

CONSTITUTION OF DIFFERENT SIGNAL PEPTIDES FOR ENHANCED THERMOSTABLE ALPHA AMYLASE SECRETION IN *Bacillus subtilis*

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Abstract. Three signal peptides of alpha amylase genes of three isolated strains: *Bacillus licheniformis* DA23, *Bacillus subtilis* D5-2, *Bacillus cereus* CN1-5 were successfully sequenced. Three predicted “Sec – type” signal peptides have a length varying from 27 (CN1-5) to 33 residues (D5-2). The secretion of alpha amylase of the recombination *B. subtilis* 168MP_{grac} strain (pHV33–P_{grac}Amy3BT2) with 71.4 ± 6.3 U/ml and the ratio of α – amylase activities to total amount of protein secretion reached 38.05 U/mg was larger than that of 168MP_{amy} with 53.2 U/ml. Base on analyzed results of PAGE and zymogram about molecular weight, alpha amylases in both strains were the same size, nearly 58 kDa. Three signal peptides were constructed in recombinant pHV33 – P_{grac} vectors. To further evaluate the efficiency of these SPs in *B. subtilis*, α -amylase activity was measured. The extracellular amylase activity of signal peptide S_{subtilisD5.2} in 168M was the highest with 76.4 ± 3.7 U/ml in four signal peptide targets. These results indicated that the promoter P_{grac} and signal peptide amylase gen S_{subtilisD5.2} tested in this study might be used for secretion α – amylase in *B. subtilis* 168M.

Keywords: *Bacillus*, signal peptide, secretion, amylase, megaprimer.

Classification number: 1.2.1; 1.3.2; 3.7.2.

1. INTRODUCTION

Amylases are important hydrolase enzymes which have been widely used since many decades. Among the amylase family, α - amylase is an industrially important one because of the ubiquitous nature, easy of production and broad spectrum of applications. This enzyme has potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries as representing approximately 30 % of the world enzyme production [1]. This enzyme randomly cleaves α -1,4- internal glycosidic linkages in starch molecules to hydrolyze them to yield glucose, maltose, dextrans and oligosaccharides. α -amylases can be obtained from animals, plants and microorganisms, especial enzyme from fungal and bacterial sources, have been focused upon and preferred to other sources for applications in industrial sectors [2, 3].

For commercial applications α -amylase is mainly derived from the genus *Bacillus*. It is estimated that *Bacillus* sp. enzymes make up about 50 % of the total global enzyme market.

These organisms not only produce an appropriate range of enzymes but also have the capacity to secrete them into the culture medium at high concentrations. Purification from the culture medium rather than from the cytoplasm considerably reduces downstream processing costs. In recent years, considerable effort has been aimed at developing *B. subtilis* as a host for the production of heterologous proteins. *Bacillus* genus is Gram-positive bacteria that secrete a large number of extracellular proteins of industrial relevance. *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. megaterium* etc. are known to be good producers of α -amylase [3, 4].

Most proteins are transported across the cytoplasmic membrane and secreted directly into growth medium of Gram positive bacteria. They are generally synthesized as precursors with a cleavable signal peptide, that is removed by signal peptidases during or shortly after translocation. The secretion of protein in *Bacillus* genus is synthesized by four pathways: Sec, Tat, Com and ABC transporter [5, 6]. Sec pathway is major secretion for approximately 300 proteins in *Bacillus* genus therefore called the general secretion pathway in this genus [7]. Signal peptide in Sec pathway usually is 18 – 40 amino acids long and although the primary structures of different signal peptides show a little similarity and don't have sequence homologous, this signal always consists of three identifiable domains as: a positively charged amino terminal (N-), a central hydrophobic (H-), and carboxyl-terminal (C-) regions [7, 8]. N region is 1-5 amino acids long, charged positive, and often has two amino acids Lys and Arg [6, 9]. The hydrophobic core (H region) with 7 - 15 amino acids takes an alpha helical conformation when it contacts with the membrane lipid phase. The C - terminal region is hydrophilic and contains the signal peptide cleavage site in the -1 and -3 positions of the signal peptide that is recognized with most of a consensus cleavage site Ala-X-Ala (71 %), V – X –A (18 %) [10]. Alpha amylase of *Bacillus* genus is secreted into growth medium by Sec pathway and then signal peptide is cleaved by Signal peptidase I.

