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Plipastatin Over-production in *Bacillus subtilis* using Site Direct Mutation.

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ABSTRACT

Construction of a plipastatin overproducing strain derivative of *Bacillus subtilis* 168 (BMG01) which harbor *sfp*$^+$ functional gene responsible for the lipopeptide production was performed using site direct mutation technique by the replacement of the *pps* weak promoter by a constitutive one P_{repU} originated from the replication gene *repU* of *Staphylococcus aureus*, whereas the plasmid construct was dedicated to promoter exchange by recombinant homologue in *B. subtilis* and the derivative strain was named *B. subtilis* BMG03. The constitutive ability of the P_{repU} promoter was proved by estimation of fengycin (plipastatin) production level of the new strain *B. subtilis* BMG03. Different factors were taken into account to set the optimal fengycin (plipastatin) production condition which drastically affect the lipopeptides synthesis and can be considered strain-dependent requirements; as volumetric oxygen transfer coefficient (k_La) and voulme mean power dissipation (P_{VL}). Under this condition, the fengycin (plipastatin) concentration of *B. subtilis* BMG03 was 507 ± 6.42 mg.L^{-1} with 1162 ± 9.82 mg.L^{-1} of surfactin, while for the mother strain BMG01 fengycin (plipastatin) concentration was 91 ± 11.2 mg.L^{-1} with 1023 ± 9.53 mg.L^{-1} of surfactin and as expected no lipopeptides production for *B. subtilis* 168 was detected. The production of strain BMG03 was compared to; *B. amyloliquefaciens* FZB42 which was produced 198 ± 16.13 mg.L^{-1} of fengycin type with 59 ± 11.7 mg.L^{-1} and 403 ± 12.14 mg.L^{-1} surfactin and mycosubtilin type, respectively. The production of *B. amyloliquefaciens* S499 was 228 ± 11.02 mg.L^{-1} of fengycin type with 173 ± 13.9 and 602 ± 11.5 mg.L^{-1} surfactin and bacillomycin type respectivelly. While, *B. subtilis* ATCC 21332 was produced 360 ± 17.35 mg.L^{-1} of plipastatin with 197 ± 12.36 mg.L^{-1} of surfactin.

Keywords: Fengycin, Bacillus subtilis, Promoter, surfactin, Lipopeptide, k_La.

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INTRODUCTION

The highly demand of microbial lipopeptides is surging due to their low toxicity and environmental friendly nature composition with the wide range of potential industrial applications and other utility in human welfare[1]. The lipopeptides production on large scale and application considered restricted by the high cost of their production, therefore the economical large scale production remains a challenge[2]. In the year 2003, the antibiotic cyclic lipopeptide daptomycin was approved in USA by Food and Drug Administration (FDA) for the treatment of skin infections caused by certain microorganisms belongs to gram-positive group[3]. The increasing of drug resistance bacteria numbers has rised a persistent need for lipopeptides as alternative antimicrobial agents to be used in dairy products and food preservation as well as in clinical applications[4]. *Bacillus* genus contain members that are considered efficient as large scale producers of these bioactive molecules[5]. The four families of *Bacillus* lipopeptides; surfactin, iturin, fengycin and kurastiken were used in biological control of plant diseases which showed antagonistic activities against various phytopathogens[6]. Other applications of lipopeptides extend beyond human health care, including use of surfactin against plant virus[7]; fengycin as fungicide for crop treatment[8] and avoiding associated effects of a conventional synthetic pesticides[9]. Lipopeptides also improve amphiphilicity and compatibility with the lipid wall of cell membranes compared to peptides, and this can enabling the delivery of actives into cells via endocytosis[10]. Lipopeptides syntheized by non-ribosomal peptide synthesis (NRPSs) or hybrid polyketide synthesis NRPSs (PKSs/NRPSs) from *B. subtilis*. Each NRPS module contains three domains; adenylation domain, PCP domain and thiolation domain. The adenylation domain (A) is responsible for activation of the selected amino acidas as an adenylate (A-domain). The NRPS Modules are multi-enzymes organized in iterative functional units and catalized different reactions which leads to peptide transformation. During 1950s and 1960s, lipopeptides group were isolated from *Bacillus* sp. and and till now more than a 100 different compounds can be described. From these lipopeptides group is the fengycin or plipastatin family which its discovery was concomitant as the German team discovered fengycin from *B. subtilis* as an anti-fungal agent[11]. While, the Japanese team discovered plipastatin from *B. cereus* as a phospholipase A2 inhibitor[12]. Only a small structural difference between fengycin and plipastatin was found; the L and D forms of tyrosine, which are in position 3 and 9, respectively, for plipastatins and 9 and 3 for fengycins and doubt still exists till now about them as well as their biological activities. Production of fengycin was also determined by *Bacillus thuringiensis*[13,14]. Fengycins or plipastatins are lipo-decapeptides with an internal lactone ring and with a β-hydroxy fatty acid chain (C_{14}- C_{18})[14], they also have two structures A and B. Fengycins are less haemolytic than surfactins and iturins but have an advantage of strong antifungal activity. Fengycin have the ability of penetration and solubilization of lipid bilayers and this is dependant on the concentration[15]. In this work, we constructed a derivative of *B. subtilis* 168 plipastatin overproducer strain by replacement of the weak native promoter P_{pps} by a constitutive one P_{repU}.

