and acidic amino acid	4d2d	80
322	A (DSM13) S (DSM8785)	Nucleotide base shuffling between recombinant alpha-amylase *B. licheniformis* DSM13 and DSM 8785. This site was in 7th beta sheet of domain A	rpFL13, 1b8b, 2b8b, 4d2d, rpET13	100 100 95 80 100
416	E to stop codon	Mutation was in domain C. Stop codon (TAA) was created by mutation.	2a11h	0
457	N457I	Mutation was in domain C Replaced by neutral and nonpolar amino acid	9g7d	30
473	H473R	Mutation was in domain C Replaced by polar and stronger basic amino acid	2b7g	100
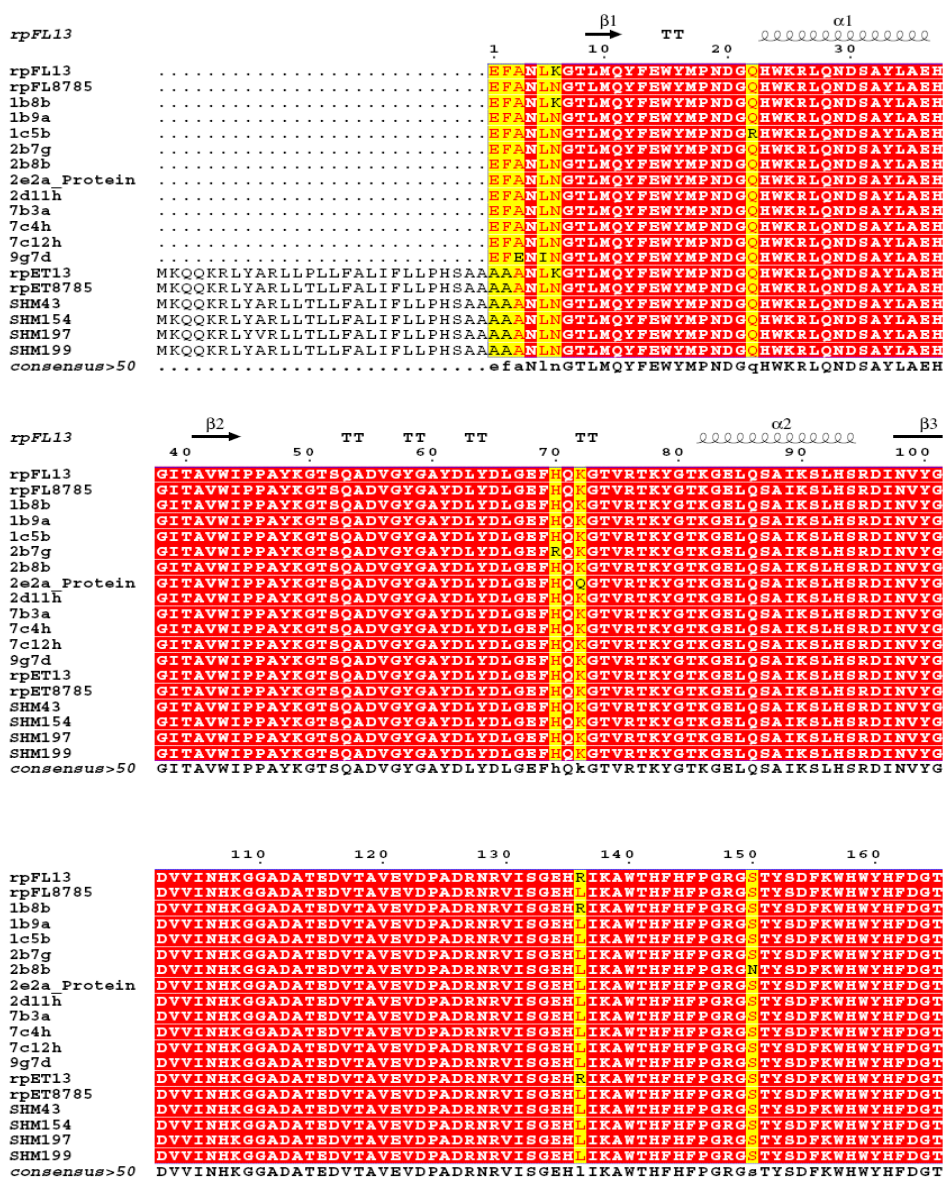
Figure 33. Secondary structure of shuffled alpha-amylases.