2. MATERIALS AND METHODS

2.1. Bacterial strains and media

Table 1. The content of recombination in Megaprimer method construction.

Strains	Flank Primer	Target gene 1	Recombinant constructs 1	Target gene 2	Recombinant constructs 2
<i>B. subtilis</i> D5.2	DASub Sig5.2	Mature α -amylase <i>B. licheniformis</i> 3BT2	DASsub3BT2Mature	Promoter Pgrac cna vector pHT43	Pgrac-DASsub3BT2Mature
<i>B. cereus</i> CN15	DACer Sig15		DAScere3BT2Mature		Pgrac-DAScere3BT2Mature
<i>B. licheniformis</i> DA23	DALich sig23		DASliche3BT2Mature		Pgrac-DASliche3BT2Mature
<i>B. licheniformis</i> 3BT2			DA3BT2full		Pgrac-DA3BT2full

Table 1 shows the content of recombination in Megaprimer method construction. The collection of *Bacillus* sp. Strains was isolated from many soil samples in different regions in LB with 1% solution starch and bacterial strains were stored at 4 °C for entire study. *E. coli* JM109,

E. coli DH5 α , *B. subtilis* 168M in the collection strains of Biomaterial Lab were used to host strains.

2.2. Additional substrates

The additional substrates for screening bacteria are gathered in Table 2.

Table 2. Additional substrates for screening bacteria in this study.

Substrates	Dilute solutions	Stock concentrations	Final concentration
Ampicillin	H ₂ O deion	50 mg/1 ml	60 μ g/ml
Chloramphenicol	Ethanol 70 % (v/v)	100 mg/1 ml	20 μ g/ml
IPTG	H ₂ O deion	1M	
Xgal	Dimethylformamide	20 mg/ml	40 μ g/ml

2.3. DNA isolation and purification

Total DNA of these strains was isolated mainly by Ausubel' method [11].

DNA plasmid isolation:

+ Isolation of plasmids from *E. coli* by Alkaline Lysis [12] and Thermo Scientific Plasmid DNA extraction kit (GeneJET miniprep kit).

+ DNA plasmid of *Bacillus* was isolated by Voskuil and Chambliss [13].

The DNA product from agarose gel was cut and purified by the GeneJET Gel extraction kit was supplied by the manufacturer.

2.4. Primer sequences

The primer sequences used in this study are presented in Table 3.

2.5. Isolation of signal peptide sequences, target gene fragment by PCR method

The study signal peptide sequences were extracted from strains by PCR method with primer sequences for signal peptide which were designed from original point of the interested α -amylase gene and conservative region I of this gene based on the published sequences in NCBI (Table 3). Standard PCR method was used to amplify the target genes with reaction temperature under optimal conditions optimal for temperature of primers.

2.6. Megaprimer

Megaprimer method is based on polymerase chain reaction and utilizes "flank" primers with two rounds of PCR [14 – 16]. In this article, Megaprimer is the method used to join an interested signal peptide with the target gene and joins promoter with this recombinant construction.

Table 3. Primer sequences in this study.

Primers	Sequences 5' → 3'
DABlisaF	ATGAAACAACAMAAACGGCTT
DABlisaR	GTGGTTGATGACYACATCCCC
DABsusisaF	ATGTTTAMAAAACGATTCAAAAC
DABsusisaR	GTATGATTGATRACCGCTC
DABcesisaF	ATGTTTAAAAAAGTAACAATAG
DABcesisaR	TTATGATTCATAACTACATC
PribaliRsig-SacII	AGCGTCCCATTAAGACTTGCCCGCGGCGCCGCTGCTGACAGAATGA
PribasuRsig-SacII	AGCGTCCCATTAAGACTTGCCCGCGGAGCGTTTTGCAGCCGCCGGG
PribaceRsig-SacII	AGCGTCCCATTAAGACTTGCCCGCGGTGCATATGCTTTACTCCC
Pri2BaliMatur3bt2	GCAAGTCTTAATGGGACGCTGA
P3BL-XbaI	TTAATCTAGACAAAGAAATTTTATAAGAAG
P3BL – EcoRI:	GAGGAAGAATTCCAAAGAAATTTTATAAGA
FprimerPgrac – EcoRI- KpnI	GGCCGAATTCTTAGCTTGGTACCAGCTATTG
RPgrac = 3' XhoI	TCATCTCGAGTTCCTCCTTTAATTGGGA
F _{3BT2mature} – SacII:	GCTCCGCGGGCAAGTCTTAATGGGA
DABsusisaF XhoI	GAGGAACTCGAGATGTTTAAAAAACGATTCA
DABcesisaF – XhoI	GAGGAACTCGAGATGTTTAAAAAAGTAACA
DABlisaF – XhoI	GAGGAACTCGAGATGAAACAACAAAAACG
FgraclinkerSig	CCCAATTAAAGGAGGAACTCGAGATGTTTAAA

