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Expression of *pps* and *fen* promoters in *Bacillus subtilis* under optimal production condition.

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ABSTRACT

The expression of P_{pps} plipastatin promoter from *Bacillus subtilis* 168, P_{fen} fengycin promoter from the wild type *B. subtilis* ATCC 21332 and P_{repU} from *Staphylococcus aureus* were studied by fusion to lacZ gene with homologue recombinant to amyE locus of strain 168; three mutant strains BMG04, BMG05 and BMG06, were constructed respectively. P_{pps} and P_{fen} expressions from these latter strains were compared to the expression of the constitutive promoter P_{repU} from *S. aureus*. At the more expressed cases under volumetric oxygen transfer coofficient k_La 0.015 s^{-1} value, the expression of P_{repU} was about 35 folds more than P_{pps} at the end of the expotentielle phase, while P_{repU} expression was about 4 folds more than P_{fen}. This work proved the strength and the efficiency of P_{repU} promoter in *B. subtilis* 168 and the possibility to remplace the weak P_{pps} promoter to obtain overproducer strain of plipastatin lipopeptide.

Keywords: *Bacillus subtilis*, Promoters, Expression, Lipopeptides, Plipastatin, Fengycin.

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INTRODUCTION

Genome project of the *B. subtilis* 168 DNA sequence revealed the presence of two large operons encode nonribosomal peptide synthetases surfactin and plipastatin [1]. Significant homology was observed between *B. subtilis* F29-3 fengycin synthetase gene [2] and the plipastatin operon from strain 168 [2, 3, 4]. Fengycin is a fungicide lipopeptide consists of the same kind of amino acids and b-hydroxy fatty acids as plipastatin [5, 6]. For the first time, plipastatin was isolated from *Bacillus cereus* BMG302-fF67 as an inhibitor of phospholipase A2 [7]. This amphiphilic cyclic peptide are composed of 10 a-amino acids linked to b-hydroxy fatty acid and their length vary from C14 to C18 [8].

The phenomenon of 'induced systemic resistance' [ISR], is the ability of some beneficial bacteria to protect plants indirectly by stimulating inducible defence mechanisms; this mechanism is systemic as root treatment with bacteria which transform the protective effects to above-ground plant parts [9]. However, *B. subtilis* was reported as one of ISR inducers [10] and plipastatins or fengycins, one of lipopeptides produced by members of this strain; it have antifungal effect on the growth of a large number of fungi as Fusarium species [11, 12] which may occur by ISR mechanism . Moreover, *B. subtilis* lipopeptides were recently studied for their versatile activity in the biocontrol of a wide range of phytopathogens [13, 14, 12].

Since the promoter plays an important role in the regulation of the transcription, it was reported that the deletion of the region between positions -55 and -42 of the fengycin synthetase operon promoter of *B. subtilis* F29-3 reduces the promoter activity by 64.5%. It was also indicated that the transcriptional fusions in the *B. subtilis* DB2 chromosome by mutating the sequence can reduces the promoter activity because the inefficiency of transcription.

By the early 1980's, the *Escherichia coli* genetics was revolutionizing by the construction of *lacZ*-gene fusions [15]. The use of β-galactosidase colorimetric assay to tag a gene product was a powerful new tool for studying gene regulation as it was difficult to quantitate it before. In 1982, The vector of Zuber and Losick allowed them to study *spoVG* gene expression [16]. Similar studies using integration vectors to create fusions with other genes was quickly followed [17, 18, 19].

The weak production of plipastatin in *B. subtilis* 168 was detected before by [20] 32 mg/l in landy Mops medium after 24 hours of culture. They also studied the surfactin promoter expression, but no further studies on plipastatin promoter expression were investigated. It was mentioned that AbrB regulator functions as a repressor of plipastatin gene expression. In *B. subtilis*, one of the most important key regulators which control gene expression during the the transitional phase is the AbrB protein [21,22]. This protein have an important role in preventing gene expression in actively growing cells and during the transition phase. This protein can reorganize the expression of more than 100 post-exponential-phase genes with different biological functions among them; antibiotic production, motility, of competence development for DNA uptake, biofilm formation, extracellular enzymes synthesis and sporulation. Although AbrB functions mainly as a repressor of gene expression, AbrB also acts as an activator of some genes [23,24] and till now we couldn't demonstrated any direct interaction of AbrB with RNA polymerase.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The strains used in this work are presented in Table 1. *B. subtilis* and Escherichia coli strains were grown aerobically in Luria-Bertani [LB] medium. The strains were grown at 30°C for *B. subtilis* and at 37°C for *Echerichia coli* JM109 in [LB] medium supplemented with ampicillin [50 µg ml^{-1}; Sigma, St. Louis, MO].

