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Improvement of poly-γ-glutamic acid (PGA) producing *Bacillus subtilis* SB-MYP-1 by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis

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Abstract

Poly-γ-glutamic acid (PGA) found in the mucilage of fermented soybean is synthesized by *Bacillus* sp. Its derivatives have been used as cryoprotectants, bitterness-relieving agents, thickeners and humectants in the ingredient industry. The wild type of *Bacillus subtilis* SB-MYP-1 isolated from fermented soybean was found to have a low efficacy to synthesize PGA during fermentation. Chemical induced mutation is generally introduced to provide microbial metabolite improvements. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was reported to have an ability to improve the microbial metabolite production. This mutagen induced nitrogenous base substitution at the replication point of protein syntheses. The work aimed to improve the *B. subtilis* SB-MYP-1 PGA production using random NTG mutagenesis. The appropriate mutation sensitivity of survival rate was selected on 0.001-10% according to 40-90 µg/ml NTG of a total 147 mutant isolates. Consequently, screened PGA-producing mutants were spotted on PGA agar and the slime diameter (mm.) of nine isolates was higher than that of the wild type ($P<0.05$). Those of nine mutant isolates were quantified for PGA production in PGA broth at 37°C for 36 h. Five isolates (NTG-17, NTG-53, NTG-88, NTG-132 and NTG-146) produced the PGA higher than the wild type ($P<0.05$). In addition, the selected five NTG mutants provided the maximum PGA production and growth steadily in L-glutamic acid supplement-submerged fermentation at 37°C during 12-72 h. The characteristics of genomic DNA band of the five mutant isolates by random primer (5'-AGTCAGCCAC-3') with a random amplified polymorphic DNA (RAPD) technique were similar to those of the wild type. However, the PGA production profile changes of NTG mutants could be exploited for the PGA-producing strain improvement.

Introduction

PGA is an unusual anionic polymer/polypeptide in which both D- and L-glutamic acids are polymerized via γ-amide linkages. It may be bio-synthesized through solid state fermentation of soybean to provide sticky mucilage, or the bioconversion pathway via *Bacillus subtilis*-submerged fermentation. The obtained purified PGA has proved to be both safe and environmentally friendly. It has potential applications in a wide range of foods, cosmetics and medicine (Ashiuchi *et al*., 2003; Jian *et al*., 2005; Shih *et al*., 2005; Tanimoto, 2010). However, the PGA production yield of the wild strain is limited (Shih and Van, 2001; Tanimoto, 2010). NTG strain improvement has been reported typically relied on induced mutation and random screening (Parekh *et al*., 2000; Xu *et al*., 2011; Siripong *et al*., 2012; Nadeem *et al*., 2013). Recently, the best mutant isolate dealt with the improvement was achieved for *B. amyloliquefaciens*-acetoin production, which was reported that the NTG mutant E-11 produced 39.6% acetoin higher than the wild type (Luo *et al*., 2014). Although methyl group was added to NTG at various positions on four nitrogenous bases of the bacterial DNA, the best mutagenicity correlated with an addition of oxygen molecule at the sixth position of guanine to create an O-6- methyl guanine, leading to direct miss-pairing resulting in G:C→A:T transitions at the next round of replication. The NTG mutagenic specificity showed a strong preference for G:C→A:T transitions (Jetawattana, 2001; Raksha *et al*., 2012).

The aims of this research were to enhance PGA productivity of the wild type *B. subtilis* SB-MYP-1 by using random NTG mutagenesis and to evaluate the PGA production yield due to L-glutamic acid supplement-submerged fermentation.

Materials and Methods

Microorganisms and culture maintenance

The wild type of *B. subtilis* SB-MYP-1 was obtained by isolation from spontaneously fermented soybean (Thai thua-nao). Biochemical characteristics

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identification was confirmed the *B. subtilis* by using API 50 CHB medium and API 20E test kit (bio Merieux Inc). Culture was maintained in sterile 30% of glycerol solution at -20°C (School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand). The wild type and mutant cultures were grown in nutrient broth (peptone 5 g/l, yeast extract 3 g/l) at 37°C for 24 h.

Chemical mutagenesis

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was obtained from TCI America (TCI-M0527).