2.7. SDS-PAGE

The SDS polyacrylamide gel electrophoresis was performed according to [22] and these proteins are stained silver by the Holtzhauer's protocols [17].

2.8. Results analysis

The result of DNA sequences were analyzed by Serial cloner programs, MEGA 5.0, BioEdit, Chromas, DNADynamo, SigmaPlot, etc. and DNA data are available online at websites [23].

3. RESULTS

In this study, three signal peptides were screened by recombination to integrate the target genes and assessed the ability to increase α -amylase secretion. The host used for this study was *B. subtilis* 168M. The target gene carried on a common object is the mature segment of the α -amylase strain 3BT2 after the cutoff of SPaseI in the Sec pathway. The experimental purpose was mapped in Figure 1.

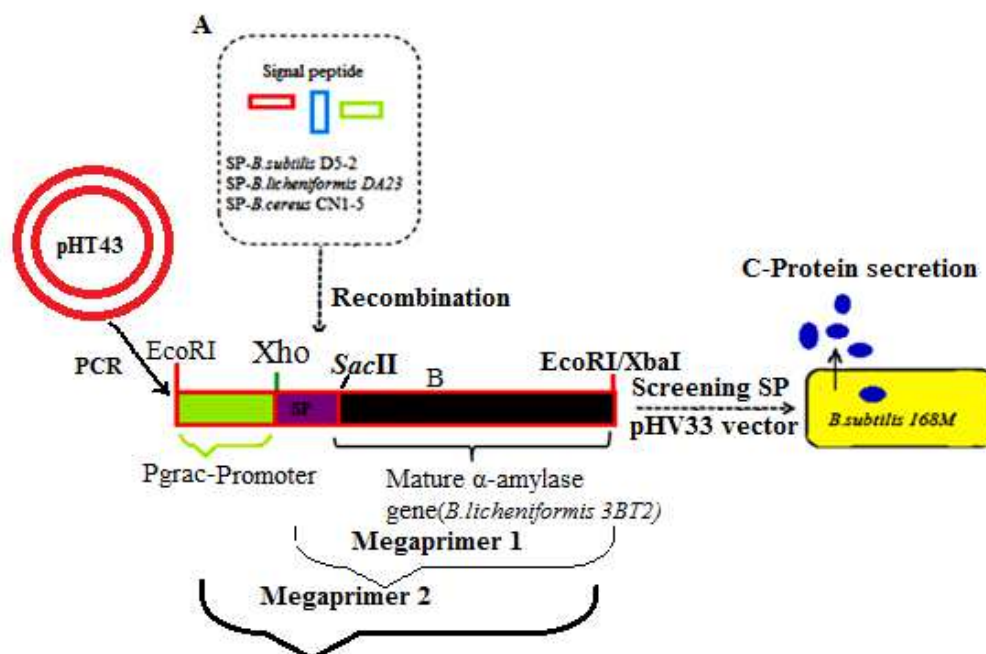


Figure 1. The diagram of the strategy to increase the ability of α -amylase secretion in 168M' host cells (A), three SPs isolated from *B. subtilis*, *B. licheniformis* and *B. cereus* by PCR methods. (B), and the mature α -amylase of strain 3BT2 used as the target protein (C). *Bacillus* 168M was used to screen signal peptide libraries and this strain was used to evaluate the level of secretion of recombinant α -amylase.