SHM43, SHM154, SHM197, and SHM199 were samples from rpET-SH library. The rest were samples from rpFL-SH library. The rpFL13 and rpET13 were recombinant alpha-amylase DSM13 in pFLAG-CTS and pET21d (+) system, respectively. The rpET8785 and rpFL8785 were recombinant alpha-amylase DSM8785 in pFLAG-CTS and pET21d (+) system, respectively. Symbols α or ℓℓℓ; alpha-helix, β; beta-sheet, TT; turn helix. Red color; same sequences, Yellow color; different sequences.The overall structure was shown in Table 5.
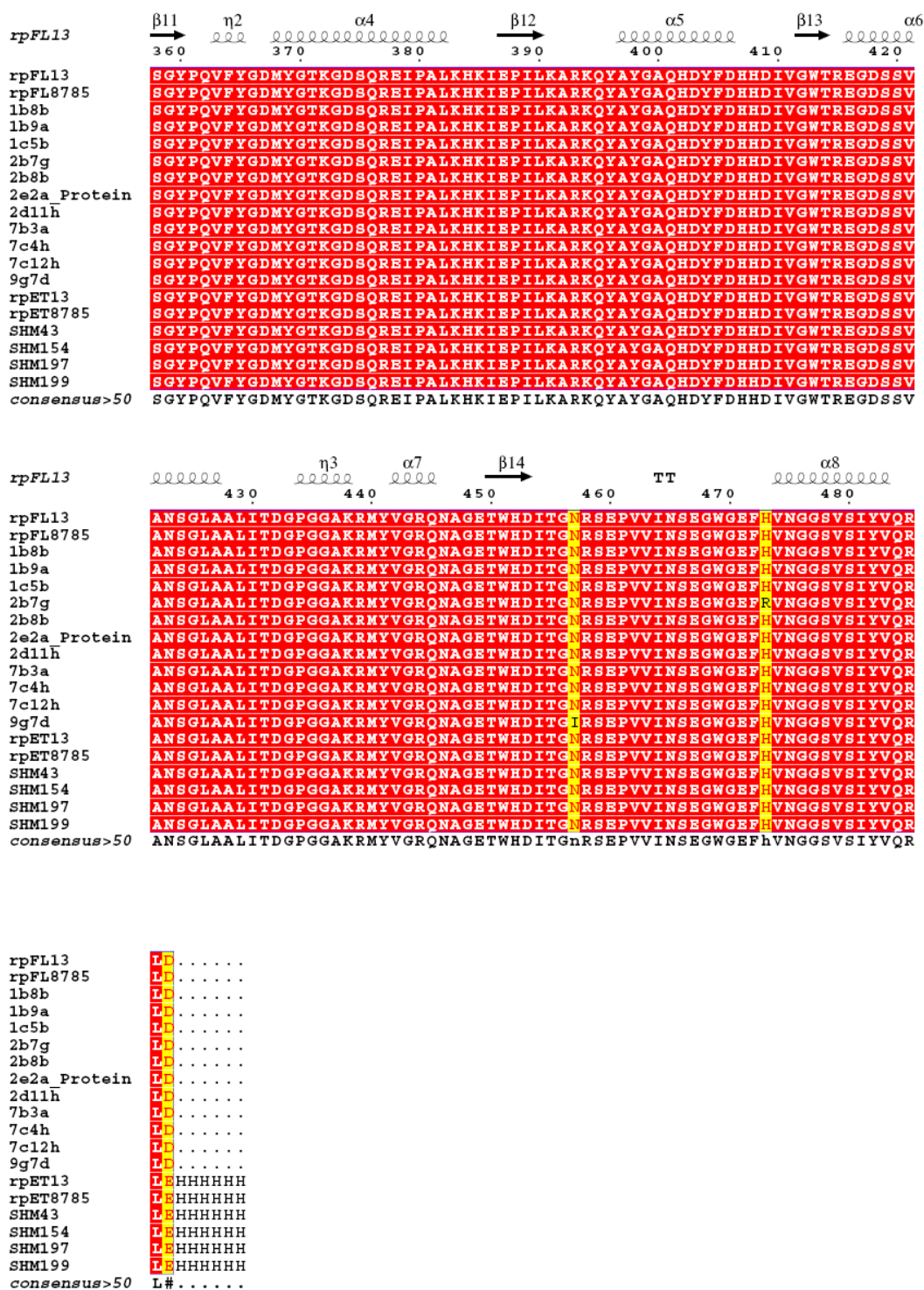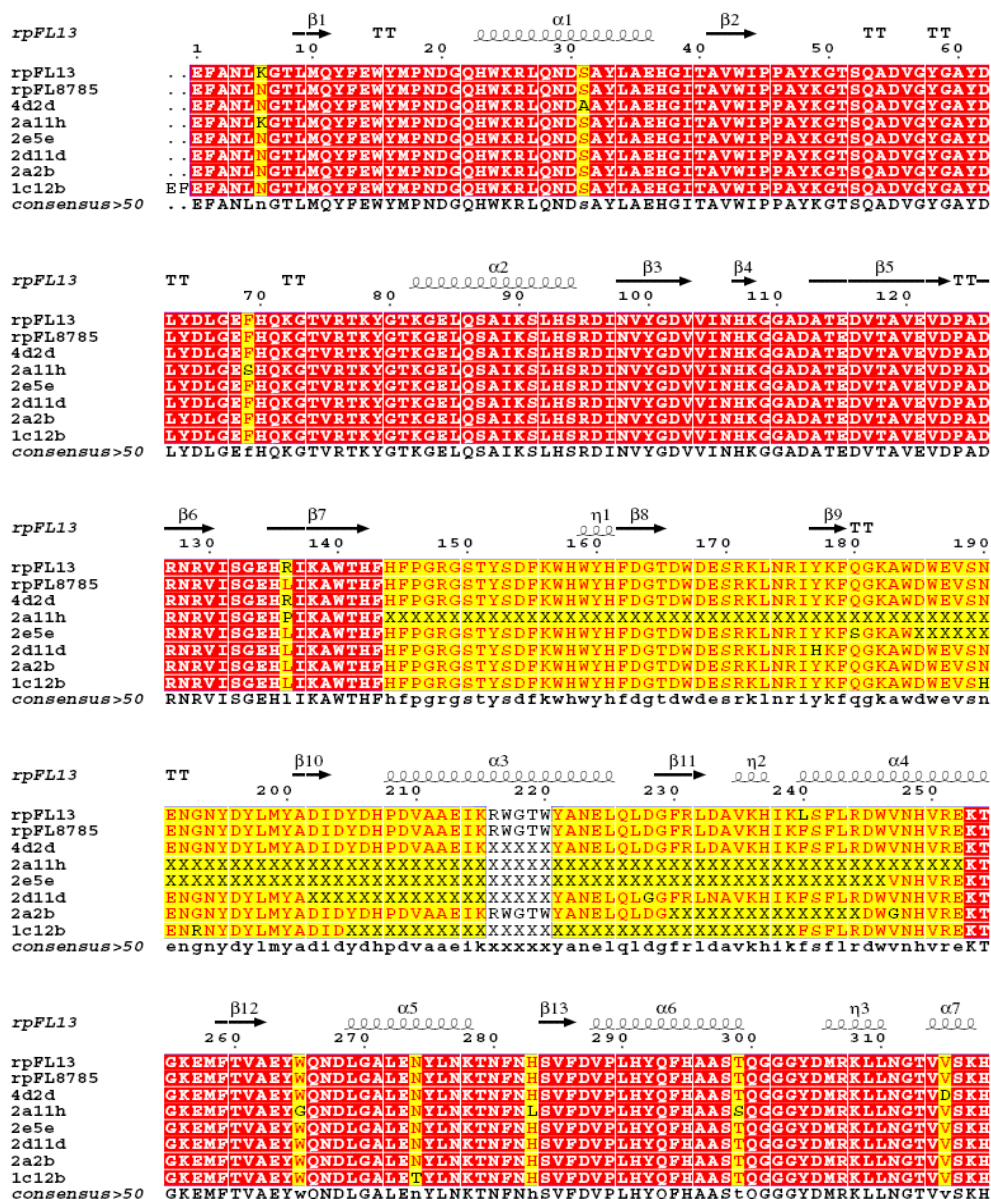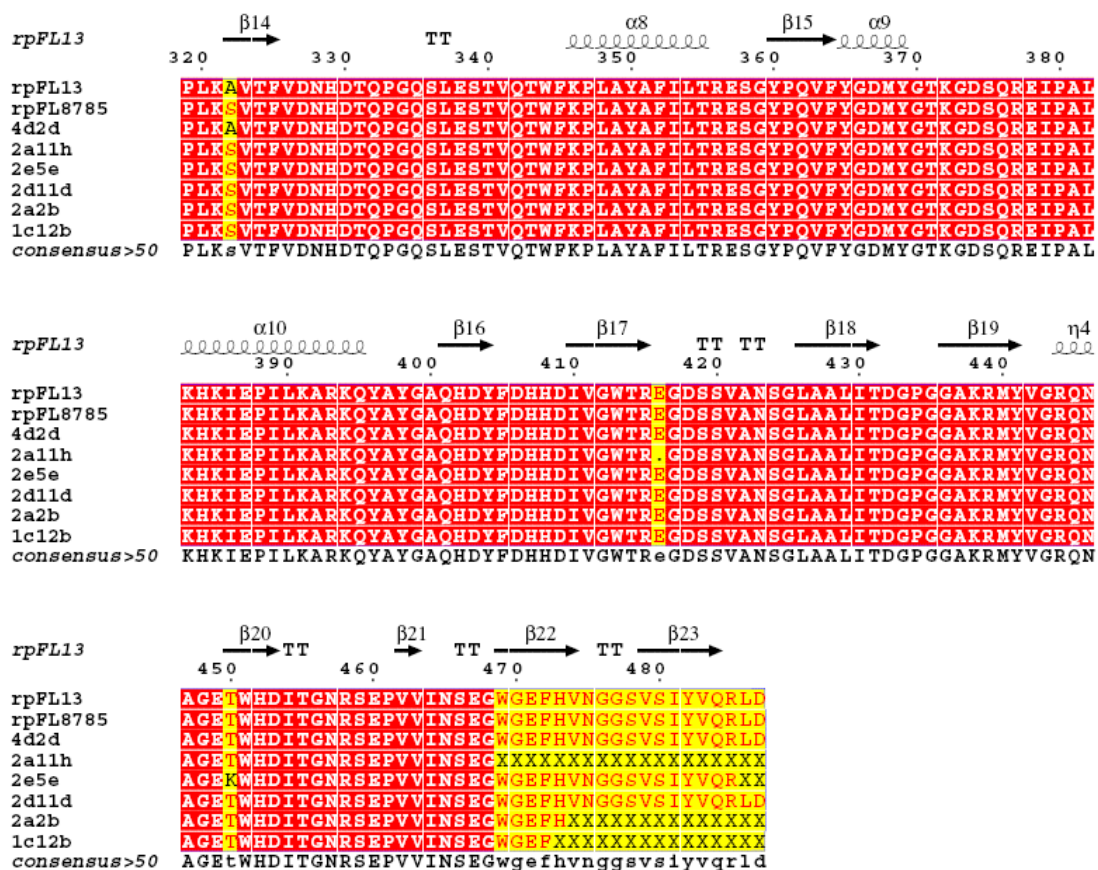
Figure 33. (Continued).

Figure 33. (Continued).

rpFL13 β1 TT α1 β2 TT TT
        1   10   20   30   40   50   60

```
rpFL13      ..EFANLKGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
rpFL8785    ..EFANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
4d2d        ..EFANLNGTLMQYFEWYMPNDGQHWKRLQNDAAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
2a11h       ..EFANLKGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
2e5e        ..EFANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
2d11d       ..EFANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
2a2b        ..EFANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
1c12b       EFEFANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
consensus>50  ..EFANLnGTLMQYFEWYMPNDGQHWKRLQNDsAYLAEHGITAVWIPPAYKGTSQADVGYGAYD
```

rpFL13 TT TT α2 β3 β4 β5 TT
        70   80   90   100   110   120

```
rpFL13      LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
rpFL8785    LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
4d2d        LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
2a11h       LYDLGESHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
2e5e        LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
2d11d       LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
2a2b        LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
1c12b       LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
consensus>50  LYDLGEfHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEVDPAD
```

rpFL13 β6 β7 η1 β8 β9 TT
        130   140   150   160   170   180   190

```
rpFL13      RNRVISGEHRIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEVSN
rpFL8785    RNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEVSN
4d2d        RNRVISGEHRIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEVSN
2a11h       RNRVISGEHPIKAWTHFXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
2e5e        RNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFSGKAWXXXXXX
2d11d       RNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIHKFQGKAWDWEVSN
2a2b        RNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEVSN
1c12b       RNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAWDWEVSH
consensus>50  RNRVISGEHlIKAWTHFhfpgrgstysdfkwhwyhfdgtdwdesrklnriykfqgkawdwevsn
```

rpFL13 TT β10 α3 β11 η2 α4
        200   210   220   230   240   250

```
rpFL13      ENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKLSFLRDWVNHVREKT
rpFL8785    ENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKPSFLRDWVNHVREKT
4d2d        ENGNYDYLMYADIDYDHPDVAAEIKXXXXXYANELQLDGFRLDAVKHIKPSFLRDWVNHVREKT
2a11h       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKT
2e5e        XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXVNHVREKT
2d11d       ENGNYDYLMYAXXXXXXXXXXXXXXXXXXXXYANELQLGGFRLNAVKHIKPSFLRDWVNHVREKT
2a2b        ENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGXXXXXXXXXXXXXXDWGNHVREKT
1c12b       ENRNYDYLMYADIDXXXXXXXXXXXXXXXXXYANELQLDGFRLDAVKHIKPSFLRDWVNHVREKT
consensus>50  engnydylmyadidydhpdvaaeikxxxxxyanelqldgfrldavkhikfsflrdwvnhvreKT
```

rpFL13 β12 α5 β13 α6 η3 α7
        260   270   280   290   300   310

```
rpFL13      GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
rpFL8785    GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
4d2d        GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVDSKH
2a11h       GKEMFTVAEYGQNDLGALENYLNKTNFNLSVFDVPLHYQFHAASSQGGGYDMRKLLNGTVVSKH
2e5e        GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
2d11d       GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
2a2b        GKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
1c12b       GKEMFTVAEYWQNDLGALETYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKH
consensus>50  GKEMFTVAEYwQNDLGALEnYLNKTNFNhSVFDVPLHYQFHAAStQGGGYDMRKLLNGTVvSKH
```

Figure 34. Secondary structure of non complete sequences of all shuffled alpha-amylases from rpFL-SH library.