Cell suspension preparation

Cell suspension was prepared by transferring 0.5 ml of stock culture to 10 ml of nutrient broth and incubated in a shaker at 200 rpm, 37°C for 24 h. Cells were harvested by centrifugation (10,000×g for 10 min at 4°C) and washed twice with 0.85% (w/v) sterile sodium chloride solution. Adjusting the cell turbidity concentration to standard McFarland No.1 (approximately 3 x 10^8 cfu/ml) with 0.85% (w/v) sterile sodium chloride solution. A suspension was serially diluted and cell load was determined by standard spreading method on nutrient agar.

Monitoring of the wild type B. subtilis *SB-MYP-1 growth profile*

Twenty micro liters of the cell suspension was transferred into 2 ml of nutrient broth, incubated in a shaking water bath at 37°C for 24 h. Growth of this bacterium was estimated every two hours starting from 0 to 14 and 24 h by standard spreading method on nutrient agar. The cell at mid log phase was considered for further NTG mutation.

Mutagenesis by NTG treatment

The wild type cell in its mid log phase was harvested and the cell suspension was prepared in 0.85% (w/v) sterile sodium chloride solution (approximately 10^8 cfu/ml). An aliquot of 1 ml of NTG concentration (at a range of 0 to 100 µg/ml in 0.1 M Tris-maleate buffer solution at pH 6.0) was then added. The culture was incubated in a shaking incubator at a speed of 200 rpm at 37°C. Mutagenic cultures were collected at 60 min (adapted from Raksha *et al.*, 2012; Siripong *et al.*, 2012) by centrifugation (10,000×g for 10 min at 4°C). The NTG-treated cell suspensions were serially diluted and cell survival was determined by standard spreading method on nutrient agar. Incubation was done in a dark incubator at 37°C for 24 h. The mutants with 0.001 to 10% survival rate were used for further experiment.

Screening and selection for the highest of PGA-producing mutants

The PGA-producing mutants were preliminary screened by a spot agar test. A single colony on nutrient agar plate was spotted on to PGA agar (L-glutamic acid 20 g/l, citric acid 12 g/l, glucose 20 g/l, NH_4Cl 7 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeCl_3 \cdot 6H_2O$ 0.04 g/l, $CaCl_2 \cdot 2H_2O$ 0.15 g/l, $MnSO_4 \cdot H_2O$ 0.104 g/l and agar 15 g/l) and incubated at 37°C in the dark for 24 h. The slime diameter of the bacterial colony was then determined. The isolate showing the largest size compared to that of the wild type was inoculated into test tube containing PGA broth (L-glutamic acid 20 g/l, citric acid 12 g/l, glucose 20 g/l, NH_4Cl 7 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeCl_3 \cdot 6H_2O$ 0.04 g/l, $CaCl_2 \cdot 2H_2O$ 0.15 g/l and $MnSO_4 \cdot H_2O$ 0.104 g/l) for secondary screening. After 36 h of incubation time, cell-free supernatant of each isolate was used for PGA yield measurement (Tahara *et al*., 1998).

PGA production in submerged culture

Aliquot of 1 ml (approximately 10^7 cfu/ml) of the highest PGA-producing mutants and the wild type was inoculated in PGA broth and incubated in a shaking incubator at 200 rpm at 37°C. Total viable count, pH values were measured and the PGA production yield was determined at 12 h intervals up to 72 h (Tahara *et al*., 1998).

Data analysis

The results of at least two independent experiments of each treatment were used to calculate the mean and standard deviation. SPSS 13.0 (SPSS Inc., Chicago, IL, USA,) was used to perform all statistical analysis. One-way analysis of variance (ANOVA) tests were followed by Fisher's least significant difference (LSD), with the overall significance level was set at 0.05.

Genetic characterization

Chromosomal DNA extraction and purification from selected mutants and the wild type were performed by using Promega Wizard Genomic DNA Purification Kit (USA). The genetic similarity between the wild type and selected mutants was analyzed by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique using a single 10-base oligonucleotide random primer (5'-AGTCAGCCAC-3'; Bio Basic Inc) as previously described (Hosoi, 2010). The reaction mixtures (15 µl) containing 10X buffer, 2 mmol/l of dNTP, 5 pmol/µl of primer, 50 ng/µl of DNA and 5 U/µl of Taq DNA polymerase (Taq- KAPA, Taq PCR Kit KK1014, USA) was made. The amplification reactions were

carried out in a PCR machine (CR- Gene Amp PCR System 9700 - PE Applied Biosystems) setting an initial denaturation at 95°C for 2 min, followed by 40 cycles of 37°C for 1 min and a final extension at 72°C for 2 min. The amplified products were electrophoresed on 1.5% agarose gel in Tris-acetate buffer and visualized under UV light after ethidium bromide staining. DNA band was observed by Gel Documentation system (MacroVue UVis-20 Hoefer).