3.1. Construction of recombinant Pgrac – SPs – amylase mature gene by Megaprimer method and conjunction with pHV33 vector for screening of amylase activity expression in *E. coli*

There are many methods to improve secretion of alpha amylase activity, but method that used in this report is replaced by signal peptide of wild alpha amylase of by isolated signal peptide alpha amylase genes from three *Bacillus* strains. Because most exported proteins are transported through the Sec pathway in *B. subtilis*, three different Sec-type signal peptides of 3 alpha amylase genes (Signal peptide of gene alpha amylase of *B. subtilis* D5-2 ($S_{\text{subtilisD5.2}}$), *B. cereus* CN1-5 ($S_{\text{cereusCN15}}$), *B. licheniformis* DA23 ($S_{\text{licheniformisDA23}}$) were cloned and fused to the α -amylase gene of *B. licheniformis* 3BT2 (lacking its native signal peptide) by megaprimer method (Figure 2) that exhibit high secretion efficiency in *B. subtilis* pHV33-Pgrac-3BT2Amyfull (pHV Amy3BT2). Four recombinant construction vectors: pHV Amy3BT2, pHV33-Pgrac- $S_{\text{cereusCN15}}$ -3BT2mature (pHV CeSig15), pHV33-Pgrac- $S_{\text{subtilisD5.2}}$ -3BT2mature (pHV SuSig5.2), pHV33-Pgrac - $S_{\text{licheniformisDA23}}$ -3BT2mature (pHV LiSig23) were transformed in *B. subtilis* 168M to form four strains as 168MSubsig52, 168MCeSig15, 168MLisig23, 168M3BT2. These strains could efficiently secrete proteins into the culture medium. Secretion of target gene in recombinant strains was estimated by total protein, activity of α -amylase and protein electrophoresis.

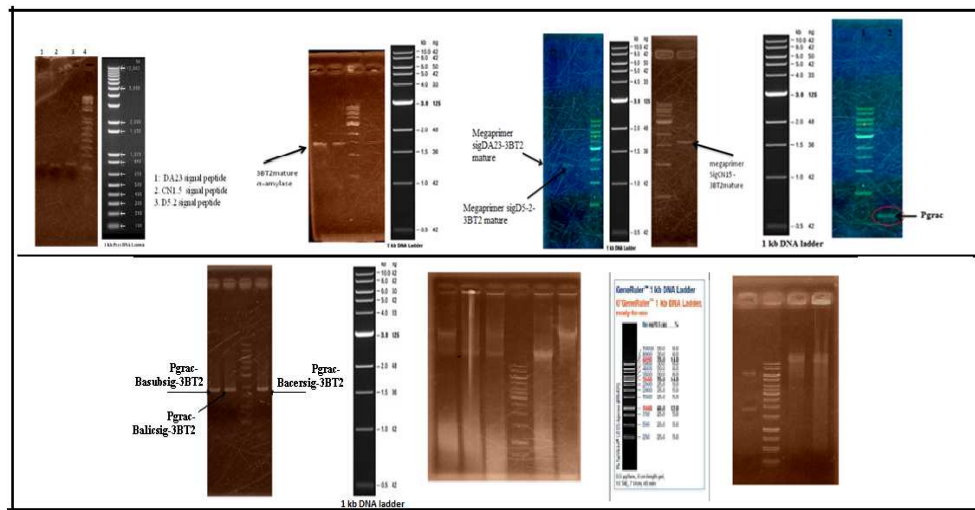


Figure 2. Construction of system to fuse target genes by Megaprimer method.

3.2. Effect of two promoters on amylase expression and secretion

The thermophilic α -amylase from *B. licheniformis* 3BT2 possessed unique traits that made it a great potential candidate for use in the industry. However, this strain produced only small amounts of α -amylase. To solve these problems, the α -amylase gene was cloned and expressed in *B. subtilis* 168M, which is an ideal food-grade host for heterologous protein expression. The promoters Pgrac and P_{Amy} were used to construct 2 different expression vectors for testing to secrete high levels of this α -amylase with initial pHV33 vector to express in *B. subtilis* 168M.

Table 4. The expression and secretion of recombinant amylase in *B. subtilis* 168M.