Molecular biology methods

Genomic DNA was prepared using the Wizard Genomic DNA Purification Kit and protocol [Promega Corp., Appl Microbiol Biotechnol Madison, USA]. Primers; were designed using the published sequence of *B. subtilis* 168 plipastatin operon [accession no. AL009126]. Known nucleic sequences of *B. subtilis* strains that are involved in the synthesis of fengycin or plipastatin operons were analysed with Needle online software [25]. All used primers was listed in table 2. The PCR conditions consisted of an initial denaturation step at 94°C

for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at melting temperature for each primer, and extension time depend on fragment size at 72°C. The final extension step was at 72°C for 2 min.

Strains construction

***B. subtilis* reconstruction with gene reporter system**

New mutant strains were constructed with *lacZ* ORF fused to their plipastatin/ fengycin promoters. This reporter gene used to compare these expressions under Optimal Plipastatin Production Conditions OPPC [26].

Table 1: Strains and plasmids

Strain or plasmid	Description	Source or reference
Bacterial strains		
Echerichia coli JM109	recA1, endA1, gyrA96, thi, hsdR17 (rK–,mK+), relA1, supE44,Δ(lacproAB), [F´, traD36, proAB, lacIqZΔM15]	Lab stock
B. subtilis 168	wild type, trpC2, sfp^0	Lab stock
B. subtilis BMG04	trpC2, amyE::P_{pps168}-lacZ cat	This study
B. subtilis BMG05	trpC2, amyE::$P_{fen21332}$-lacZ cat	This study
B. subtilis BMG06	trpC2, amyE::P_{repU}-lacZ cat	This study
B. subtilis ATCC 21332	Wild-type	Lab stock
Plasmids		
pGEM-T Easy	Cloning vector, Ap^r	Promega
pDG1661	Integration vector, spoVG-lacZ Ap^r, Cm^r, Spc^r	Guéroult-Fleury et al. (1996)
pBG106	P_{repU}-neo fragment, Ap^r, Nm^r	Leclère et al. (2005)
pMG105	*Hind*III-*EcoR*I P_{PPS} 168 fragment ligated within EcoRI site of pDG1661	This study
pMG106	*Hind*III-*EcoR*I P_{fen} ATCC 21332 fragment ligated within EcoRI site of pDG1661	This study
pMG107	EcoRI P_{repU} fragment from pBG106 ligated within EcoRI site of pDG1661	This study

Table 2: Different primers used for the construction and verification of *B. subtilis* strains

Primers	Primer sequence	Tm (C)
P_{pps} Promoter fwd	5'TAATAGCGGGAGACCTGTTTTC'3	50
P_{pps} Promoter rev	5'AAAAAGCAGAAAAATGACAATAAAAGA'3	
*amyE*_fwd	5'CTTCGGTATGTGATTGTGAAGC'3	60
amyE rev	5'GCAGACAATATCAGCATCCTTG'3	

Promoters expression under Optimal Plipastatin Production Conditions OPPC

The influence of oxygen transfer on plipastatin/ fengycin production under two different regimes covering the tested oxygen transfer conditions in the same power dissipation with two different volumetric oxygen transfer coefficient k_La values; k_La 0.06 s^{-1} and k_La 0.015 s^{-1} was applicated as described [26] in landy modified medium at 30 C˚.

α- amylase activity

This activity was determined by growing *B. subtilis* colonies overnight on LB plates containing 1% soluble starch and then staining the plates with a potassium iodide and iodine solution.