MATERIALS AND METHODS

Bacterial strains and culture condition.

The bacterial strains and plasmids used in this study are presented in (Table 1), The cultures collections of *B. subtilis subsp. subtilis* str 168 and its drivatives *B. subtilis* BMG01 and BMG03 (plipastatin overproducer) in addition to *B. subtilis* ATCC 21332; *B. amyloliquefaciens* S499; *B. amyloliquefaciens* FZB42 were used to co-produce lipopeptides families[16]. Cultures were performed in 0.01 S^{-1} of oxygen supply (K_La) condition (50 mL standard Erlenmeyer flask; shaking frequency of 200 min^{-1} and the relative filling volumes (*Rv*) of 0.4 $mL.mL^{-1}$ liquid/flask), this condition was tested in each *Bacillus* strain, the cultures were performed during 48 h of fermentation (stachinary phase) at 30°C in modifed Landy MOPS medium with glutamic acid, presented results are means of triplicate experiments ± standard deviation[16,17]. The *Echerichia coli* DH5α strain was grown at 37°C in [LB] medium supplemented with ampicillin [50 µg ml^{-1}; Sigma, St. Louis, MO][18].

Table 1. Bacterial strains and plasmids used in the study.

Bacterial strains	Description	Source
***B. subtilis* ATCC 21332**	**Wild type**	**Lab stock**
***B. amyloliquefaciens* S499**	**Wild type**	**ProBioGEM***

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***B. amyloliquefaciens* FZB42**	**Wild type**	**ProBioGEM***
***B. subtilis* 168**	**trpC², sfp⁰**	**Lab stock**
***B. subtilis* BMG01**	A mutant derivative from *B. subtilis* 168, trpC2, sfp+	**Hussein and Fahim[17]**
***B. subtilis* BMG03**	A mutant derivative from *B. subtilis* BMG01, trpC2, *amyE* :: *sfp+ cat* , (P*repU- neo*)::*ppsA*	**This study**
***E. coli* DH5α**	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ⁻	**Lab stock**
Plasmids	Description	Source
pGEM-T Easy	Cloning vector, Ap^r	Lab stock
pBG106	P*repU*-neo fragment, Ap^r, Nm^r	Leclère et al.[19]
pMG108	1.332 kb *SalI-Not*I P*repU*-neo fragment from pBG106 inserted into double digested *SalI-Not*I pGEM-T Easy	**This study**
pMG109	**1.120 kb *dacC* fragment cloned into pGEM-T Easy**	**This study**
pMG110	1.120 kb *Sac*I- *SalI dacC* fragment from pMG109 inserted into pMG108	**This study**
pMG111	1.340 kb *pps*A fragment cloned into pGEM-T Easy	**This study**
pMG112	1.340 kb *AatII- NcoI* fragment from pMG111 inserted into pMG110	**This study**

ProBioGEM: Laboratoire des Procédés Biologiques, Génie Enzymatique et Microbien, France.

