Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(6):549-555

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Enhancement of adenosine production by over expression of *purA* in *Bacillus subtilis* XGL

Chenglin Zhang, Shanshan Du, Lei Guo, Qingyang Xu, Xixian Xie and Ning Chen*

*College of Biotechnology, Tianjin University of Science and Technology, Tianjin, China
National and Local United Engineering Lab of Metabolic Control Fermentation Technology, Tianjin, China
Chenglin Zhang and Shanshan Du are Co-first authors

ABSTRACT

Adenylosuccinate synthetase (AdSS), encoded by purA, is generally considered as one of the rate-limiting enzymes involved in the de novo biosynthesis of adenosine. In this study, effect of purA overexpression on adenosine production was investigated and Bacillus subtilis XGL-XY and XGL-A were constructed. B. subtilis XGL-XY harboring a recombinant plasmid pBEA containing purA showed 25.5 % and 29.2% increased adenosine production and yield but a decline in cell growth. Considering the metabolic burden caused by pBEA, an additional purA gene under the control of P43 promoter was integrated into the B. subtilis XGL genome in purA locus by single crossover, resulting in B. subtilis XGL-A. The strain exhibited improved adenosine production, yield and similar cell growth with B. subtilis XGL. Furthermore, lowered IMP concentration was detected in B. subtilis XGL-XY and B. subtilis XGL-A. Fed-batch fermentation assay showed that, compared with B. subtilis XGL, 29.4 % and 18.3 % increase in adenosine production and yield and up to 17.3 g/L and 0.207 g/g glucose was achieved by B. subtilis XGL-A. Above all, it can be concluded that AdSS is a crucial enzyme in adenosine synthesis and purA overexpression could drove more metabolic flux from IMP to adenosine synthesis and thus enhance adenosine production.

Keywords: *purA*, adenosine, adenylosuccinate synthetase, *Bacillus subtilis*, IMP

INTRODCUTION

Adenosine is one of purine nucleoside involved in many enzymatic reactions and plays a vital role in neurotransmission and anti-inflammatory responses[1-3]. In addition, adenosine is crucial for coronary circulation and protection, and can be used to effectively terminate certain supraventricular tachycardia events involving the atrioventricular node in the re-entry pathway[5-6].

The main methods used for adenosine production include chemical synthesis, RNA degradation and microbial fermentation. However, industrial production of adenosine is mainly achieved by microbial fermentation at present due to its significant low-cost and less-contaminating advantage. *Bacillus subtilis* has a long tradition as a safe and stable producer of purine nucleosides[7-9]. Moreover, the bacteria exhibited stronger pentose-phosphate pathway flux than other bacteria and therefore more 5-phosphoriboxyl-1-pyrophosphate (PRPP, major precursor for purine nucleoside synthesis) could be accumulated[10]. Thus, *B. subtilis* is considered to have high potential for adenosine production on industrial scale.

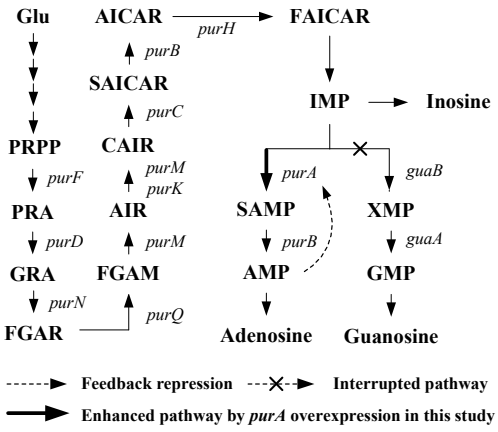

Fig. 1 Adenosine biosynthesis pathway in *B. subtilis*

Glu, glucose; PRPP, phosphoribosyl pyrophosphate; PRA, 5-phosphoribosyl amine; GAR, glycinamide hydrochloride; FGAR, formylgly cinamide ribotide; FGAM, formylglycinamidine ribotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-aminoimidazole-4-carboxylate ribotide; SAICAR, 5-aminoimidazole -4-(N-succinylocarboxamide) ribotide; AICAR, 5-aminoimidazole-4-carboxamide ribotide; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; IMP, inosine monophosphate; SAMP, adenylosuccinic acid; AMP, Adenosine monophosphate,; XMP, xanthosine monophosphate; GMP, guanosine monophosphate

In the de novo purine nucleotide biosynthetic pathway, adenylosuccinate synthetase (AdSS, encoded by *purA*) is one of the rate-limiting enzymes, which catalyzes the first of two reactions from the IMP branch point to AMP (Fig. 1) and transcription of *purA* is feed-back repressed by AMP[11]. It is reported that *purA* deletion resulted in significant accumulation of inosine or a guanosine[11-13]. So it is likely that an increased quantity of AdSS might overcome the feedback repression and improve the flux of IMP to AMP, and therefore contribute to a further increase in IMP use for adenosine biosynthesis. However, little information is available about the contribution of AdSS to adenosine production.