β-Galactosidase activity test

1 ml of culture medium was incubated for 20-60 min on ice to stop growth. After centrifuging 2 min at 12000 g, the pellet was suspended in 0.5 ml of Z buffer, and then was lysed with 30 µl of chloroform under strong agitation for 15 sec. Then both the tube and the ONGP fresh solution were incubated at 37ºC for 5 min. 200µl of the substrate [ONGP] was added to each tube and the time is measured, 0.5 mL 1 M Na_2CO3 was added to stop the reaction after appearance of yellow colour. Samples were Vortex and, the reaction time was time noted precisely.

1 mL of each sample was transferred to an eppendorf tube and centrifuged 5 min at 12000 g to remove debris and chloroform. The optical density at 420 nm was recorded. The activity was calculated according to the following equation:

$$\text{Miller Units} = \frac{1000 \times (OD_{420})}{T \times V \times (OD_{600})}$$

OD_{420} reflects subtilistrate hydrolysis
OD_{600} reflects cell density
T = time of the reaction in minutes
V = volume of culture used in the assay in ml.

RESULTS AND DISSCUSSION

Expression analysis of plipastatin/fengycin promoters by fusion to *lacZ* gene

The efficiency of the constitutive promoter P_{repu} was proved before in *B. subtilis* ATCC 6633 [27, 14]. It was important to verify of this efficiency in *B. subtilis* 168 before undertake the replacement of the native promoters of plipastatin/ fengycin operons. The srength of P_{repu} was compared to that of P_{pps} promoter from *B. subtilis* 168 and P_{fen} promoter from *B. subtilis* ATCC 21332 by analyzing the strength these promoters using a gene reporter system which constitutes an extensive toolbox for the study of promoter regulation. The three promoters; P_{pps}, P_{fen} and P_{repu} were fused to the *lacZ* gene with chloramephnicole as a resistance gene and inserted in *B. subtilis* 168 by homologue recombinant to amyE locus. The resulting plasmids were used to transform *B. subtilis* 168, leading to strains BMG04, BMG05 and BMG06, respectively. In these strains, β-galactosidase or β-gal activities [expressed in Miller units] were used to study the expression levels of the lipopeptides plipastatin/ fengycin synthetases operons using the OPPC.

Plipastatin/ fengycin promoter sequence comparison from *B. subtilis* 168 and ATCC 21332

Plipastatin/fengycin promoters from *B. subtilis* 168 and ATCC 213321 strains were sequenced after PCR amplification and cloning.. The product size was 382 bp from total promoter region 385 bp. The sequencing and alignment results revealed 96.9% of identity with 100% identity in the two promoter boxes -10 F[TATAGT], -35 F [TTGTAC] and RBS sites, respectively. Strain ATCC 21332 promoter sequence showed absence of ten sequenced nucleotides compared to strain 168.

Plipastatin/ fengycin operons seems to have several and complicated expression regulators, whereas sigma F factor is one of regulators control this operon. sigma F factor gene consider member of some regulons, one of them are AbrB regulons which are transcription repressor of plipastatin operon [28]. Another pleiotropic regulator *degQ* was introduced in *B. subtilis* YB8 strain into strain 168 expressing *sfp* gene and resulted in a 10-fold increase in the plipastatin production [29]**.** Fengycin promoter of *B. subtilis* F29-3 have UP element between positions -55 and -39, which is critical for fengycin synthesis and its deletion reduces the promoter activity by 64.5 [30], but in this study the absence of the ten sequenced nucleotides observed for the promoter of ATCC 21332 may due to the relative increase in this promoter activity than this of strain 168 .

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Since the regulation of fengycin or plipastatin transcription are complicated and may have other factors other than the UP element, further work are requested to elucidate fully how these factors affect their synthesis.

Figure 1: Needle EMBOSS alignment of P_{pps} 168 and Pfen ATCC 21332 sequenced promoters, twelve bases differences [in red colour], no differences in -10 F, -35 F boxes and RBS sites.

