Factors contributing to the enhanced production of protease and lipase in *Bacillus pumilus* SG2 mutant

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Abstract. A random mutagenesis on a wild strain *Bacillus pumilus* SG2 using physical and chemical agents was performed previously and a mutant *Bacillus pumilus* SGMM8 which produced comparatively higher levels of protease and lipase was isolated. This study was attempted to analyse the causes underlying the enhanced enzyme production by the mutant. Substrate uptake, repression/de-repression studies and determination of K_m and V_{max} were done to understand if de-repression and enhanced affinity to substrate are the plausible reasons for enhanced enzyme activity. It was observed that the protease production was not repressed at high glucose concentrations in the mutant. The K_m and V_{max} of SG2 lipase were 2.265 mg/ml and 158 U/ml respectively and those of SGMM8 lipase were 1.619 mg/ml and 159 U/ml respectively and thus enhanced affinity may be the underlying cause for enhanced lipase activity. The genome sequence of the mutant enzymes were analysed and transversion mutations were identified in the coding regions. The mutations in the signal peptide of the protease and near the active site of the lipase may have caused increased enzyme production/activity.

Keywords: *Bacillus pumilus*, K_m/V_{max} , lipase, protease, repression studies, strain improvement

INTRODUCTION

Economical industrial production of microbial enzymes can be achieved by the following approaches: developing a suitable medium for fermentation, optimizing the parameters for cultivation and refining the fermentation process and improving the productivity of the strains. However, the strain improvement approach offers more enzyme yield. The techniques used to modify the strains genetically in order to increase the production of a desired product are collectively referred to as strain improvement. Strain improvement is achieved by either mutant selection or recombinant DNA technology (Mahadik *et al.*, 2004; Sangeetha *et al.*, 2011).

We had earlier reported the isolation of an improved mutant of *Bacillus pumilus* SG2. The parent strain was subjected to physical mutagenesis with UV irradiation and chemical mutagenesis using ethyl methane sulphonate and the most potent mutant that produced remarkably higher quantities of protease and lipase was isolated. The enzymes of the mutant were partially purified and characterized (Sangeetha *et al.*, 2011). However, the factors which contributed to improved enzyme production were not reported earlier. Alterations involving either enhancement or decrement in enzyme production/activity can be attributed to many factors like repression/

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derepression and cell membrane permeability (Stulke and Hillen, 1999). Strain improvement processes result in alterations in the base pairs as well.

This study was carried out to understand the factors contributing to the enhanced enzyme production by the mutant SGMM8. Hence uptake and repression studies and enzyme kinetic studies were performed. The study also aimed at sequencing the genes encoding protease and lipase in the parent strain and the mutant for comparison and identification of base alterations that had contributed to the enhanced enzyme activity in the non-genetically modified mutant.

MATERIALS AND METHODS

Bacterial strains, growth medium and conditions. The parent strain *Bacillus pumilus* SG2 was isolated from an industrial effluent and was subjected to strain improvement by physical mutagenesis using UV light followed by chemical mutagenesis using ethyl methane sulphonate. An improved strain which exhibited enhanced production of protease and lipase was isolated and designated as SGMM8. The production medium consisted of (w/v) 0.04% CaCl_2 , 0.02% MgCl_2 , 1% glucose, 0.5% NaCl, 0.3% yeast extract and 1% tributyrin (in sodium phosphate buffer, pH 9.0). Overnight culture (5 ml) ($\text{O.D}_{600}=1.0$) of parent strain *Bacillus pumilus* SG2 and its hyper-producing mutant SGMM 8 were inoculated into separate 100 ml medium and incubated on a rotary shaker (180 rpm) for 48 h at 37°C.

Enzyme kinetics. The proteolytic activity at different concentrations (2-10 mg/ml) of casein and lipolytic activities at different concentrations (2-10 mg/ml) of tributyrin were studied using methods described by Rahman *et al.* (2005) and Kordel *et al.* (1991) respectively. The K_m and V_{max} values were determined from the Lineweaver-Burk plot.