The rpFL13 and rpET13 were recombinant alpha-amylase DSM13 in pFLAG-CTS and pET21d (+) system, respectively. The rpET8785 and rpFL8785 were recombinant alpha-amylase DSM8785 in pFLAG-CTS and pET21d (+) system, respectively. Symbols α or ℓℓℓ; alpha-helix, β; beta-sheet, TT; turn helix. Red color; same sequences, Yellow color; different sequences. The overall structure was shown in Table 5.

Figure 34. (Continued)**.**

CHAPTER VI

CONCLUSIONS

1. Two recomninat alpha-amylases from *B. licheniformis* DSM13 and DSM8785 were over-expressed in bacterial expression system by using pET21d (+) or pFLAG-CTS expression vector, resulting in the secretion of alpha-amylases by unsign *Bacillus* signal peptide or *E. coli* OmpA signal peptide. Moreover, the efficiency of purification was achieved by using multiple histidine sequences.

2. The optimal temperature and optimal pH of alpha-amylases rpFL13-10xHis and rpFL8785-10xHis were 70°C and pH at 7, repectively. The half life ($t_{1/2}$) of both recombinant alpha-amylase were 30 min at 60 ^{o}C at pH 7.

3. Both recombinant alpha-amylases rpFL13-10xHis and rpFL8785-10xHis were not calcium-dependent enzyme. However, the addition of calcium ions enhanced the activity and stability of enzymes.

4. Both recombinant alpha-amylases were good hydrolyzing enzymes by using cassava starch as substrate. The specific catalytic constants in the hydrolysis of cassava starch were higher than hydrolysis of soluble starch, which were 1.23 and 1.18 folds by using two recombinant alpha-amylases from rpFL13-10xHis and rpFL8785-10xHis, respectively.

5. The construction of shuffled alpha-amylase genes by DNA shuffling was successful to create the diversity of alpha-amylase genes. The shuffled no. 2b8b, 2a11h, and 4d2d contained shuffled amino acid sequences between both

B. licheniformis alpha-amylase DSM13 and DSM 8785. The mutagenesis of shuffled no. 1b9a was occurred in conserved regions and calcium binding site, which involed in catalytic activity of alpha-amylase.

6. The screening under six different conditions were done by high throughput. However, the expected property of shuffled alpha-amylase was not detected.

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APPENDICS

APPENDIX I

REAGENTS AND EQUPMENTSREAGENT

1.1 REAGENT

All chemicals were molecular grade or analytic grade.

1. Reagent for PCR amplification.

   - 25 mM dNTP mix (New England BioLabs)

   - 10X *Pfu* buffer (New England BioLabs)

2. Reagent for agarose gel electrophoresis

   - Agarose low EEO. Molecular biology grade (Research organics)

   - 25 bp DNA ladder (Invitrogen)

   - 1X TAE Buffer

   - 1 Kb Ladder marker DNA (Bio Lab)

   - 6X Loading dye

   - Staining solution; 0.5 μg/ml ethidium bromide in distilled water

3. Reagent for transformation

   - Chemical competent cells

   - Electrocompetent cells

   - LB agar plate

   - LB agar plus 1%(w/v) soluble starch

4. Reagent for SDS-PAGE and Zymogram

   - Protein sample buffer (see Appendix I)

- 30% polyacylamide (BioRAD)

- 1.5 M Tris-HCl pH 8.8

- 0.5 M Tris-HCl pH 6.0.8

   - 10% (w/v) Amonium persulfate

   - 10% (w/v) Sodium dodecylsulfate

   - 1% (w/v) soluble starch

5.Enzymes

   - *Taq* DNA polymerase (New England BioLabs)

   - *Pfu* DNA polymerase (New England BioLabs)

   - *T4* ligase (New England BioLabs)

   - *Hind*III (New England BioLabs)

   - *EcoR*I (New England BioLabs)

   - *Xho*I (New England BioLabs)

   - *Nco*I (New England BioLabs)

   - DNaseI (Fermentas)

6.Reagent for SDS-PAGE and Zymogram

   - Protein sample buffer

   - 30% polyacylamide (BioRAD)

   - 1.5 M Tris-HCl pH 8.8

   - 0.5 M Tris-HCl pH 6.8

   - 10% Amonium persulfate

   - 10% Sodium dodecylsulfate

7.Reagent for enzymes purification

- Ni-NTA resins (QIAGEN)

- Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole. pH 8.0)

- Wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 40 mM imidazole. pH 8.0)

- Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole. pH 8.0)

8.Reagent for analysis of enzyme activity

- 3,5-dinitrosalisylic acid (Sigma)

- 1 N NaOH (Sigma)

- 1% (w/v) soluble starch

- 1% (w/v) cassava starch

1.2 EQUIPMENTS

1. Thermomixer (Eppendorf)

2. BioRad Mini Protean II Cell (BioRAD)

3. Microtiter plate reader (Sunrise)

4. Electrotoporater (Eppendorf)

5. BioRAD Mini Protein II Cell (BioRAD)

6. pH meter (Hanna instrument)

APPENDIX II

STANDARD AND DATA

2.1 STANDARD CURVE FOR COLORIMETRIC METHOD
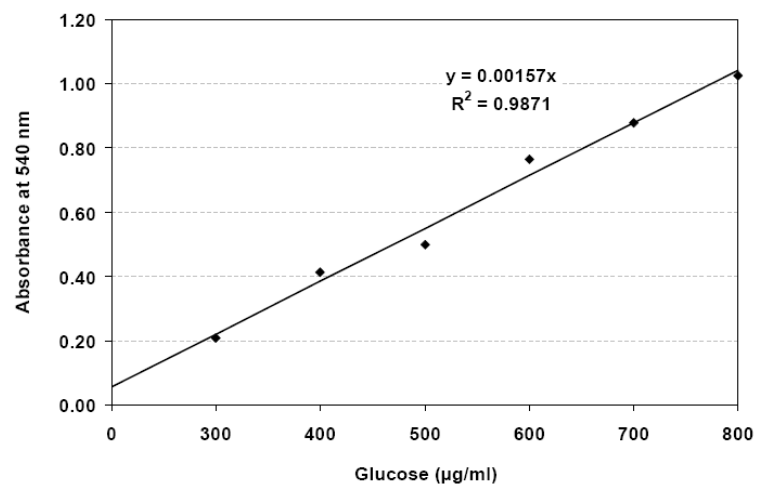
Figure 1A. Standard curve of glucose concentration analysis using 3,5-dinitrosalisylic acid.
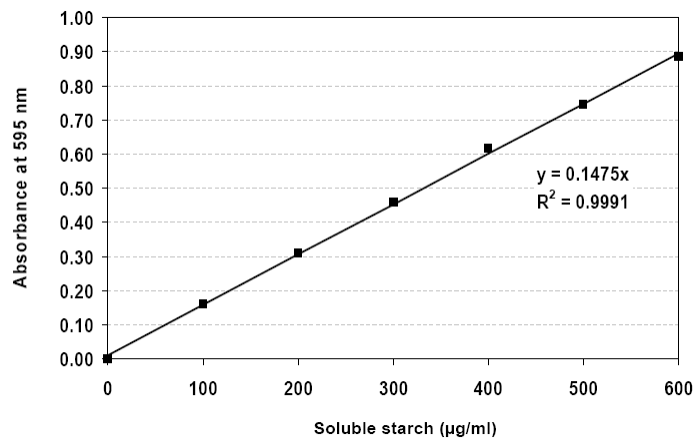
Figure 2A. Standard curve of soluble starch concentration analysis using iodine method.
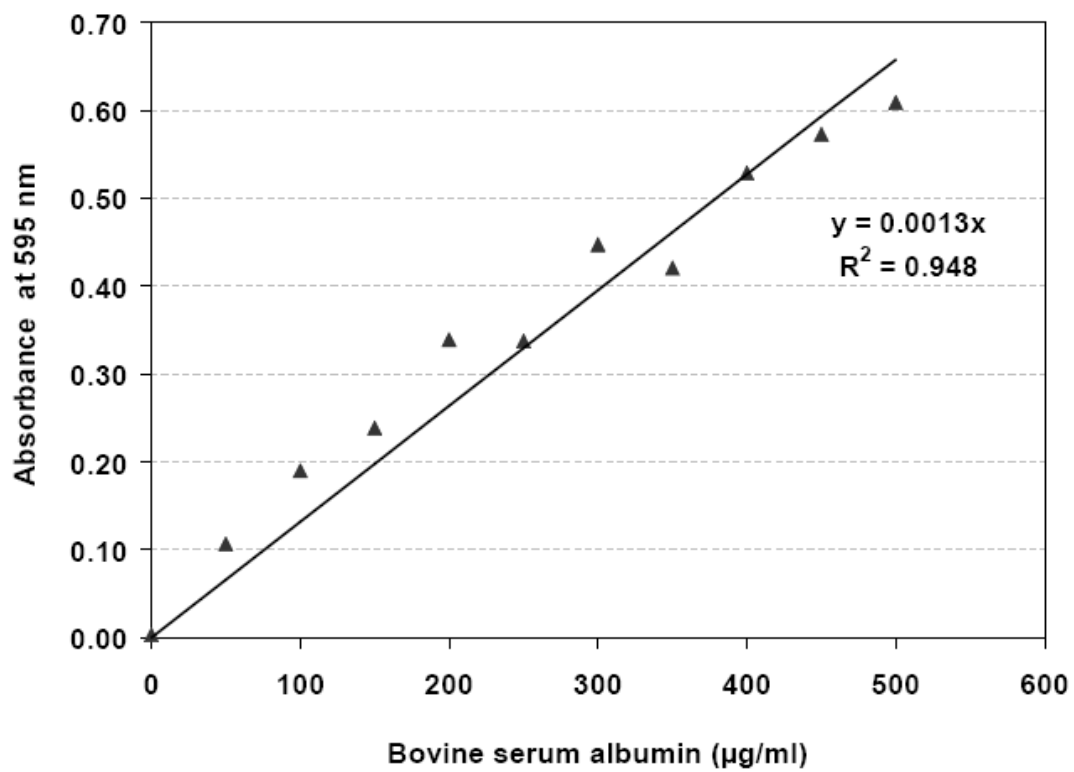
Figure 3A. Standard curve of bovine serum albumin concentration analysis using