Results and Discussion

The monitoring of the wild type B. subtilis *SB-MYP-1 growth profile*

The mid log phase of *B. subtilis* SB-MYP-1 started at the 6 h (7.72 log cfu/ml; data not shown) which related to the phase that many reports were indicated the chemical induced mutagenesis to which the gene sensitive was at the mid log phase (Renault and Heslot, 1987; Cavin *et al*., 1989; Doan and Obbard, 2012). So, the sixth hour of *B. subtilis* SB-MYP-1 growth phase was considered for further used in NTG mutagenesis on PGA-producing studies.

NTG mutagenesis, screening and selection for the highest of PGA-producing mutants

After exposure of *B. subtilis* SB-MYP-1 to NTG mutagen, 147 isolates of which less than 10% survival was appeared at the 40-90 µg/ml of NTG (data not shown). NTG effectively influenced DNA damage in the bacterial cell, which was depended on NTG concentration. (Stephen and David, 1986; Siripong *et al*., 2012). PGA production of those isolates was screened by spotting on PGA agar. Slime diameter was measured and found that 9 out of 147 isolates was higher than that of the wild type ($P<0.05$) (Figure 1A). These nine mutant isolates were proved for the PGA production yield in PGA broth, and five isolates (NTG-17, NTG-53, NTG-88, NTG-132 and NTG-146) provided higher yield than that of the wild type ($P<0.05$) at 36 h (Figure 1B).

PGA production in submerged culture

Growth profile of the wild type *B. subtilis* SB-MYP-1 and five mutant isolates (NTG-17, NTG-53, NTG-88, NTG-132 and NTG-146) in L-glutamic acid supplement-submerged fermentation were presenting to the log phase and stationary phase at 12-48 h (Figure 2) while pH value slightly decreased from 7.0 to 5.6-5.8 (Figure 3). In addition, PGA production time (at 12 h) of the mutant isolates was earlier than the wild type (Figure 4). However, the maximum PGA yield of both the wild type and mutant isolates was found at 36-72 h. When considering on the PGA potential of

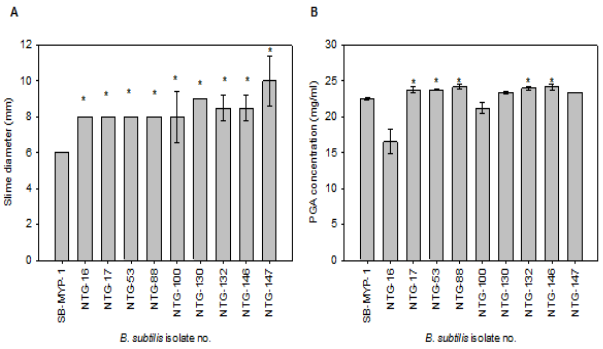

Figure 1. Slime diameter of *B. subtilis* NTG mutants and the wild type (SB-MYP-1) on PGA agar (A) and PGA concentration of the cell-free supernatant from *B. subtilis* NTG mutants and the wild type (B)

Data representing the average of slime diameter and PGA concentration are mean ± standard deviation of triplicate independent experiments

(*) indicates a significant difference ($P<0.05$) by one-way ANOVA with LSD test were compared to those of the wild type (SB-MYP-1)

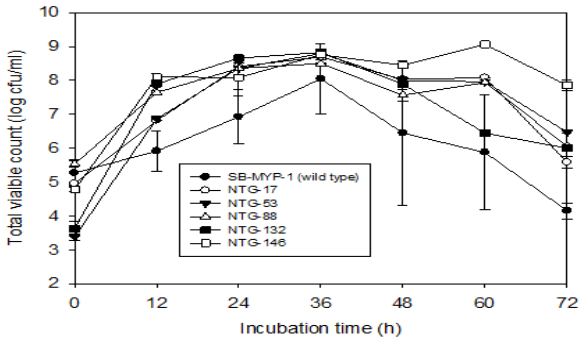

Figure 2. The growth profile of the wild type and mutant strains of *B. subtilis* in PGA broth using a shaking incubator of 200 rpm at 37°C