Uptake and repression studies with glucose and inorganic phosphate. Glucose and phosphate uptake by the parent and mutant cells were studied by determining the residual concentrations of glucose and phosphate in the

culture medium at regular intervals. Glucose was measured using the method described by Miller (1959) and phosphate was measured using the method described by Fiske and Subbarow (1925).

The catabolite repression of the synthesis and secretion of SGMM8 protease and lipase by glucose, inorganic phosphate (KH_2PO_4) at different concentrations (0-2% w/v) was also studied. For the investigation on repression by glucose, the production medium with varying concentrations of glucose were inoculated with 1.0% (v/v) of overnight culture ($\text{O.D}_{600}=0.6$) and incubated at 37°C in shaking incubator at 120 rpm for 28 hours. The production medium containing varying concentrations of inorganic phosphate was used for study on repression by phosphate. At the end of the incubation period, the liquid cultures were centrifuged at 10000 g for 15 min and the supernatants were subjected to protease and lipase assay. The proteolytic activity was measured by the photometric method described by Rahman *et al.* (2005). One unit (U) of protease activity is equivalent to 0.5 μg of tyrosine liberated by 1.0 ml of enzyme solution under the assay conditions. The lipase activity was assayed by the photometric method described by Kordel *et al.* (1991). One unit (U) of lipase activity is equal to 1 μmol of *p*-nitrophenol liberated per min under the assay conditions.

Amplification and sequencing of *alp* and *lip* genes of SG2 and SGMM8. DNA isolation from SG2 and SGMM8 was performed based on the method of Tripathi and Rawal (1998). The PCR amplification was carried out in Ericomp Delta cyclor I system (Ericomp, Inc, CA) and PTC-100 (MJ Research Inc. USA) thermocycler machines. The *alp* and *lip* gene amplification was performed using the primers described by Rahman *et al.* (2007) and Sangeetha *et al.* (2014) respectively. The primers used for *alp* gene amplification were forward: 5'- ATG TGC GTG AAA AAG AAA AAT GTG -3' and reverse: 5'- TTA GTT AGA AGC TGC TTG AAC GTT -3'. The reaction conditions were as follows: one denaturation step at 94°C for 4 min, 30 cycles of annealing of primers at 60°C for 45 sec, extension at 72°C for 2 min except for the final cycle for which extension proceeded for 5 min. The primers and the reaction conditions used for *lip*

gene amplification were reported earlier (Sangeetha *et al.*, 2014).

Gene sequencing. The direct gene sequencing was carried out by the method of Sanger *et al.* (1997) using DTCS quick start Dye terminator kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The original sequences of the parent and mutant strains were submitted to GenBank, NCBI.

RESULTS AND DISCUSSION

Uptake and repression studies. To investigate if the enhanced enzyme production by the parent SG2 and mutant SGMM8 was due to enhanced synthesis and/or secretion, uptake and repression studies with glucose and phosphate were performed. Glucose uptake by the parent SG2 and the hyper-producing mutant SGMM8 was not similar. Strong catabolite repression was observed with glucose concentrations higher than 1% (w/v). Protease production by the parent was repressed from 126 to 10 U/ml while the lipase production was repressed from 94 to 12 U/ml with increase in concentrations of glucose from 1% (w/v) to 2% (w/v) (Figure 1). The mutant exhibited seven fold higher activities of protease and lipase than the parent. The production of both protease and lipase by the parent and the mutant gradually increased with increase in glucose concentrations from 0 to 1% (w/v) and the production was optimum when the concentration of glucose was 1% (w/v). However, uptake of phosphate by both the parent and the mutant was similar. The decreased uptake or consumption rate of glucose could be attributed to a cis/trans acting mutation (Fickers *et al.*, 2003). Repression studies show that enzyme production by the mutant was not repressed at 1.5% (w/v) of glucose although a decline in enzyme activity is observed (Figure 1).

Catabolic genes and operons in Gram-positive bacteria are subjected to carbon catabolite repression (CCR) by glucose and other metabolized carbon sources. The mechanisms underlying glucose repression include catabolite repression which involves global regulators, inducer exclusion and induction prevention.