Bradfords solution.

2.2 DATA

Table 1A. Activity of recombinant alpha-amylases on difference pH values by using 1%(w/v) soluble starch as substrate (based on equal amount of protein).

Buffer	pH values	Activity (U/ml)			
		Without 2mM $CaCl_2$		With 2mM $CaCl_2$	
		rpFL13	rpFL8785	rpFL13	rpFL8785
50 mM Sodium acetate	2	9.97	9.38	21.53	10.26
	3	12.53	11.71	32.29	18.90
	4	17.34	14.54	51.49	25.39
	5	38.66	23.77	92.12	28.43
	6	68.38	38.60	132.73	47.36
50 mM Potassium phosphate	6	59.48	33.73	127.15	39.34
	7	95.48	46.44	171.06	65.85
	8	66.02	29.46	95.28	49.01
	9	43.91	17.60	62.31	36.39
50 mM Glycine NaOH	9	37.77	16.63	52.22	35.42
	10	19.31	13.06	26.80	15.39
	11	8.35	10.03	23.00	10.11
Buffer	**pH values**	**(%) Relative activity**			
		Without 2mM $CaCl_2$		Without 2mM $CaCl_2$	
		rpFL13	rpFL8785	rpFL13	rpFL8785
50 mM Sodium acetate	2	10.44	9.82	22.55	10.75
	3	13.13	12.26	33.82	19.80
	4	18.16	15.23	53.92	26.59
	5	40.49	24.89	96.48	29.77
	6	71.62	40.43	139.01	49.60
50 mM Potassium phosphate	6	62.29	35.33	133.17	41.20
	7	100.00	48.64	179.16	68.96
	8	69.15	30.85	99.79	51.33
	9	45.99	18.44	65.26	38.11
50 mM Glycine NaOH	9	39.56	17.42	54.70	37.09
	10	20.23	13.68	28.07	16.12
	11	8.74	10.50	24.09	10.59

Table 2A. Percentage of remained activity of alpha-amylases on pH stability analysis for 30 min (based on equal amount of protein).

		Activity (U/ml)						
		pH condition						
Enzymes	**Initial**	**3**	**4**	**6**	**7**	**8**	**10**	**11**
rpFL8785-10xHis	172.51	13.36	13.15	120.16	128.80	121.20	16.37	12.71
rpFL8785-10xHis plus 2mM CaCl2	172.51	12.86	14.01	141.13	149.62	139.36	18.87	14.51
rpFL13-10xHis	196.83	14.63	13.83	152.51	163.42	155.79	15.22	14.86
rpFL13-10xHis plus 2mM CaCl2	196.83	14.10	23.00	174.39	187.60	166.49	13.48	13.80
		(%) Remained activity						
		pH condition						
Enzymes	**Initial**	**3**	**4**	**6**	**7**	**8**	**10**	**11**
rpFL8785-10xHis	100.0	7.7	7.6	69.7	74.7	70.3	9.5	7.4
rpFL8785-10xHis plus 2mM CaCl2	100.0	7.5	8.1	81.8	86.7	80.8	10.9	8.4
rpFL13-10xHis	100.0	7.4	7.0	77.5	83.0	79.1	7.7	7.6
rpFL13-10xHis plus 2mM CaCl2	100.0	7.2	11.7	88.6	95.3	84.6	6.8	7.0

Table 3A. Activity of recombinant alpha-amylases on different temperatures by using 1%(w/v) soluble starch as substrate for 20 min with different sampling time (based on equal amount of protein).

Activity (U/ml)								
(^{0}C)		30				40		
Sampling time (min)	0	5	10	20	0	5	10	20
rpFL8785-10xHis	0.6	19.5	19.8	39.6	0.6	13.3	21.2	48.4
rpFL8785-10xHis plus 2mMCaCl$_2$	0.6	18.1	37.1	66.4	0.6	20.0	49.5	64.5
rpFL13-10xHis	0.6	30.4	68.8	105.3	0.6	41.3	69.8	129.5
rpFL13-10xHis plus 2mMCaCl$_2$	0.6	52.1	74.2	146.9	0.6	48.3	107.6	174.6
(^{0}C)		50				60		
Sampling time (min)	0	5	10	20	0	5	10	20
rpFL8785-10xHis	0.6	16.2	26.3	55.9	0.6	19.2	39.2	69.9
rpFL8785-10xHis plus 2mMCaCl$_2$	0.6	22.2	47.0	87.7	0.6	28.9	77.1	104.6
rpFL13-10xHis	0.6	35.2	59.3	134.2	0.6	67.6	101.9	121.8
rpFL13-10xHis plus 2mMCaCl$_2$	0.6	58.3	119.1	187.9	0.6	81.3	139.0	172.9

Table 3A. (Continued).

	Activity (U/ml)							
(^{0}C)	70				80			
Sampling time (min)	0	5	10	20	0	5	10	20
rpFL8785-10xHis	0.6	63.5	76.2	78.8	0.6	40.3	63.7	69.1
rpFL8785-10xHis plus 2mMCaCl$_2$	0.6	94.6	108.0	109.5	0.6	47.3	66.0	84.0
rpFL13-10xHis	0.6	118.5	128.7	132.2	0.6	87.4	88.1	86.5
rpFL13-10xHis plus 2mMCaCl$_2$	0.6	163.5	181.8	186.4	0.6	85.0	86.8	89.8
(^{0}C)	90				100			
Sampling time (min)	0	5	10	20	0	5	10	20
rpFL8785-10xHis	0.6	30.2	36.6	61.7	0.6	19.6	22.8	20.1
rpFL8785-10xHis plus 2mMCaCl$_2$	0.6	39.7	56.1	80.2	0.6	34.1	54.6	48.1
rpFL13-10xHis	0.6	66.5	76.0	82.0	0.6	37.4	45.6	41.2
rpFL13-10xHis plus 2mMCaCl$_2$	0.6	66.5	67.2	82.0	0.6	44.4	48.0	48.4

Table 4A. Activity of recombinant alpha-amylases on different temperatures by using

1% (w/v) soluble starch as substrate at selected sampling time (based on equal amount of protein).

	Activity (U/ml)							
(^{0}C)	30	40	50	60	70	80	90	100
Time (min)	20	20	20	20	5	5	5	5
rpFL8785-10xHis	39.6	48.4	55.9	69.9	254.1	161.1	120.8	78.2
rpFL8785-10xHis plus 2mMCaCl$_2$	67.5	74.4	87.8	105.1	378.4	189.3	158.8	136.2
rpFL13-10xHis	105.3	118.5	121.8	129.5	536.8	314.1	230.7	149.4
rpFL13-10xHis plus 2mMCaCl$_2$	146.9	163.5	172.9	174.6	751.6	339.8	266.1	177.8
	(%) Relative activity							
(^{0}C)	30	40	50	60	70	80	90	100
rpFL8785-10xHis	10.5	12.8	14.8	18.5	67.1	42.6	31.9	20.7
rpFL8785-10xHis plus 2mMCaCl$_2$	17.8	19.7	23.2	27.8	100.0	50.0	42.0	36.0
rpFL13-10xHis	27.8	31.3	32.2	34.2	141.9	83.0	61.0	39.5
rpFL13-10xHis plus	38.8	43.2	45.7	46.1	198.6	89.8	70.3	47.0

2mMCaCl$_2$

Table 5A. Percentage of remained activity of recombinant alpha-amylases on Analysis of temperature stability for 30 min at pH7 (based on equal amount of protein).