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EMBOSS_001         1 TAGCGGGAGACCTGTTTTCCAAGAGAAAACAGGTTTTTTTATGTCTGTAA     50
                     ||||||.||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 TAGCGGCAGACCTGTTTTCCAAGAGAAAACAGGTTTTTTTATGTCTGTAA     50

EMBOSS_001        51 TAACGCTTTGTCGAACCTTCCAACAATTAGAAGAATGATGCACAAAAGAA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 TAACGCTTTGTCGAACCTTCCAACAATTAGAAGAATGATGCACAAAAGAA    100

EMBOSS_001       101 AATTAGTCCGATTAAAAAGTAGTGTATAAGACCTTATGTAAAACAATACT    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 AATTAGTCCGATTAAAAAGTAGTGTATAAGACCTTATGTAAAACAATACT    150

EMBOSS_001       151 TTTATCACTTTATATCCGGAAATTTGATAAAAAAAGAAACGAAAAATAAA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       151 TTTATCACTTTATATCCGGAAATTTGATAAAAAAAGAAACGAAAAATAAA    200

EMBOSS_001       201 GAAATAATACTATATTCAAAAAAAATGTTATAAAAATTTAATGTTTTTTA    250
                     |||||||||||||||| ||||||||||||||||||||||||||||||||||
EMBOSS_001       201 GAAATAATACTATATTC-AAAAAAATGTTATAAAAATTTAATGTTTTTTA    249
                       -35                      -10
EMBOSS_001       251 TTGTACTTTAAAAAATATCCGTTAATATAGTGCATATATGGATTATATAG    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       250 TTGTACTTTAAAAAATATCCGTTAATATAGTGCATATATGGATTATATAG    299

EMBOSS_001       301 TCATATAATTTCTTTTATTGTCATTTTTCTGCTTTTTTCTACATTTTCTT    350
                     ||||||||||||||||||||||||||||||||||           |||||||
EMBOSS_001       300 TCATATAATTTCTTTTATTGTCATTTTTCTGCT----------TTTTCTT    339
                                          RBS
EMBOSS_001       351 ATCCTCTTATTATGAGAACTGGAGGGAATCCG    382
                     ||||||||||||||||||||||||||||||||
EMBOSS_001       340 ATCCTCTTATTATGAGAACTGGAGGGAATCCG    371
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Verification of *B. subtilis* derivative strains construction

To verify that the promoter was inserted in the appropriate sense within the plasmid pDG1661, *Sph*I and *EcoR*I double digestion was performed to release the *cat* gene [1280 bp]. If the promoter was in the anti-sense direction, a fragment of 1620 bp [*cat* gene + P_{pps}] and [*cat* gene + P_{fen}] would have been obtained. The constructions of these plasmids are in the right direction.

The new plasmids pMG105, pMG106 and pMG107 were used to transform *B. subtilis* 168 by the natural competence method. The colonies were obtained on the LB petri dishes supplemented with chloramphenicol. One mutant strain was selected for each construction and named BMG04, BMG05 and BMG06, respectively. After the extraction of genomic DNA, the verification of the new strains was effected by PCR using *amy*E primers to detect an insertion within the amylase gene of the recombinant plasmid. An amplicon of about 5 kb from the three *B. subtilis* derivatives was detected compared to the 3 kb fragment from the mother strain 168, indicating that the fragments bearing P_{pps}– *lacZ,* P_{fen}– *lacZ* and P_{repu}– *lacZ* plasmid were successfully integrated into the new strains.

As the insertion of 3kb fragment occurred in the *amy*E gene locus, amylase activity was checked. Strain 168 showed an amylase activity, whereas strains BMG04, BMG05 and BMG06 can't hydrolyse the starch.

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Comparison of P_{pps}, P_{fen} and P_{repU} promoters strength in the *B. subtilis* 168