Negative regulation of the transcription of catabolite-repressive genes involves a trans-acting repressor protein called catabolite control protein, Ccp A and cis-acting elements referred to as *cres*, catabolite responsive elements (Deutscher *et al.*, 2002; Inacio *et al.*, 2003).

Figure 1. Glucose repression studies. The values expressed are mean $\pm$ S.D of three independent experiments.

The enhanced production of SGMM8 protease and lipase is therefore most likely due to decreased levels of glycolytic intermediates when the glucose uptake by the hyper-producing mutant was decreased. The glucose uptake by the bacteria was studied by determining the residual glucose in the culture medium. The glucose uptake by the parent was found to be 18.6 $\mu\text{g/g/h}$ while the glucose uptake by the mutant was 10.4 $\mu\text{g/g/h}$ (Data not shown). The documents that report the relationship between glucose uptake capacity and catabolite repression are very few. Fickers *et al.* (2003) have reported enhanced lipase production by *Y. lipolytica* mutant which exhibited lipase production uncoupled from catabolite repression from glucose and the glucose uptake capacity by the mutant was reduced 2.5 fold compared to the wild type strain. Christiansen and Nielsen (2002) have reported decreased protease production by *B. clausii* due to enhanced glucose uptake.

Increase in enzyme synthesis was observed when the phosphate concentration was increased from 0 to 0.5% (w/v) (Data not shown). Thus 0.5% (w/v) was the optimum concentration for both protease and lipase production. Phosphate plays a vital role as an effector of a large number of enzymatic reactions of primary metabolism, including the synthesis of DNA and RNA, protein and carbohydrate metabolism, cellular

respiration and control of ATP levels. The optimal phosphate requirement by the mutant was the same as that of the parent.

***K_m* and *V_{max}* of protease and lipase.** The protease and lipase produced by the parent and mutant strains were purified and the purity of the enzymes was confirmed as reported earlier (Sangeetha *et al.*, 2010). The *K_m* and *V_{max}* values were determined for the protease and lipase produced both by the parent and the mutant *B. pumilus* strains. The protease of both SG2 and SGMM8 had a *V_{max}* value of 120 U/ml of protein. The *K_m* values of the protease produced by the wild and mutant were similar, 0.9 and 0.88 mg/ml respectively. The lipase produced by the parent and the mutant strains exhibited slightly different *K_m* and *V_{max}* values. The SG2 lipase had a *K_m* value of 2.265 mg/ml and a *V_{max}* of 158 U/ml while the values of *K_m* and *V_{max}* of SGMM8 lipase were 1.619 mg/ml and 159 U/ml respectively (Figure 2). The decreased *K_m* value exhibited by SGMM8 lipase indicates that the enzyme had increased affinity for its substrate when compared to the lipase of the wild strain.

Figure 2. Line-weaver Burk plot for the determination of *K_m* and *V_{max}* of SG2 and SGMM8 lipases.

Protease and lipase genes. The coding regions of protease and lipase of the parent and the mutant strain were compared to determine whether the enhancement in enzyme production in the mutant could be attributed to the alterations in the nucleotide sequences. The coding regions of SG2 and SGMM8 protease and lipase were amplified. We had earlier reported the molecular characterization of the lipase produced by *B. pumilus* SG2 (Sangeetha *et al.*, 2014). The coding regions of the enzymes were sequenced and the sequences were submitted to GenBank under the following accession numbers: *B. pumilus* SG2 Protease: GQ398415; *B. pumilus* SG2 Lipase: GQ398414; *B. pumilus* SGMM8 Protease: GU143024; *B. pumilus* SGMM8 Lipase: GU143025.

A nucleotide sequence comparison between the *alp* gene of SG2 and SGMM8 revealed two base substitutions. The gene encoding the protease of the improved strain SGMM8 exhibited two G-C to A-T transversion mutations; the first mutation was at nucleotide 5 near the start region while the second was at nucleotide 785 in the coding region (Figure 3). Translation studies of the amplified genes revealed that the cysteine 2, present next to the first amino acid methionine in the signal peptide, was replaced by tyrosine. Also, the glycine 262 present before the oxyanion residue asparagine was replaced by glutamate. Comparison of nucleotide sequences of the *lip* gene of SG2 and SGMM8 indicated a GC-AT transversion mutation at nucleotide 500 in the coding region (Figure 4). The amino acid at position 167 was valine in SG2 lipase and was substituted by isoleucine in the mutant enzyme. This mutation was present near the active site aspartate 164 and thus probably contributed to enhanced lipase activity.