Molecular Biology Methods

Primers design and Polymerase Chain Reaction (PCR) conditions

Primers were designed for partial *dacC* and partial *ppsA* genes involved in fengycin (plipastatin) lipopeptide operon synthesis using the published sequence of *B. subtilis* 168 plipastatin operon (accession no. AL009126). Primers designed to have artificial sites suitable with pGEM-t easy vector restriction sites (Fig. 1), whereas *dacC* fwd have *SacI* artificial site bold underlined bases and *SalI* underlined in *dacC* rev, *pps*A fwd have *NcoI* artificial sites bold underlined and *SphI* sites underlined in *ppsA* rev (Table 2). *B. subtilis* genomic DNAs strains were isolated using DNA purification kit (Wizard®Genomic, Promega). PCR conditions started with initial denaturation step at 94°C for 2 min and followed by 35 cycles of 45 sec at 94°C, annealing step at 50°C for 30 sec and elongation step at 72°C for 90 sec.

Table 2. Primers used in new *B. subtilis* strain derivative construction.

Primers	Sequence	Product size
dacC fwd	5-GCACTTTCCGGG**GAGCTC**-3	1120bp
dacC rev	5-TTAGATAA**GTCGAC**AACCAACTCTG-3	
*pps*A fwd	5-TAAC**CCATGG**CCAAAGGAGA-3	1340bp
ppsA rev	5- CGAAACA**GCATGC**GTCTTGT-3	

Agarose gel electrophoresis

Agarose was dissolved in 100 mL 0.5X TBE buffer pH 8.8. Gels were coloured by addition of Gel Red 10 µL/100 mL. Gels were run in an electrophoresis unit using 0.5X TBE buffer pH 8.8 at 100 volts for two hours. Gels were photographed using Gel Documentation system.

Gel Extraction

The obtained fragments were extracted and purified using Zymoclean™ Gel DNA Recovery Kit (Epigenetics Company).

Cloning and transformation protocol

Cloning and transformation protocol was performed as described into pGEM®-T and pGEM®-TEasy Vector Systems Technical manual from QIAGEN as follows; 2 µL of each ligation reaction added to a sterile (17×100 mm) polypropylene tube or a 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 0.1

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ng uncut plasmid for determination of the transformation efficiency of the competent cells. The *E. Coli* JM109 high efficiency competent cells tube removed of frozen from storage and placed in an ice bath until just thawed (about 5 min), then 50 µL of cells are transferred carefully into each tube prepared before. The tubes are flicked gently to mix and place them on ice for 20 min, then heat-shock for 45-50 s in a water bath at exactly 42°C. The tubes are returned immediately to ice for 2 minutes; after that, 950 µL room-temperature SOC medium (20 g bactotryptone, 5 g yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose and 20 mM Mg^{+}(10 mM $MgSO_4$ + 10 mM $MgCl_2$) are added to the tubes containing cells transformed with ligation reactions and 900 µL to the tube containing cells transformed with uncut plasmid. The tubes are incubated for 1.5 h at 37°C with shaking (~150 rpm) and 100 µL of each transformation culture are plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. The plates incubated overnight (16-24 h) at 37°C. If 100 µl are plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1×10^8 cfu/µg DNA.