	Activity (U/ml)								
Enzymes	**Initial**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
rpFL8785-10xHis	138.9	134.7	115.9	85.4	67.6	17.6	13.7	16.2	14.0
rpFL8785-10xHis plus 2mM CaCl$_2$	138.9	135.7	126.8	96.2	76.6	37.7	20.6	19.6	17.4
rpFL13-10xHis	187.5	168.9	162.2	108.9	100.6	25.7	13.4	13.8	14.5
rpFL13-10xHis plus 2mM CaCl$_2$	187.5	173.3	171.1	149.1	140.7	64.4	30.0	18.3	13.9
	(%) Remained activity								
Enzymes	**Initial**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
rpFL8785-10xHis	100.00	96.94	83.40	61.47	48.67	12.69	9.83	11.70	10.08
rpFL8785-10xHis plus 2mM CaCl$_2$	100.00	97.67	91.30	69.26	55.11	27.15	14.80	14.09	12.55
rpFL13-10xHis	100.00	90.06	86.46	58.05	53.65	13.71	7.14	7.37	7.75
rpFL13-10xHis	100.00	92.39	91.21	79.50	75.05	34.32	15.99	9.73	7.41

plus 2mM

CaCl$_2$

Table 6A. Activity of recombinant alpha-amylases on analysis of temperature
stability at four different temperatures at pH 7 with different sampling
times (based on equal amount of protein).

Temperature(^{0}C)				37			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	138.9	139.0	135.9	135.0	134.7	129.0	114.9
rpFL8785-10xHis plus 2mM CaCl$_2$	138.9	139.6	136.3	135.0	135.7	125.3	116.1
rpFL13-10xHis	187.5	184.9	175.6	172.3	168.9	154.6	137.2
rpFL13-10xHis plus 2mM CaCl$_2$	187.5	185.4	174.2	172.8	173.3	158.6	153.2
Temperature(^{o}C)				60			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	138.9	129.0	86.5	76.9	67.6	61.2	39.0
rpFL8785-10xHis plus 2mM CaCl$_2$	138.9	134.7	120.5	101.4	76.6	63.6	58.4
rpFL13-10xHis	187.5	134.2	123.7	113.8	100.6	82.2	58.9
rpFL13-10xHis plus 2mM CaCl$_2$	187.5	171.9	157.1	145.9	140.7	105.0	79.4
Temperature(^{0}C)				75			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	138.9	65.8	50.0	25.4	18.0		
rpFL8785-10xHis plus 2mM CaCl$_2$	138.9	82.8	55.7	32.2	20.1		
rpFL13-10xHis	187.5	120.1	81.4	29.5	20.8		
rpFL13-10xHis plus 2mM CaCl$_2$	187.5	129.5	65.4	48.8	22.1		
Temperature(^{0}C)				100			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	138.9	32.9	21.4	16.2	14.0		
rpFL8785-10xHis plus 2mM CaCl$_2$	138.9	38.2	20.4	11.5	12.3		
rpFL13-10xHis	187.5	45.4	23.0	14.1	14.5		
rpFL13-10xHis plus 2mM CaCl$_2$	187.5	69.7	51.8	13.9	13.9		

Table 7A. Percentage of remained activity of recombinant alpha-amylases on analysis of temperature stability at four different temperature, at pH 7 with different sampling times (based on equal amount of protein).

Temperature(^{0}C)				37			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	100.0	100.0	97.8	97.2	96.9	92.9	82.7
rpFL8785-10xHis plus 2mM CaCl$_2$	100.0	100.5	98.1	97.2	97.7	90.2	83.6
rpFL13-10xHis	100.0	98.6	93.6	91.9	90.1	82.5	73.1
rpFL13-10xHis plus 2mM CaCl$_2$	100.0	98.9	92.9	92.2	92.4	84.6	81.7
Temperature(^{0}C)				**60**			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**	**60**	**120**
rpFL8785-10xHis	100.0	92.9	62.3	55.4	48.7	44.0	28.1
rpFL8785-10xHis plus 2mM CaCl$_2$	100.0	97.0	86.8	73.0	55.1	45.8	42.0
rpFL13-10xHis	100.0	71.6	66.0	60.7	53.6	43.8	31.4
rpFL13-10xHis plus 2mM CaCl$_2$	100.0	91.6	83.8	77.8	75.0	56.0	42.3
Temperature(^{0}C)				**75**			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**		
rpFL8785-10xHis	100	47.36	36	18.3	12.97		
rpFL8785-10xHis plus 2mM CaCl$_2$	100	59.58	40.1	23.2	14.434		
rpFL13-10xHis	100	64.03	43.4	15.8	11.116		
rpFL13-10xHis plus 2mM CaCl$_2$	100	69.03	34.9	26	11.808		
Temperature(^{0}C)				**100**			
Sampling time (min)	**0**	**5**	**10**	**15**	**30**		
rpFL8785-10xHis	100.0	23.7	15.4	11.7	10.1		
rpFL8785-10xHis plus 2mM CaCl$_2$	100.0	27.5	14.7	8.3	8.8		
rpFL13-10xHis	100.0	24.2	12.3	7.5	7.8		
rpFL13-10xHis plus 2mM CaCl$_2$	100.0	37.2	27.6	7.4	7.4		

Table 8A. Products formation (reducing sugar) of recombinant alpha-amylases by using 2 substrates at various concentrations.

		Product formation (µmol) from 2 substrates at each sampling time (min)					
Substrate (mg/ml)		Soluble starch as substrate			Cassava starch as substrate		
		0	10	20	0	10	20
rpFL13-	0	0.02	4.16	8.54	0.02	4.07	4.75
10xHis	5	0.02	4.81	9.28	0.02	4.54	5.47
	10	0.02	5.46	10.01	0.02	5.01	6.19
	15	0.02	6.11	10.51	0.02	5.48	6.91
	20	0.02	6.81	10.63	0.02	6.80	6.97
	25	0.02	4.16	8.54	0.02	4.07	4.75
rpFL87	0	0.02	4.61	6.78	0.02	4.73	9.52
85-	5	0.02	5.03	7.46	0.02	5.25	9.81
10xHis	10	0.02	5.45	8.15	0.02	5.76	10.12
	15	0.02	5.87	8.79	0.02	6.27	10.40
	20	0.02	6.60	9.15	0.02	7.00	10.52
	25	0.02	4.61	6.78	0.02	4.73	9.52

Table 9A. Activity and velocity of recombinant alpha-amylases by using 2 substrates at various concentrations (based on equal amount of protein).

Substrate concentration (mg/ml)		Velocity (µmol/min) at 10 min		Activity (U/ml) at 10 min	
		Soluble starch	Cassava starch	Soluble starch	Cassava starch
rpFL13-	0	4.163	4.612	409.06	402.81
10xHis	5	6.115	5.868	433.00	423.86
	10	8.181	8.056	458.19	445.21
	15	9.667	9.360	483.37	467.98
	20	9.683	9.335	483.78	468.15
	25	4.163	4.612	409.06	402.81
rpFL878	0	4.069	4.735	471.75	417.67
5-10xHis	5	5.485	6.273	472.05	432.53
	10	9.435	8.353	472.46	452.52
	15	9.464	9.360	473.22	467.98
	20	9.494	9.361	473.70	467.86
	25	4.163	4.735	409.06	417.67

Table 10A. Kinetic parameters of recombinant alpha-amylases by using 2 substrates from 3 kinetic equations.

Equations	Parameters	rpFL13-10xHis		rpFL8785-10xHis	
		Soluble starch	Cassava starch	Soluble starch	Cassava starch
Michaelis-Menten	K_m (mg)	15.87	9.695	16.12	13.42
	V_{max} (µmole/min)	14.54	1.109	14.85	9.814
	$k_{cat} = (V_{max} / _{[E]in\ reaction})$ (s-1)	91.51	6.98	94.56	62.49
	k_{cat}/K_m = (s-1/mg)	5.77	0.72	5.87	4.66
Lineweaver-burk	K_m (mg)	13.53	9.01	15.04	8.68
	V_{max} (µmole/min)	15.26	12.56	15.93	12.74
	$k_{cat} = (V_{max} / _{[E]in\ reaction})$ (s-1)	96.03	79.04	101.42	81.09
	k_{cat}/K_m = (s-1/mg)	7.10	8.77	6.74	9.34
Edie-Hofstee	K_m (mg)	13.72	9.25	11.48	8.85
	V_{max} (µmole/min)	15.41	12.79	14.1	12.88
	$k_{cat} = (V_{max} / _{[E]in\ reaction})$ (s-1)	96.99	80.50	89.78	82.01
	k_{cat}/K_m = (s-1/mg)	7.07	8.70	7.82	9.27