Strains	Amylase		Total Protein Secretion (μ g/ml)	The ratio of α – amylase activities to total amount of protein secretion (U/mg)
	Agar diffusion (mm)	DNSA (U/ml)		
168MPamy	14	53,2 \pm 5,5	1763,5 \pm 15	30,17
168MPgrac	21	71,4 \pm 6,3	1876,4 \pm 25	38,05
168M	1	2,1 \pm 0,3	1798,7 \pm 30	1,17

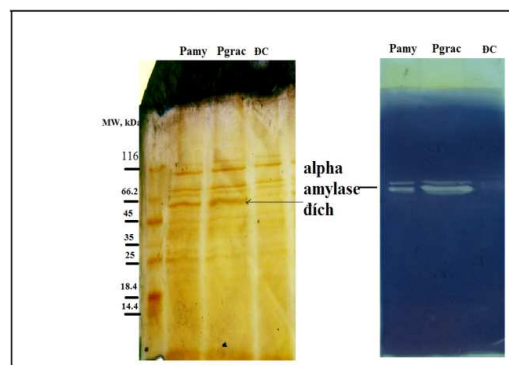


Figure 1. Effect of promoter on amylase secretion of recombinant strains.

The results in Table 4 and Figure 3 showed that secretion efficiency of the constructed pHV33 vector with promoter P_{grac} was higher than that of the vector with promoter P_{amy}. To compare the activity alpha amylase of the recombination *B. subtilis* 168MP_{grac} strain (pHV33–P_{grac}–Amy3BT2) and *B. subtilis* 168MP_{amy} strain (pHV33–P_{amy}–Amy3BT2, recombinant vector construction of pHV33 containing the P_{amy} promoter of *B. subtilis* 168M and alpha amylase gene of *B. licheniformis* 3BT2 was noticed a little activity of 53.2 U/ml after 36 h. It was less 1.3 fold than activity of the recombination strain containing gene cluster of P_{grac} promoter and target gene (71.4 U/ml) at the same time. These results indicated that, P_{grac} promoter in this study might be useful for the expression and secretion of target gene in *B. subtilis* and so it was chosen for further research. Zymogram analysis and amylase activity of two recombination strains showed that the crude extracts of alpha amylase by ethanol were analyzed by PAGE. The alpha amylase was clearly detected in both strains with the same molecular weight nearly 58 kDa. Ying *et al.* expressed high levels of the hyperthermophilic α -amylase from *Thermococcus* sp. HJ21 in *B. subtilis*, the promoters P_{grac}, P_{xylA}, P₄₃, and P_{hag} were used to construct four different expression vectors for testing [18].

3.3. Evaluation of expression efficiency of alpha amylase of recombinant constructors in *B. subtilis* 168M

To determine the expression efficiency of the four SPs on the amylase secretion, for plasmid pHV33–P_{grac}–3BT2Amyfull (pHV_{Amy}3BT2), pHV33 – P_{grac} – S_{cereusCN15} – 3BT2mature (pHV_{Ce}Sig15), pHV33 – P_{grac} – S_{subtilisD5.2}–3BT2mature (pHVSuSig5.2), pHV33 – P_{grac} – S_{licheniformisDA23} – 3BT2mature (pHV_{Li}Sig23) were transformed in *B. subtilis* 168M. These recombinant strains were named as 168M3BT2, 168MCeSig15, 168MSubsig52, 168MLisig23, respectively.

Table 5. Effect of different SPs on α -amylase expression and secretion.

Strains	α – amylase activities (U/ml)			Total Protein Secretion (mg/ml)	The ratio of α – amylase activities to total amount of protein secretion (U/mg)
	Agar diffusion (mm)	Cell extraction	Liquid culture		
168MSubsig52	23±1.5	3.1±0.3	76.4±3.7	1896.4±35.0	40.9±3.13
168MCeSig15	16.5±2	4.9±0.7	47.7±4.6	1767.2±21.6	26.9±2.23
168MLisig23	21±1.5	6.3±0.6	71.3±3.2	1824.7±27.1	38.7±0.72
168M3BT2	20.5±2	4.1±0.4	68.6±5.1	1787.3±20.2	37.5±1.42