The P_{pps}, P_{fen} and P_{repU} promoters expression of BMG04, BMG05 and BMG06 strains were compared under two different k_La values; k_La 0.06 s^{-1} and k_La 0.015 s^{-1} and cultivated in landy modified medium at 30 C°. As showed figure 2, P_{pps} promoter have gradual increase through the 24 h of cultivation and the maximum expression [β-gal activity] value was 90 Miller units [main standard deviation SD ± 7.2] at k_La 0.015 s^{-1} followed by k_La 0.06 s^{-1} [58 Miller units [SD± 6.3]. The same behaviour was mentioned to both P_{fen} and P_{repU} promoters, but P_{fen} showed more strengh in expression than P_{pps} and reached to maximum expression of 510 Miller units [SD ± 10.1] at k_La 0.015 s^{-1} followed by k_La 0.06 s^{-1} [137 Miller units [SD ± 7.4]. The P_{repU} promoter strength in *B. subtilis* 168 proved its efficiency and strength as it seem to be strong at the both k_La values, at 0.015 s^{-1} the expression was 2006 Miller units [SD ± 13.6] followed by [1420 Miller units [SD ± 11.8] at k_La 0.06 s^{-1}.

Figure 2: Plipastatin[P_{pps}], fengycin [P_{fen}]and P_{repU} promoters expression in *B. subtilis* 168 by fusion to *lacZ* gene measured in miller units, during 24 hours of culture in Landy modified medium, whereas [●P_{fen}], [■P_{pps}],[▲P_{repU}], [A] under k_La 0.06 s^{-1} and [B] under k_La 0.015 s^{-1}.

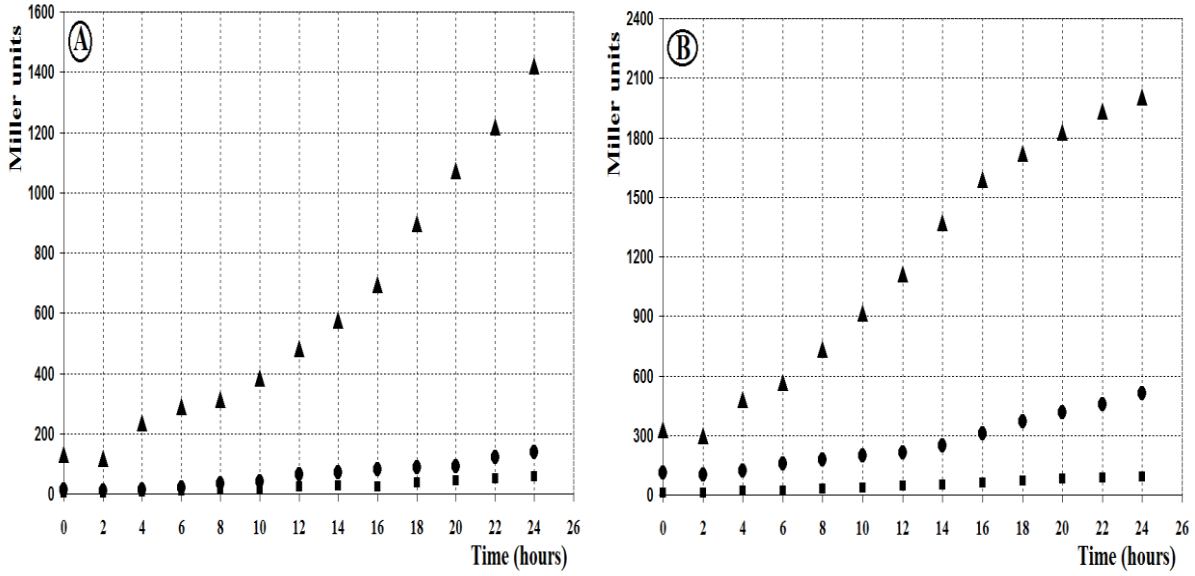
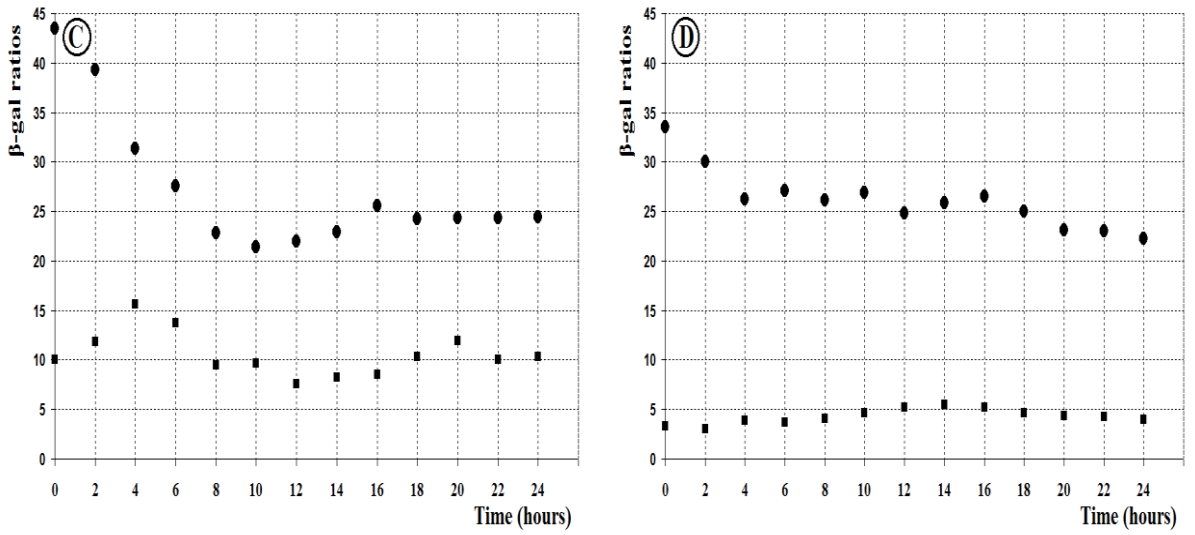