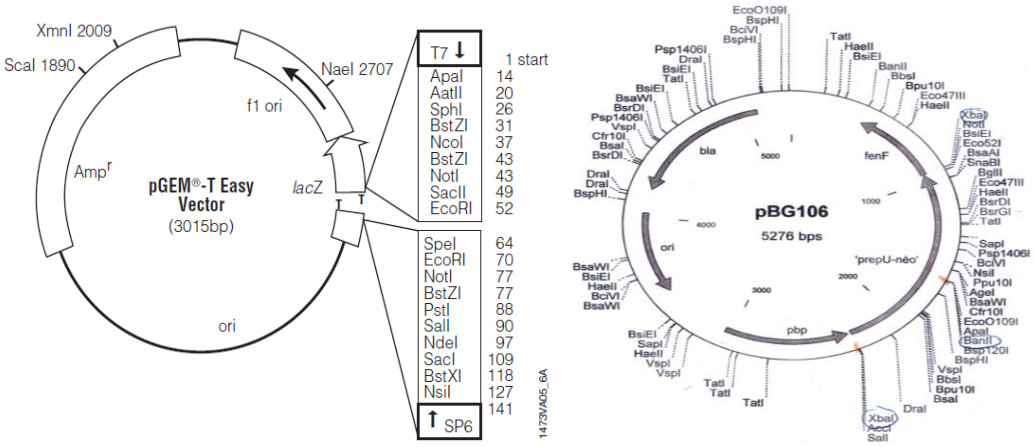

Figure 1. Vectors maps used in the plipastatin overproducer *B. subtilis* strain.

Construction protocol with the constitutive promoter p_{repU}.

Both pBG106 and pGEM-T Easy vector (Fig. 1) were *SalI* and *NotI* digested. The P_{repU}-neo cassette was released from pBG106 and inserted between *SalI* and *NotI* sites of pGEM-T Easy Vector to obtain pMG108. *dacC* fragment was generated by PCR (1120 bp) (Fig. 2), which have two artificial sites *SacI and SalI* was ligated to pGEM-T Easy Vector. The ligation mixture was transformed into *E. coli* DH5α cells. The resulting plasmid was named pMG109. Both pMG108 and pMG109 were *SacI and SalI* double digested, the *dacC* fragment was then inserted between the *SacI and SalI* sites of pMG108 to obtain pMG110. *ppsA* fragment was then generated by PCR (11340bp) (Fig. 2) with two artificial sites of *NcoI* and *SphI. ppsA* fragment was cloned into pGEM-T Easy vector and the resulting plasmid named pMG111. Both pMG111 and pMG110 plasmids were double digested with *NcoI* and *SphI* to obtain and *ppsA* fragment which was inserted between the *NcoI* and *SphI* sites of pMG110 and the final plasmid construction was named pMG112. While, the final plasmid construction was used to transform *B. subtilis* BMG01 (*B. subtilis* 168 derivative by insertion of *sfp*+ gene) according to Hussein and Fahim[17].

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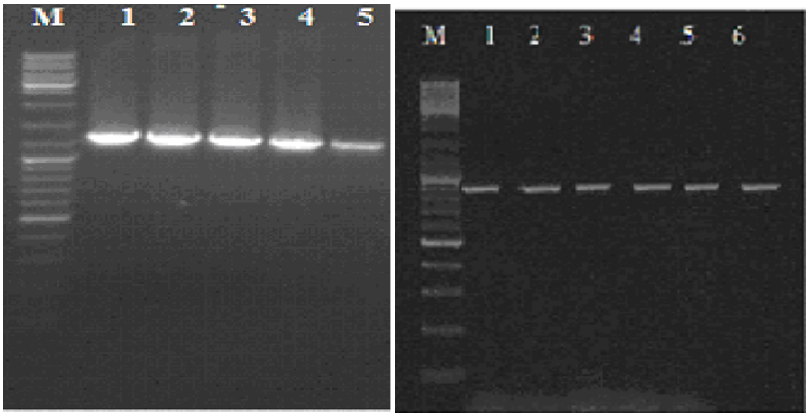

Figure 2. PCR generated *dacC* and *ppsA* partial fragments from *B. subtilis* 168 with 1120 and 1340 bp sizes, respectively.

Transformation by natural competence for *B. subtilis.*

An overnight culture of the strain was prepared in LB medium (10 mL) and incubated at 37ºC under 150 rpm shaking frequancy.The next day, about (0.7 O.D 600 nm) was calculated of the overnight culture, and was centrifuged at 4000 rpm for 10 min, and then the pellet was washed with sterilized H_2O and re-centrifuged. 5 mL of MS1 solution was added to the pellet and incubated about 5 h at 37ºC (150 rpm). The culture was diluted (1/10 volume) with MS2 solution and incubated 90 min at 37ºC (150 rpm). About 5 µL of plasmid (100 ng.µL^{-1}) was added to the competent cells and the mixture incubated for 30 min minimum in a water bath at 37ºC, samples were then plated on LB with the selective antibiotic 20 µg.mL^{-1} neomycin and incubated for 24 h at 37ºC.