In the present work, the *purA* gene was overexpressed in an adenosine producer in the form of recombinant plasmid or by integrating to genome. Its effect on cell growth, glucose consumption, and adenosine production is studied.

EXPERIMENTAL SECTION

Strains and plasmids

Strains and plasmids used in this study are listed in Table 1.

Table 1 Strains and plasmids used in this study

Strains	Relevant characteristics	Source
B. subtilis XGL	Adenosine-producing mutant, His^{-a}, Xan^{-}, $8\text{-}AG^{r}$, SG^{r}	Laboratory stock
B. subtilis XGL-XY	*B. subtilis* XGL haboring pBEA	This study
B. subtilis XGL-A	*B. subtilis* XGL with additional *purA* gene at upstream of *purA* locus	This study
Escherichia coli DH5α	F-, Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*	Laboratory stock
Plasmids		
pKS1	Replication thermosensitive derivation of the rolling circle plasmid pWV01 (Kan^{r}, Em^{r})	[14]
pBE43	Amp^{r}, Km^{r}, containing the constitutively strong promoter P43	Laboratory stock
pBEA	pBE43 with *purA*	This study
pKSEA	pKSEA with P43 and *purA*	This study

a: His^{-}, Xan^{-}, $8\text{-}AG^{r}$ and SG^{r} represents xanthine and histidine deficiencies and 8-azaguanine and sulfaguanidine resistance, respectively.

Culture media and growth conditions

B. subtilis and *E. coli* cells were grown in Luria - Bertani (LB) medium. If needed, antibiotics were added at appropriate concentrations.

For batch fermentation, the *B. subtilis* strain was first grown on slant medium (2 g glucose, 5 g yeast extract, 10 g beef extract, 10 g peptone, 2.5 g NaCl, 10 mg xanthine, 10 mg histidine, 30 g agar per liter of distilled H_2O, pH 7.0) for 24 h at 32℃, and was then transferred to seed medium (20 g glucose, 5 g yeast extract, 25 g corn syrup, 0.4 g

$MgSO_4$, 1 g KH_2PO_4, 5 g monosodium glutamate, 8 g peptone, 5 g urea, 30 mg xanthine, 30 mg histidine per liter of distilled H_2O, pH 7.0) and cultured on a shaker for 8 h at 32℃, 200 r/min. The seed culture was then inoculated into fermentation medium (80 g glucose, 16 g yeast cream, 16 g monosodium glutamate, 7 g $(NH_4)_2SO_4$, 3 g K_2HPO_4, 10 g corn syrup, 30 mg xanthine, 30 mg histidine per liter of distilled H_2O, pH 7.0) at a ratio of 10% (v/v) and cultured on a shaker for 48 h at 34℃, 200 r/min.

For fed batch fermentation, 10% (v/v) of seed culture was inoculated into 5-L jar bioreactor containing 3L of fermentation medium. Fermentation was carried out at 34℃, pH was automatically controlled at 6.7, glucose concentration was maintained at 25 g/L by adding 80% glucose (w/v) and DO level was controlled at 20% - 30% by adjusting agitation speeds.

Primers

Primers used are listed in Table 2.

Table 2 Primers used in this study

Primers	Sequence (5'-3')	Description
purA-A	GGCGGTACCGAAAGGTTAACGGAGGTGCACGG (*Kpn* I)	*purA* amplification
purA-B	GGCCTGCAGTTAGTTCGCACGGTACACACTGCGA(*Pst* I)	
P43-S	TAGCTGCAGGAGCTCAGCATTATTGA (*Pst* I)	P43-*purA* amplification by overlap PCR
P43-A	CCGTGCACCTCCGTTAACCTTTCGCTATCACTTTATATTTTA	
purA-U	TAAAATATAAAGTGATAGCGAAAGGTTAACGGAGGTGCACGG	
purA-D	TGTGGTACCTTAGTTCGCACGGTACACACTGCGA (*Kpn* I)	
DJH-S	AACTTACCCGCCATACC	Identifying *purA* integration
DJH-A	GTCTTAACCGCTTGACC	
16S-S	TGGTAGTCCACGCCGTAAACGA	Internal reference for RT-qPCR
16S-A	TGTCAGAGGATGTCAAGACCTGGTAAG	
purA-1	CGGAAATAACGCAGGGCATACAATC	Determinating *purA* trancrption by RT-qPCR
purA-2	ATGAAGATACGCAAGCTCTGTGACTAATG	