The results in Figure 4 and Table 5 showed that efficiency expression of alpha amylase of four signal peptides was dissimilarity among these strains. The extracellular amylase activity of signal peptide S_{subtilisD5.2} in recombinant 168MSubsig52 was highest with 76.4 ± 4.3 U/ml and that of signal peptide S_{amy CN1-5} in 168MCeSig15 was lowest in four strains with 47.7 ± 4.6 U/ml. The secretion efficiency of 168MSubsig52, 168MCeSig15 and 168MLisig23 containing three signal peptides S_{amy D5-2}, S_{amy DA23} and S_{amy CN1-5} were approximately 112 %, 104 % and 70 % alpha amylase activity of 168M3BT2, respectively. The optimal signal peptide S_{subtilisD5.2} was

used for further experiment. SPs play a very important role in the transport of secretion proteins by the Sec pathway because they interact with SecA proteins, the signal recognition particle (SRP) and they have recognized site for signal peptidases [5]. The interaction between SP and mature protein is also known to affect the secretion of proteins [5, 9]. Therefore, an effective signal peptide for the secretion of any target protein is the most important choice. Several reports were done for identifying effective signal sequences [19]. In the same species, the secretion efficiency of proteins with different signal peptides was different effects and the secretion process may be affected by these SPs [20, 21].

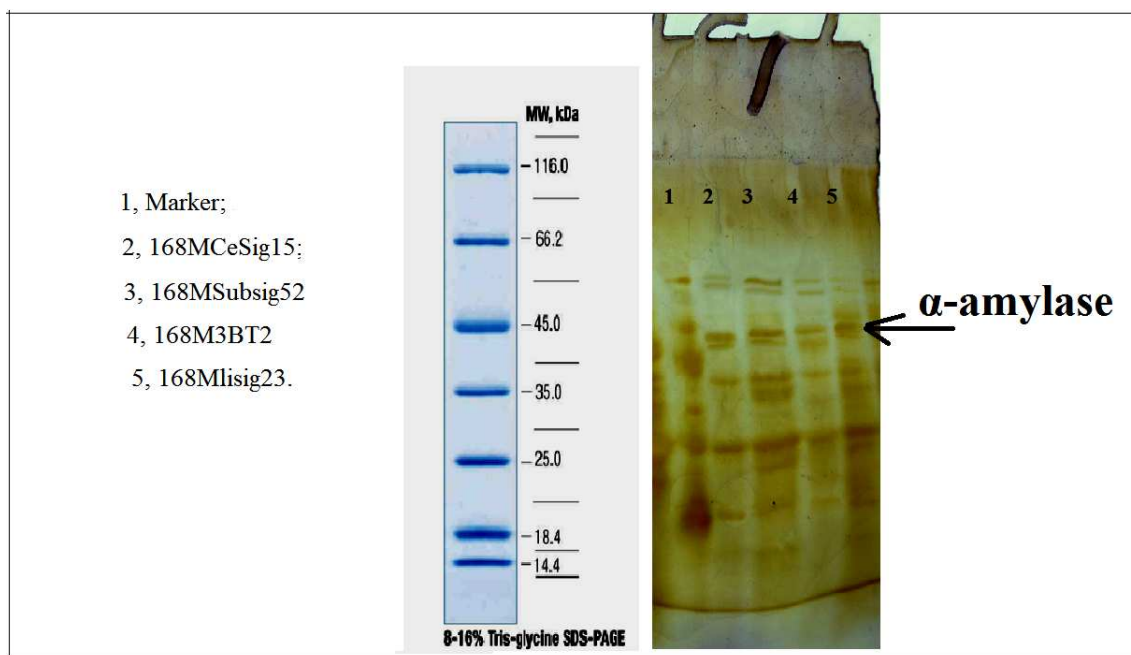


Figure 2. Effect of different SPs on alpha amylase secretion.

4. CONCLUSION

In this study, the effect of replacement SP alpha amylase of *B.licheniformis* 3BT2 on secretion one in *B. subtilis* was investigated by removing the SPs alpha amylase gene of other species in the same genus of *Bacillus* as *B. cereus*, *B. subtilis*, *B. licheniformis*. Four recombinant target genes transformed in *B.subtilis* 168M to evaluate for effect on the expression of amylase activity and secretion of protein of pHV33-Pgrac-3BT2Amyfull (pHV Amy3BT2), pHV33 – Pgrac – S_{cereus}CN15 - 3BT2mature (pHVCeSig15), pHV33 – Pgrac – S_{subtilis}D5.2-3BT2mature (pHVSuSig5.2), pHV33 – Pgrac – S_{licheniformis}DA23 - 3BT2mature (pHVLiSig23),. The results showed that the vector containing the alpha amylase SP of *B. subtilis* 168 and Pgrac promoter was found to have the highest transcriptional activity and produce the highest amylase activity.

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