Quantitative analysis of lipopeptides

Both Plipastatin (Fengycin) and other lipopeptides were quantified using High Performance Liquid Chromatography (HPLC) after their extraction from 1 mL supernatant of culture by C_{18} cartridges (Extract-clean SPE 500 mg, Grace Davison-Alltech, Deerfield, IL, U.S.A). The lipopeptide adsorbed on the C_{18} was then eluted in 8 mL pure methanol. The eluted lipopeptides were dried by rotating vapour and speed vacum followed by re-dissolving in 200 µL of pure methanol. The surfactin concentration was determined by reverse phase C_{18} HPLC (600 s, Waters, Milford, MA, U.S.A) equipped with a Merck C_{18} column (5 mm; Darmstadt, Germany)[16]. The total yield was collected before analysis by (HPLC) in C_{18} column (5 µm; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was isocritical acetonitrile-water-trifluoroacetic acid solvent system (80:20:0.5, 55:45:0.5, 45:55:0.5 [vol/vol/vol] for surfactin and 40:60:0.5 [vol/vol/vol] for fengycin or plipastatins and iturin, respectively. (20 µl) of collected samples were injected and then eluted at a flow rate of one ml.min^{-1}. Surfactin (Sigma), fengycins or plipastatins, iturins were purchased with purity of 98% as standards. The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules[16].

RESULTS AND DISCUSSION

B. subtilis BMG03 constuction and verification.

P_{repU}, a promoter originating from *Staphylococcus aureus*, was inserted in plasmid named pBG106 (Leclère *et al.*, 2005) which led to increase in iturin (mycosubtilin) production 15 folds in the resulting strain *B. subtilis* BBG100 than the mother strain *B. subtilis* ATCC 6633. In our previous work, the strength and the efficiency of P_{repU} promoter in *B. subtilis* 168 have been proved to remplace the weak P_{pps} promoter, whereas at the more expressed cases under optimal fengycin (plipastatin) condition, the expression of P_{repU} was about 35 folds more than P_{pps} at the end of the expotentielle phase[18]. The construction was designed firstly by generation of *dacC* (1120bp) and *ppsA* (1340bp) fragments from *B. subtilis* 168 by PCR and then were cloned in pGEM-T Easy vector. The resulting plasmids were named pMG109 and pMG111, respectively. The final construction plasmid pMG112 used to transform *B. subtilis* BMG01 for plipastatin overproduction construction *B. subtilis* BMG03 (Fig. 3).

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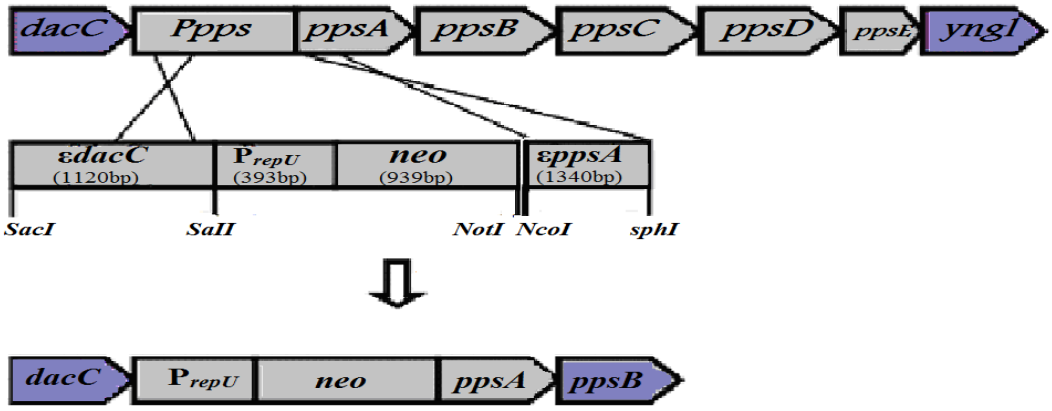

Figure 3. *pps* promoter replacement by the P_{repU}-*neo* cassette in *B. subtilis* BMG01.