2.3 KINETIC PARAMETER PLOTS
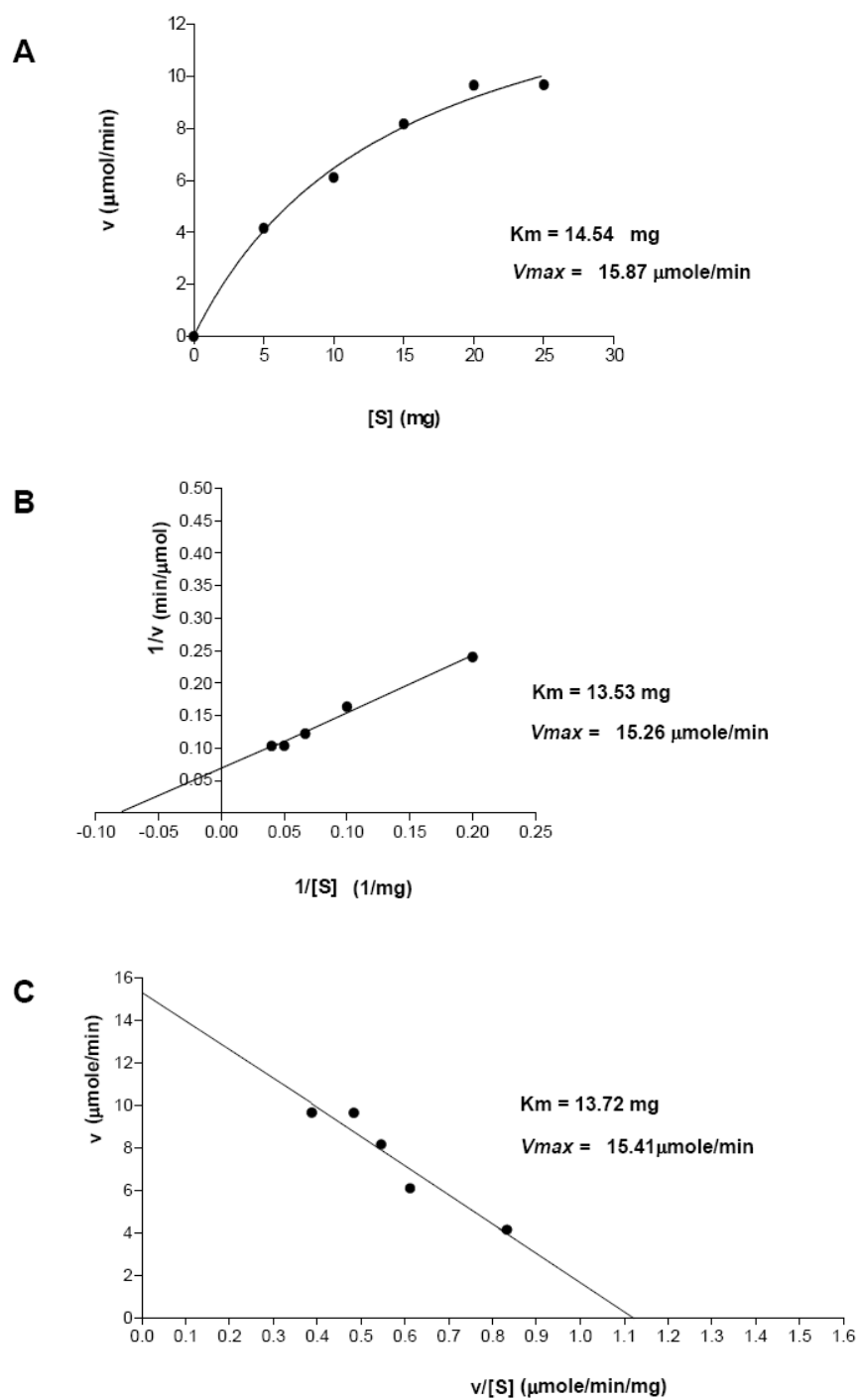
Figure 4A. Kinetic equation plots for soluble starch hydrolysis by rpFL13-10xHis

(A) Michaelis-Menten plot, (B) Lineweaver-burk plot, and
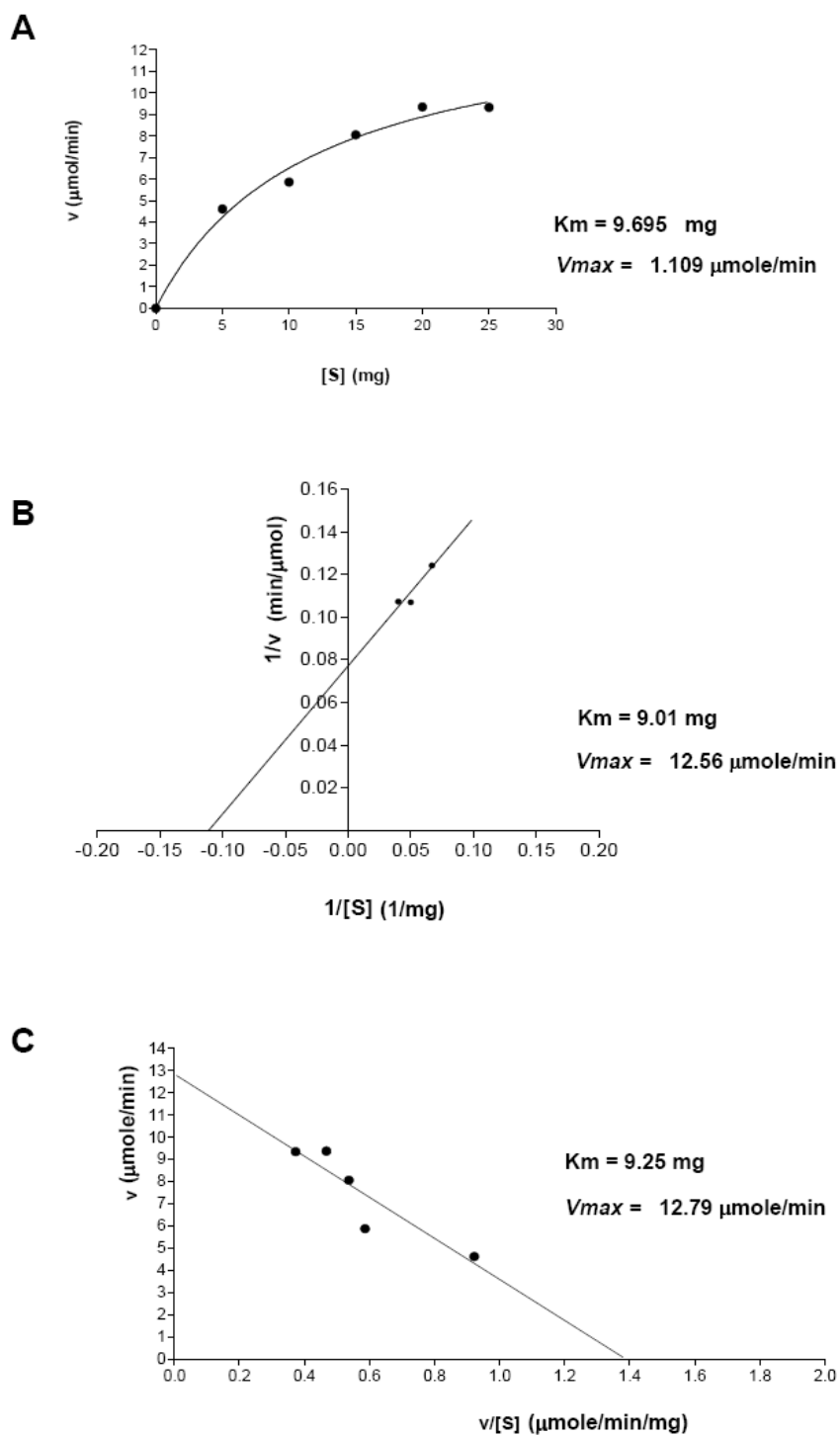
(C) Edie-Hofstee plot.

Figure 5A. Kinetic equation plots for cassava hydrolysis by rpFL13-10xHis

(A) Michaelis-Menten plot, (B) Lineweaver-burk plot, and
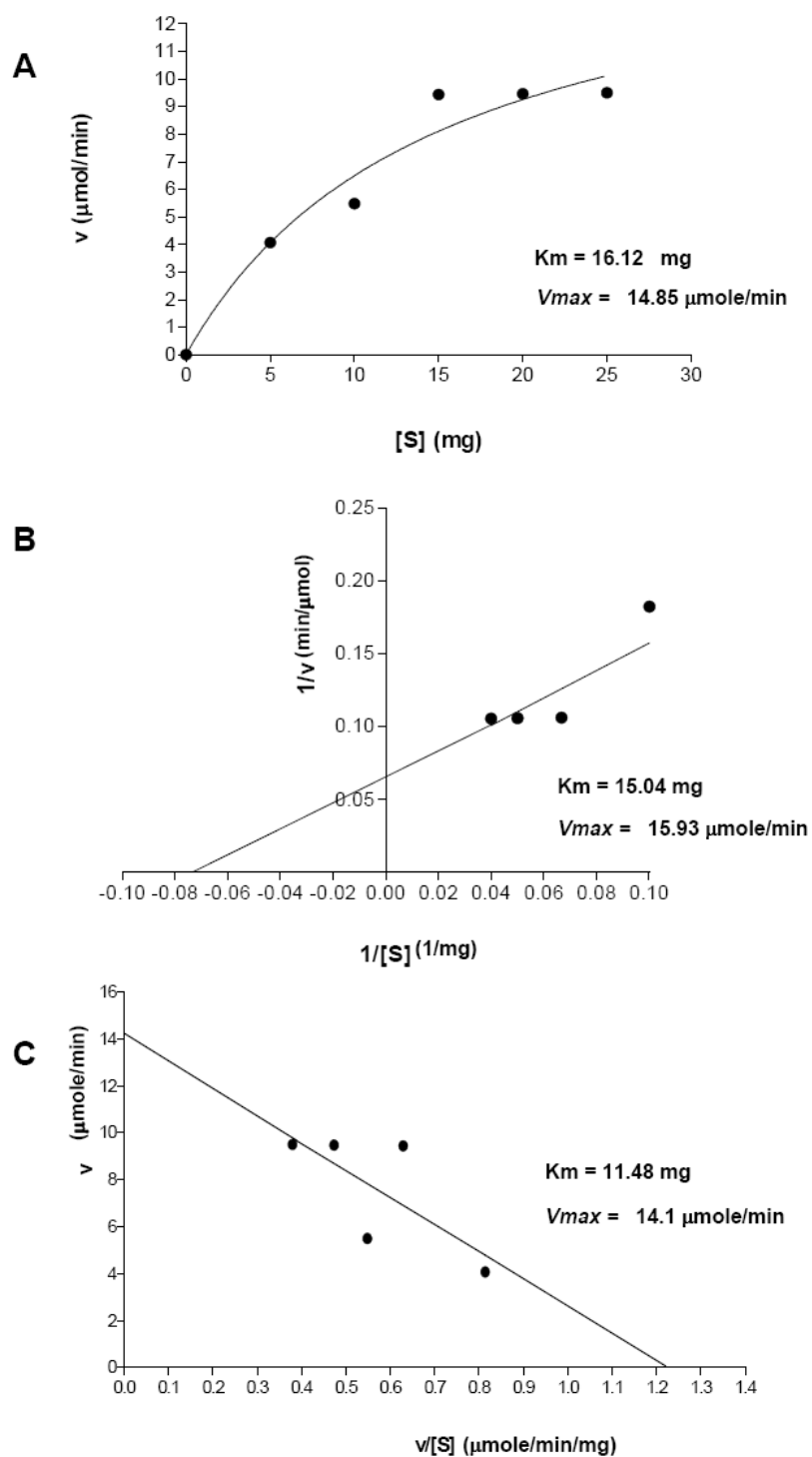
(C) Edie-Hofstee plot.