Data representing the average of total viable count are mean ± standard deviation of duplicate independent experiments

five NTG mutants, it was higher and more stable than that of the wild type at 24-72 h (Figure 4). Scientific evidence of the *B. subtilis*-PGA fermentation data was related to the *Bacillus* sp. grown on PGA medium composition that including carbon sources, nitrogen sources and trace elements compositions. PGA was synthesized from L-glutamic acid (2%), citric acid (1.2%), ammonium sulfate and other trace elements (0.04-7.0%) via tricarboxylic acid (TCA) cycle pathway. Nevertheless, intracellular multi-enzymes pathways of *B. subtilis* were the predominant of the PGA syntheses, such as the glutamate dehydrogenase (GD) pathway, glutamine synthetase (GS) pathway and 2-oxoglutarate aminotransferase (GOGAT) pathway (Shih and Van, 2001; Shih *et al*., 2005). The NTG efficiency has been stimulated to have a relatively wide spectrum of microbial mutations by alkylating pyrimidines and purines, which the G:C

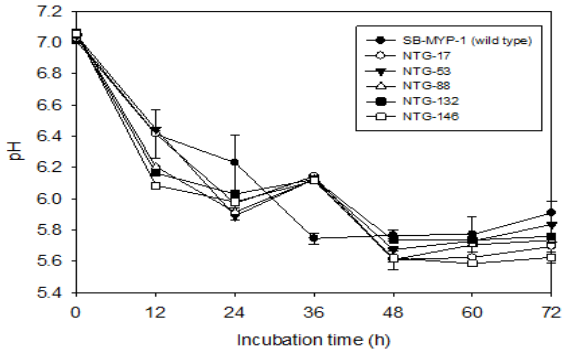

Figure 3. The pH value of the wild type and mutant strains of *B. subtilis* in PGA broth using a shaking incubator of 200 rpm at 37°C

Data representing the average of pH value are mean ± standard deviation of duplicate independent experiments

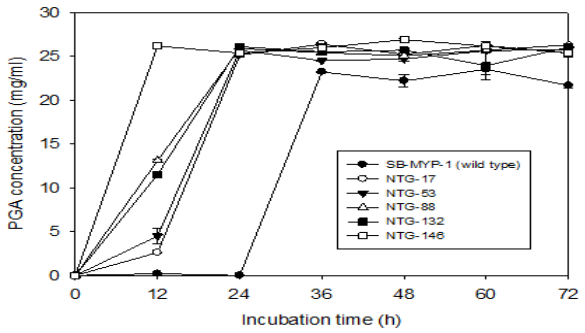

Figure 4. PGA concentration of the wild type and mutant strains of *B. subtilis* in PGA broth using a shaking incubator of 200 rpm at 37°C

Data representing the average of PGA concentration are mean ± standard deviation of duplicate independent experiments

→ A:T transition is more prevalent than that of the A:T → G:C. Especially, NTG improvement could leading to have a potentiality of their multi-enzyme activity or the active site for the binding of the substrate for metabolite conversion and yield increases (Gunka *et al*., 2010; Prabu *et al*., 2011; Raksha *et al*., 2012).

Genetic characterization

The RAPD band pattern of five mutant isolates which were similar to those of the wild type (Figure 5) which according to the results of Jetawattana (2001), which had reported an improved acetic acid production of *Acetobacter pasteurianus* SKU1108 by NTG mutagenesis. However, the PGA production profile of five NTG mutants based on L-glutamic acid supplement-submerged fermentation is prospective for the PGA-producing strain development.

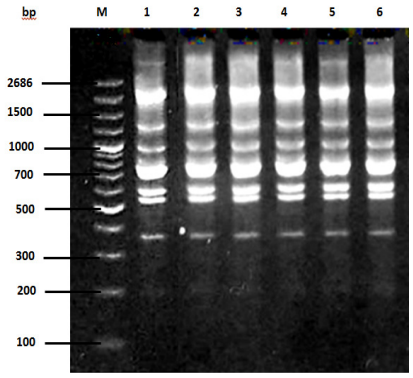

Figure 5. RAPD DNA fragments amplified with random primers. Lane M indicates 100 base pairs of DNA marker; lane 1 SB-MYP-1 (wild type); lane 2 NTG-17; lane 3 NTG-53; lane 4 NTG-88; lane 5 NTG-132 and lane 6 NTG-146

Conclusions

This study has demonstrated that random mutagenesis with NTG could successfully use for improving the PGA production. The PGA production of five *B. subtilis*-NTG mutant isolates (NTG-17, NTG-53, NTG-88, NTG-132 and NTG-146) was higher than that of the wild type in L-glutamic acid supplement-submerged fermentation. It is expected that the high yield of five NTG mutants potential and applied for the large scale of PGA production.

Acknowledgement

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