The verification of *B. subtilis* BMG03 new strain construction was performed by PCR using the two primers located up and downstream from the inserted cassette *dacC* fwd and *ppsA* rev (Table 2). The PCR generated large fragment of about 3.79 kb for the new *B. subtilis* BMG03 strain, compared to 2.8 kb for the mother strain *Bacillus subtilis* 168. The fragment was sent to sequencing and the result showed that the P_{pps} promoter of the new strain *B. subtilis* BMG03 was replaced by the P_{repU}-*neo*.

Estimation of fengycin (plipastatin) production.

In order to test the fengycin (plipastatin) production level of the new strain *B. subtilis* BMG03 to prove the constitutive ability of the new promoter P_{repU}-*neo*, the fermention process was performed with the strain *B. subtilis* BMG03 and its mother strain *B. subtilis* BMG01 to verify the overproducing ability of plipastatin production. A lot of studies have pointed out different factors for their effect on lipopeptide production that this effect can be strain-dependent requirements[19,20]. as well as volumetric oxygen transfer coefficient (k_La) and voulme mean power dissipation (P_{VL}) could drastically affect the lipopeptides synthesis, take into account these coursers to set the optimal fengycin (plipastatin) production condition[16,17]. Under this condition, the co-production ± standard deviation (SD) was realized, the fengycin (plipastatin) concentration of *B. subtilis* BMG03 was 507 ± 6.42 $mg.L^{-1}$ of plipastatin with 1162 ± 9.82 $mg.L^{-1}$ of surfactin, while for the mother strain BMG01 was 91 ± 11.2 $mg.L^{-1}$ with 1023 ± 9.53 $mg.L^{-1}$ of surfactin and as expected no lipopeptides production for *B. subtilis* 168 was detected (Fig 4).

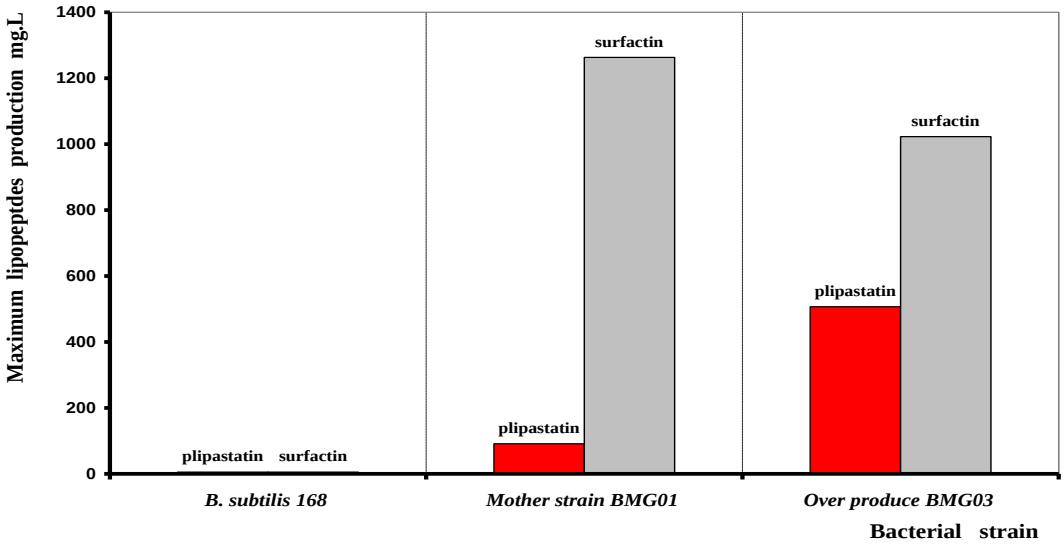

Figure 4. Lipopeptides production of *B. subtilis* 168, *B. subtilis* BMG01 and *B. subtilis* BMG03 under the optimal fengycin (plipastatin) production condition.