Figure 6A. Kinetic equation plots for soluble starch hydrolysis by rpFL8785-10xHis

(A) Michaelis-Menten plot, (B) Lineweaver-burk plot, and
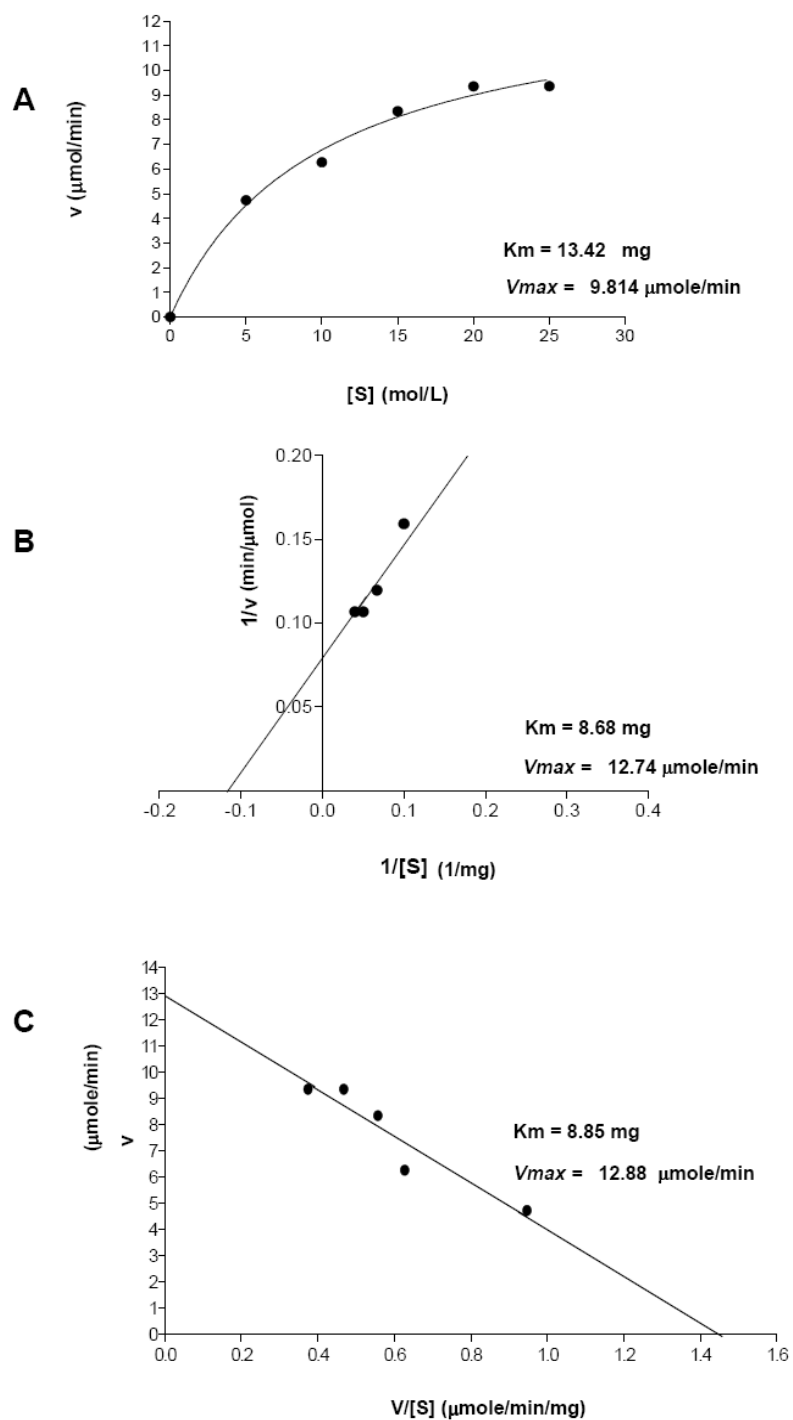
(C) Edie-Hofstee plot.

Figure 7A. Kinetic equation plots for cassava starch hydrolysis by rpFL13-10xHis

(A) Michaelis-Menten plot, (B) Lineweaver-burk plot, and

(C) Edie-Hofstee plot.

2.4 MOLECULAR WEIGHT DETERMINATION
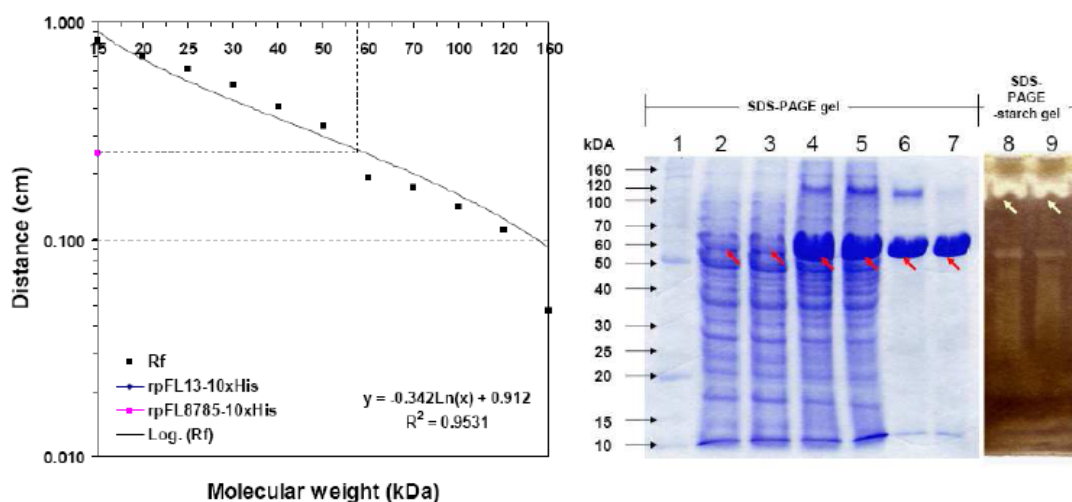
Figure 8A. Molecular weight determination of two recombinant alpha-amylases**.**

Land 1: molecular weight protein marker.

Land 2: rpFL13-10xHis at 0 hr of induction.

Land 3: rpFL8785-10xHis at 0 hr of induction.

Land 4: rpFL13-10xHis at 18 hr of induction.

Land 5: rpFL8785-10xHis at 18 hr of induction.

Land 6: purified alph-amylase from rpFL13-10xHis.

Land 7: purified alpha-amylase from rpFL8785-10xHis.

Land 8: activity of purified alpha-amylase from rpFL13-10xHis.

Land 9:activity of purified alpha-amylase from rpFL8785-10xHis**.**

Table 11A. The R_f of mobile phase (sample dry) and protein samples.