Figure 3:The ratios between β-galactosidase activity in *B. subtilis* 168 by fusion to *lacZ* gene measured in miller units, whereas [●] is the ratio between β-gal activity of P_{repU} / β-gal activity of P_{pps}, [■]is the ratio between β-gal activity of P_{repU} / β-gal activity of P_{fen}, [C] under k_La 0.06 s^{-1} and [D] under k_La 0.015 s^{-1}.



To arrising the high expression of P_{repU} comparing to both P_{pps} and P_{fen}, the ratio between β-gal activity of P_{repU} and β-gal activity of P_{pps} activity was determined at the two cases [0.015 s^{-1} and k_La 0.06 s^{-1}] and proved the weakness of P_{pps} expression in *B. subtilis* 168 compared to the high strength of P_{repU}. Figure 3 [C] under k_La 0.06 s^{-1} revealed that the expression of P_{repU} is about 43 folds more than P_{pps} at the beginning of the cultivation, then it decrease to reach 23 folds at the end the expotentielle phase. After 10 hours of culture, the ratio of expression continue arrising to reach about 26 folds at 16 hours. This revealed that the expression of gene dependent on P_{pps} promoter repressed at the end of the expotentielle phase of growth, which aren't the same for P_{repU}, whereas its expression is efficient and strong from the beginning of the growth. The ratio between β-gal activity of P_{repU} and β-gal activity of P_{fen} activity was determined at the same case [0.06 s^{-1}]. P_{fen} was found to be more strong than P_{pps} expression in *B. subtilis* 168, whereas the expression of P_{repU} is about 15 folds more than P_{fen} at the first 6 hours of the growth and start to decrease till reach to about 8 folds at 12 hours of culture.

Figure 3 [D] under k_La 0.015 s^{-1} showed 35 folds of activity for P_{repU} than P_{pps} compared to 4 folds of activity for P_{repU} than P_{fen} at the beginning of the cultivation, while the activity reached to 23 folds for P_{repU} than P_{pps} at the end of 24 hours of growth.

These results proved strength and efficiency activity of the P_{repU} promoter in *B. subtilis* 168, so it could be used to remplace P_{pps} promoter in the strain producer derivative BMG01, which was constructed before [31]. This was proved before by [14] who remplace the native mycosubtilin promoter of *B. subtilis* ATCC 6633 by the constitutive promoter P_{repU}, which enhanced the production 15 folds in the resulted strain BBG100. Another replacement for the weak plipastatin promoter P_{pps} of our strain 168, with other strong promoter *amyQ* was obtained after the insertion of the functional *sfp* gene from *Bacillus amloliqufaciens* S499 at the *amyE* locus of strain168 chromosome and lead to strain 2504 and caused enhancement in plipastatin production from traces to 434 mg/l [32].