The alterations in the specific activity of the SG2 protease and lipase may reflect the effect of physical and chemical mutagenesis. The specific activity of protease remained unaltered and thus the two transversion mutations had no impact on the catalytic activity of the enzyme. However, specific activity of SGMM8 lipase showed a marginal increase when compared to SG2 lipase. This could be attributed by the base substitution in the coding region which resulted in a neutral mutation by replacing the residue valine 136 with isoleucine. Interestingly, Christiansen *et al.* (1994)

have patented *B. lentus* alkaline protease variants with enhanced stability. Two of these variants have valine residues replaced with isoleucine. The signal peptide of the protease of SGMM8 had a base substitution at the amino acid position -30 (with respect to the first amino acid of the pro-peptide) present on the N-terminal region of the peptide. There exists a strong preference in the amino acid residues around the signal peptide cleavage site. Enhanced secretion of *B. subtilis* β -lactamase was observed when the amino acids at positions 27 and 28 were substituted with alanine using site directed mutagenesis (Nakamura *et al.*, 1988).

Thus the enhanced proteolytic and lipolytic activity observed in the mutant SGMM8 could be attributed to the enhanced secretion of protease following a mutation in the signal peptide and the enhanced activity of lipase as a consequence of a mutation in the coding region.

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-35          -10
aaaaattcaggtctactcttattttgctctatctctatttaaaatcaagaataaagc
aaacggatcattctaatagactacggattattattctcaataagaaagggctgtgga -1
SD
atgtgcgtgaaagaagaaatgtagtgaaggtgtttatgggtgtctctctctctttt 60
M *C V K K K N V M T S V L W A V P L L F
tcagcagggtttggaggctcgatggcaaatgccgaactgccctcaagtcagaaagcgaa 120
S A G F G G S M A N A E T A S K S E S E
aaaagctatctcgttgccttaagctctgccaccaaaaacagctctaaagaaacaggca 180
K S Y I V G F K A S A T T N S S K K Q A
gtgacaaaaatggtgggaattagaaaaacaataccgctctattaatgctgcacaaagt 240
V T Q N G G K L E K Q Y R L I N A A Q V
aagatgtctgaacacggcgcaaaaaaactgaacacagcactagcattgctttagtagaa 300
K M S E Q A A K K L E H D P S I A Y V E
gaagacacaaagcagaagcatatgcccaacgcctcttatggtatccctcaaatcaaa 360
E D H K A E A Y A Q T V P Y G I P Q I K I 2
gctccagcgtacacgctcaaggttataaaggctcaacgtcaagtagctgtcttgat 420
A P A V H A Q G Y K G A N V K V A V L D 32
actggaatccacgcgcacacccgtaactaaatgttcagggcggtgctagcttctccct 480
T G I H A A H P D L N V A G C A S F V P 52
tcagagccaaatgccacacaaagctcaatcaacgggaactcatgtagcgggaacatt 540
S E P N A T Q D F Q S H G T H V A G T I 72
gctgccttgataacacgattggtgttcttgggtgtgcgaacgcctctctgtatgcc 600
A A L D N T I G V L G V A P S A S L Y A 92
gttaagattatagatcgttacggcgatggaacaatcagctggattatcagcgggtattgaa 660
V K V L D R Y G D G Q Y S W I I S G I E 112
tgggctgttgcacaaatcagtgatgtaacatagcttagcggcggaacaaacggctcc 720
W A V A N N M D V I I N M S L G C P N G S 132
acagcgttataaaatgctgtagacacagcgaataacggcgagtagtgtgctgtgccga 780
T A L K N A V D T A A N N R G V V V V A A 152
gcagggaattcaggttcaactggctctactagcaccgtagggtatcctgcgaataacgac 840
A *G N S G S T G S T S T V G Y P A K Y D 172
tctacaattgctgttgcaacgtgaacacaaatgacagaaactgtctcttagcgcga 900
S T I A V A N V N S N N V R N S S S S A 192
ggctctgaattagatgtttctgcacctggtacatogattttaagtaacagtaacagcgt 960
G P E L D V S A P G T S I L S T V P S S 212
ggatatacgtcttatacgggaacatcaatggcatctctcatgtagcaggagcagcagcg 1020
G Y T S Y T G T S M A S P H V A G A A A 232
cttatccttttaagatcgaatctatcaacgtctcaggttcgtcagcgcttagaanaa 1080
L I L S K Y P N L S T S Q V R Q R L E N 252
acggcaacacggcttgtaattcgttctattatggaaaaggatttaacgttcaagcg 1140
T A T P L G N S F Y Y G K G L I N V Q A 272
gcttctaacgggtggcaaaaatccggcgaaactggccggatttttatttattgcttt 1200
A S N G W Q K N P A N W A G F F I Y C F 292
ttcagatgttctctctttt
F D V S S F
1239
298