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On the other hand, the results at the various study conditions, *B. amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type). *B. subtilis* strains produced two families of lipopeptides surfactin and fengycin (plipastatin type)[16]. At the same optimal fengycin (plipastatin) production condition, the obtained production of *B. amyloliquefaciens* FZB42 were 198 ± 16.13 mg.L^{-1} of fengycin type with 59 ± 11.7 mg.L^{-1} and 403 ± 12.14 mg.L^{-1} surfactin and mycosubtilin type, respectively. The production of *B. amyloliquefaciens* S499 were 228 ± 11.02 mg.L^{-1} of fengycin type with 173 ± 13.9 and 602 ± 11.5 mg.L^{-1} surfactin and bacillomycin type respectivelly. While, *B. subtilis* ATCC 21332 were 360 ± 17.35 mg.L^{-1} of plipastatin with 197 ± 12.36 mg.L^{-1} of surfactin (Fig. 5).

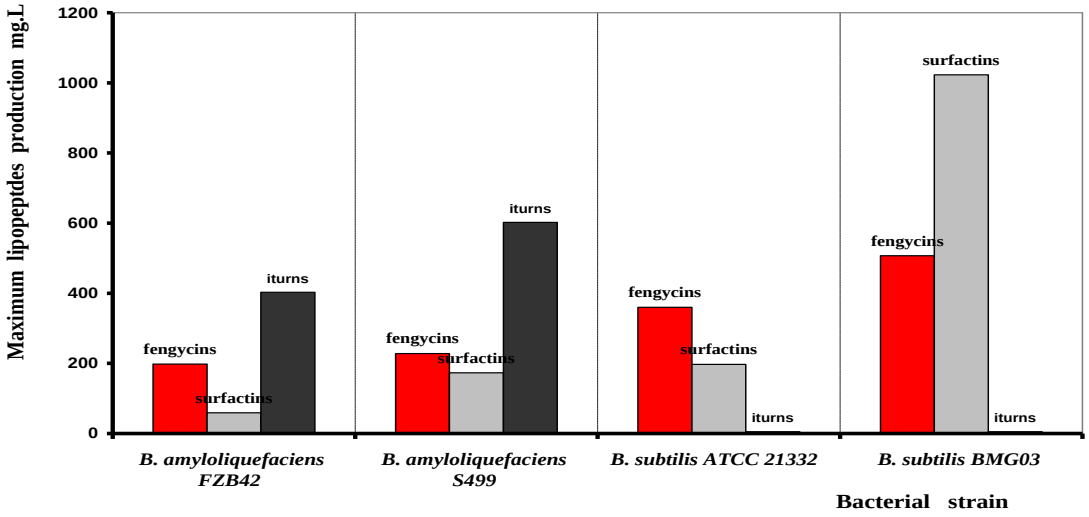

Figure 4. Lipopeptides production of *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* S499, *B. subtilis* ATCC 21332 and *B. subtilis* BMG03 under the optimal fengycin (plipastatin) production condition.

In addtion to that, it was planned to construct mono-plipastatin overproducer strain from the new strain *B. subtilis* BMG03 by the interruption of surfactin operon to maximize plipastatin production, but as mentioned Ongena et al.[21], that the interruption of surfactin operon haven't affect positively or significantly on plipastatin production. They constructed a strain *B. subtilis* 2508 derivative of 168 by the replacement of the weak P_{pps} promoter by the P_{amyQ} which produced 697 mg.L^{-1} surfactin and 434 mg.L^{-} fengycin (plipastatin) and when they interrupted surfactin operon the resulting derivative strain *B. subtilis* 2504 produced 452 mg.L^{-1} plipastatin only. Therefore, we haven't interrupted surfactin operon in our work as no increase in plipastatin production will be obtained. Moreover, Coutte et al.[22] was mentioned that the plipastatin operon interruption was strongly reduced in vitro antifungal properties and, enhanced specific surfactin production (1470 mg.L^{-1}), haemolytic activity and spreading behaviour of the strain.

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