REFERENCES

[1] Kunst FN, Ogasawara I, Moszer et al. Nature 1997 ; 390: 249–256.
[2] Chen CL, Chang LK, Chang YS, Liu ST and Tschen JSM. Mol Gen Genet 1995; 248:121–125.
[3] Tognoni A, Franchi E, Magistrelli C, Colombo E, Cosmina P, and Grandi G. Microbiology 1995; 141:645–648.
[4] Tosato V, Albertini AM, Zotti M, Sonda S, and Bruschi CV. Microbiology 1997;143: 3443–3450.
[5] Steller S, Vollenbroich D, Leenders F, Stein T, Conrad B, Hofemeister J, Jacques P, Thonart P, and Vater J. Chem Biol 1999; 6:31–41.
[6] Vanittanakom N, Loeffler W, Koch U, and Jung G. J Antibiot 1986; 39:888–901.
[7] Umezawa H, Aoyagi T, Nishikiori T, Okuyama A, Yamagishi Y, Hamada M, and Takeuchi T. J Antibiot 1986; 39:737–744.
[8] Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, and Thonart P. Appl Biochem Biotechnol 2001; 91: 551–561.
[9] Van Loon LC, Bakker P, and Pieterse CMJ. Annu Rev Phytopathol 1998 ; 36: 453–483.
[10] Kloepper JW, Ryu CM, and Zhang SA. Phytopathology 2004; 94: 1259–1266.
[11] Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, De Pauw E, and Thonart P. Appl Biochem Biotechnol 1999; 77: 223–233.
[12] Ongena, M. and Jacques, P. Trends Microbiol 2008; 16: 115–125.
[13] Souto GI, Correa OS, Montecchia MS, Kerber NL, Pucheu NL, Bachur M, and Garcia AF. J Appl Microbiol 2004; 97: 1247–1256.
[14] Leclère V, Béchet M, Adam A, Guez JS, Wathelet B, Ongena M, Thonart P and Gancel, F. Appl Environ Microbiol 2005; 71: 4577–4584.
[15] Casadaban MJ, Martinez-Arias A, Shapira SK, Chou J. Method Enzymol 1983; 100:293-308.
[16] Zuber P and Losick R. Cell 1983; 35:275-283.
[17] Ferrari E and Howard SMH, Hoch JA. In J A 1985; 180-184.
[18] Piggot PJ and Curtiss CAM. J Bacteriol 1987; 169:1260-1266.
[19] Lewandoski M and Smith I. Plasmid 1988; 20:148-154.
[20] Coutte F, Leclère V, Béchet M, Guez JS, Lecouturier D, Chollet-Imbert M, Dhulster P and Jacques P. J Appl Microbiol 2010; 109:480-491.

[21] Phillips ZE and Strauch MA. Cell Mol Life Sci 2002; 59: 392–402.
[22] Strauch MA and Hoch JA. Mol Microbiol 1993; 7: 337–342.
[23] Kim HJ, Kim SI, Ratnayake-Lecamwasam M, Tachikawa K, Sonenshein AL and Strauch M. J Bacteriol 2003;185: 1672–1680.
[24] Fisher SH, Strauch MA, Atkinson MR and Wray LV. J Bacteriol 1994; 176: 1903–1912.
[25] Needleman SB and Wunsch CD. J Mol Biol 1970; 48:443-453.
[26] Fahim S, Dimitrov K, Gancel F, Vauchel P, Jacques P, Nikov I. Bioresource technology 2012; 126: 1-6.
[27] Tsuge K, Akiyama T, Shoda M. J Bacteriol 2001; 183:6265-6273.
[28] Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, Bidnenko E, *et al*. Science 2012; 2 :335.
[29] Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M. Antimicrob Agents Chemother 1999; 43:2183-2192.
[30] Ke WJ, Chang BY, Lin TP, Liu ST. J Bacteriol 2009; 191:4615-4623.
[31] Hussein W and Fahim S. Int J cuur Micobiol App Sci 2015; 4(11): 177-184.
[32] Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P. Environ Microbiol 2007; 9:1084-1090.