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Figure 3. Nucleotide and deduced amino acid sequence of SG2 protease. The Shine-Dalgarno sequence is underlined and the oxyanion asparagine is italicized. The bases that underwent GC-AT transversion are represented in bold-black. The arrows indicate the cleavage sites for signal and pre-peptide.

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cggtcaataaagggaaggag -1
SD
atgattcgatttaagaaaaagagttgtcaaatcttctgttgccttgccttagtgataggt 60
M I R F K K K S L Q I L V A L A L V I G 20
tcaatggccttcattccagcgaagaagcgaagcggtgagcgaatccgggttgtagtg 120
S M A F I Q P K E A K A A E H N P V V M 40
gtgcattggcgtgggtggtgctcttataacttgccttcgatcaaacgatacttggtatca 180
V H G M G G A S Y N F A S I K R Y L V S 60
cagggatgggatcaaaaccaaactttttgcaatcgatttcagacaaaacaggcaataac 240
Q G W D Q N Q L F A I D F I D K T G N N 80
ctaaaacatggccgaggtctctctagattcgtgaagatgtagcagcaaaacggggcgc 300
L N N G P R L S R F V K D V L A K T G A 100
aaaaaagtagatattgtggctcatagtagtgggcggtggaacacggttatactattaaa 360
K K V D I V A H S M G G A N T L Y Y I K 120
aacctagacggcgagataaaattgaaacgcgtctcacacttggtggcggaacgggactc 420
N L D G G D K I E N V V T L G G A N G L 140
gtatcactcagagcattaccaggcaccgacccaataacaaaattctttacacttctgtc 480
V S L R A L P G T D P N Q K I L Y T S V 160
tatagctcagcggatctcatcctcgtcaacagctctctcgttaattggcgcaagaac 540
Y S S A D L I *I V N S L S R L I G A R N 180
gtctcctcaccggcgttgacatctcgtctattaaacctcaagcgaagcgaaggggtat 600
V L I H G V G H I G L L T S S Q V K G Y 200
gtgaaagaaggattgaatggcgaggat
V K E G L N G G G
628
209

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Figure 4. Nucleotide and the deduced amino acid sequence of SGMM8. The Shine-Dalgarno sequence and the oxyanion site conserved regions have been underlined. The isoleucine that substituted valine has been represented in bold-black.

CONCLUSION

The study involved the analysis of factors which contributed to the improved production of protease and lipase by the improved strain *Bacillus pumilus* SGMM8. The mutant was moderately relieved off the repressive effects of glucose and phosphate. Thus de-repression could be highlighted as the possible reason for the enhanced enzyme production. Also, the decreased K_m value exhibited by SGMM8 lipase indicates that the enzyme had increased affinity for its substrate when compared to the lipase of the wild strain. The *alp* and *lip* genes encoding the protease and lipase enzymes of both the parent and the mutant strains were sequenced. The enhanced enzyme activities exhibited by the hyper-producing mutant could be attributed to a transversion mutation induced in both the genes.

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