MW (kDa)	15	20	25	30	40	50	60	70	100	120	160
D1 (cm)	5.15	4.4	3.9	3.3	2.6	2.1	1.2	1.1	0.9	0.7	0.3
D2 (cm)	5.2	4.4	3.9	3.3	2.6	2.1	1.25	1.1	0.9	0.7	0.3
Rf	0.815	0.693	0.614	0.520	0.409	0.331	0.193	0.173	0.142	0.110	0.047

Samples	Distance2	Distances2	R_f
rpFL13-10xHis	1.55	1.6	0.248
rpFL8785-10xHis	1.6	1.6	0.252
Dry distance	6.3	6.4	1

2.5 SEQUENCE ANALYSIS
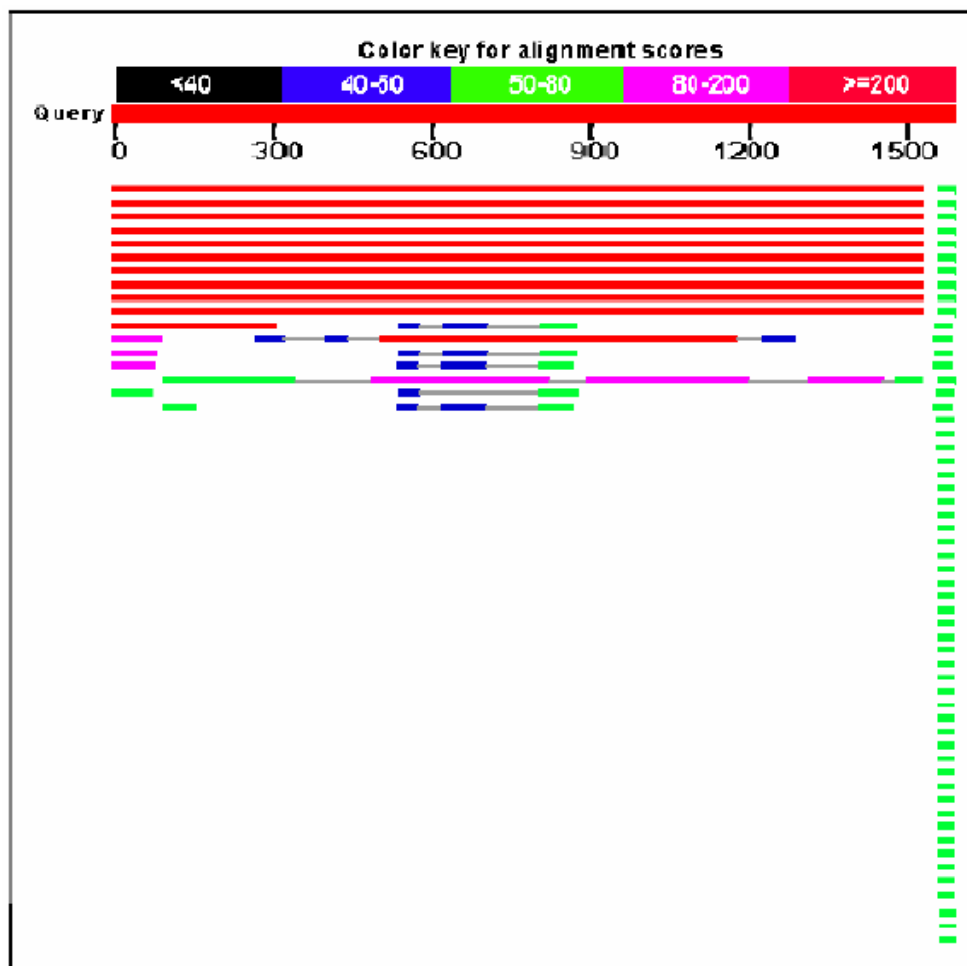

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                                                         Score     E
Sequences producing significant alignments:            (Bits)  Value

gi|5459335|emb|AJ243541.1|CVE243541  Cloning vector pAMY-em1 cat,   3037   0.0
gi|5459332|emb|AJ243540.1|EVE243540  Expression vector pERM-ex1 e   3037   0.0
gi|142510|gb|M13256.1|BACAMYS  B.licheniformis amyS gene            3037   0.0
gi|39551|emb|X03236.1|BLAAMYLG  Bacillus licheniformis 584 alpha-   3005   0.0
gi|142479|gb|M38570.1|BACAMYABL  B.licheniformis alpha-amylase ge   3005   0.0
gi|56160984|gb|CP000002.2|  Bacillus licheniformis ATCC 14580, co   2997   0.0
gi|52346357|gb|AE017333.1|  Bacillus licheniformis DSM 13, comple   2997   0.0
gi|48766831|gb|AY630336.1|  Bacillus licheniformis strain ATCC...   2997   0.0
gi|57335423|emb|AJ786636.1|  Bacillus licheniformis yjeA gene ...   2997   0.0
gi|27903806|gb|AF438149.1|  Bacillus licheniformis from Iran h...   2260   0.0
```

Figure 9A. Blastn analysis of alpha-amylase from *B. licheniformis* DSM 8785.

Figure 10A. Blastn analysis of alpha-amylase from *B. licheniformis* DSM 13.

BIOGRAPHY

Miss Sirima Sukasem was born on January 2, 1976 in Ratchaburee, Thailand. She graduated with a Bachelor degree from Department of Biotechnology, Faculty of Technology, Khon Kaen University, in 1998. In 1999, she continued her Master degree at same faculty and then, she graduated in 2003. She had an experience at Scienctific Promotion Limited in position of sale perspon and product specialist for one year. She had opportunity to study Doctor of Philosophy in School of Biotechnology, Institute of Agricultural Technology at Suranaree University of Technology. During her study, she had experience on her research for one year and had opportunity to study for doctoral program for 6 courses (11 credits) at Division of Food Biotechnology, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Science, Vienna, Austria. She had experience on oral and poster presentation. The oral presentation was in the title of "*Cloning and expression of Thermostable Bacillus licheniformis alpha-amylase in Escherichia coli.*" On 23-25 March, 2004 at Fermentation Research Center for Value Added Agricultureal Prodeuct, Khon Kaen, Thailand**.** Her poster presentation was at the 10th International congress: Genetic of Industrial Microorganism. Prague, Czeck, 24-28 June, 2006. The title was "*Secretion of Bacillus* hydrolytic *enzymes in Escherichia coli expression system.*" She conducted to research in the topic of production and improvement of alpha-amylase by DNA shuffling.

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ทุนการศึกษาที่ได้รับ	
ชื่อวิทยานิพนธ์ :	

ภาษาอังกฤษ PRODUCTION AND IMPROVEMENT OF ALPHA-AMYLASE
BY DNA SHUFFLING

ภาษาไทย การผลิตและพัฒนาคุณสมบัติของเอนไซม์อัลฟา-อะไมเลสด้วย
เทคโนโลยีการสลับสับเปลี่ยนดีเอ็นเอ

สอบวัดคุณสมบัติ 900002 : QUALIFYING EXAMINATION
ผ่านเมื่อภาคการศึกษาที่ 1 ปีการศึกษา 2547

สอบภาษาต่างประเทศ 900003 : FOREIGN LANGUAGE EXAMINATION (ENGLISH)
ผ่านเมื่อภาคการศึกษาที่ 3 ปีการศึกษา 2547

รายชื่อผลงานวิจัยที่ได้รับการตีพิมพ์เผยแพร่:

- รายชื่อบทความวิจัยที่ได้รับการตีพิมพ์ในวารสารวิชาการระดับชาติ/นานาชาติ

  Yamabhai, M., Emrat, S., **Sukasem, S**., Pesatcha, P., Jaruseranee, N., Buranabanyat, B.
  (2007) Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia
  coli* expression systems. **J. Biotechnology.** 133 (1), 50-57

- รายชื่อบทความวิจัยที่ได้รับการตีพิมพ์ในการประชุมวิชาการระดับชาติ/นานาชาติ

  Yamabhai, M., **Sukasem, S**, Pesatcha, P., Jaruseranee, N., Buranabanyat, B. and Emrat, S.
  (2006) Secretion of *Bacillus* hydrolytic enzymes in *Escherichia coli* expression
  system. The 10th International congress: Genetic of Industrial Microorganism.
  Prague, Czeck. 24 - 28 June, 20, 2006

  Sukasem, S. and Yamabhai, M. Cloning and expression of Thermostable *Bacillus
  licheniformis* alpha-amylase in *Escherichia coli*. The 1st International Conference
  on Fermentation Technology for Value Added Agricultural Products

- รายชื่อบทความวิจัยอยู่ในขั้นตอนรอดำเนินการตรวจจากอาจารย์ที่ปรึกษา เพื่อที่จะทำการ
ตีพิมพ์ในวารสารวิชาการระดับชาติ/นานาชาติ

Sukasem, S. and Yamabhai, M.(2007) The Property improvement of *Bacillus licheniformis* alpha-amylase by DNA shuffling.

Presentation

Oral presentation

Sukasem, S. and Yamabhai, M. Cloning and expression of Thermostable *Bacillus licheniformis* alpha-amylase in *Escherichia coli*. The 1st International Conference on Fermentation Technology for Value Added Agricultural Products

Poster presentation

Yamabhai, M., **Sukasem, S**, Pesatcha, P., Jaruseranee, N., Buranabanyat, B. and Emrat, S. (2006) Secretion of *Bacillus* hydrolytic enzymes in *Escherichia coli* expression system. The 10th International congress: Genetic of Industrial Microorganism. Prague, Czeck. 24 - 28 June, 20, 2006

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International Publications

Suginta, W., Vongsuwan, A., Songsiriritthigul, C., Prinz, H., Estibeiro, P., Duncan, R. R., Svasti, J. & Fothergill-Gilmore, L. A. (2004). An endochitinase A from *Vibrio carchariae*: cloning, expression, mass and sequence analyses, and chitin hydrolysis. *Arch. Biochem. Biophys*. 424, 171-180.

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<u>Songsiriritthigul, C.,</u> Kobdaj A. & Suginta W. The active site residues Trp275 and Trp397 are important for the binding selectivity of chitinase A to soluble substrates. Second Annual Symposium of Protein Society of Thailand "Odysseys in Protein Research". Bangkok, Thailand, 20-21 September, 2007.

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