Construction of *B. subtilis* XGL-XY

A 1.3-kb structural gene of *purA* was amplified by PCR using the primers purA-A and purA-B based on the published sequence, then was digested by *Pst* I and *Kpn* I and was cloned into downstream of the constitutively strong promoter P43 in the expression vector pBE43, resulting in pBEA. Then the plasmid pBEA was electroporated (1.15-kV, 200 Ω, 25-μF electric pulse in a prechilled 0.2-cm-diameter cuvette) into *B. subtilis* XGL to obtain *B. subtilis* XGL-XY.

Construction of *B. subtilis* XGL-A containing an additional *purA* gene at the *purA* locus

An integration plasmid pKSEA was obtained through the overlapping PCR. Briefly, PCR was applied to amplify the P43 promoter and the *purA* gene with the primers P43-S/P43-A and purA-U/purA-D, repectively, and overlapping PCR was performed to obtain the fragment combining P43 and *purA*, the resulting fragment was digested with *Pst* I and *Kpn* I and then was cloned to pKS1 to obtain pKSA.

Plasmid pKSA was electroporated into *B.subtilis* XGL as the method described by Konstantin et al (2005) to obtain *B. subtilis* XGL-A [14]. PCR analysis with the primers DJH-S/DJH-A together with sequencing was used to identify the integration of the *purA* gene in *purA* locus.

RNA isolation and RT-qPCR assay

Total RNA was isolated from *B. subtilis* cells using the RNAiso Plus kit (Takara, Dalian, China) according to the manufacturer's protocol. RT-qPCR assays were carried out using the UltraSYBR Two Step qRT-PCR Kit (CWBIO, Beijing, China) according to the manufacturer's procedure. The 16S rDNA was used as internal reference. Assays were independently performed more than three times under identical conditions. The fold change of each transcript in each sample relative to the control sample was normalized to the internal control gene and calculated according to the comparative Ct method[15].

Enzyme activity assay

A total of 10 mL of cells were harvested during their mid-log phase in the fermentation medium by centrifugation at 4℃, and crude cell extract was prepared as described[16]. Adenylosuccinate synthetase were detected as described[17]. Protein content was determined according to the Bradford method and specific activity was expressed as nmol min^{-1} mg^{-1} protein..

Genetic stability of *B. subtilis* XGL-A

Stability of *B. subtilis* XGL-A was assessed by examinating percentage of erythromycin (Em) -resistant cells in the culture population. Fermented culture was obtained at the end of fermentation and was spread on LB plate after a suitable dilution. Following incubation at 32℃ for about 24 h, colonies were replica-plated onto nonselective and a selective LB-Em agar plate, respectively. After incubation for 48 h at 32℃, viable cell counts were performed on LB and LB-Em plates, which were used to calculate the percentage of cells retaining the plasmid integrated to *B. subtilis* XGL-A[18].

Analytical procedure

Cell growth was monitored by optical density with a spectrophotometer at 600 nm. Glucose concentration was determined by an SBA Biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, China). Adenosine was measured using an HPLC equipped with a Kromasil C18 column (250 mm × 416 mm, 5μm, Agilent) with an eluent containing 10% acetonitrile at 1.0 ml/min at 30℃, and purine compounds were detected at 259 nm. Intracellular concentration of IMP was measured as described[18].

Statistical analysis

Every experiment was repeated three times, and data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to determine significant difference, and the statistical significance was defined as $p \leqslant 0.05$.

RESULTS AND DISCUSSION

Effect of *purA* overexpression on adenosine production by *B. subtilis* XGL-XY

Successful construction of *B. subtilis* XGL-XY by transforming pBEA to *B. subtilis* XGL was identified by PCR with primers purA-A and purA-B. To make sure *purA* gene was transcribed and expressed in *B. subtilis* XGL-XY, RT-qPCR was performed and the activity of AdSS was detected. As shown in Fig. 2, the transcription level of *purA* in *B. subtilis* XGL-XY was about 12.2-fold higher than that in *B. subtilis* XGL. In addition, the activity of AdSS was elevated up to 5.2-fold (Fig. 3) during the exponential phase, indicating successful expression of *purA* in *B. subtilis* XGL-XY.

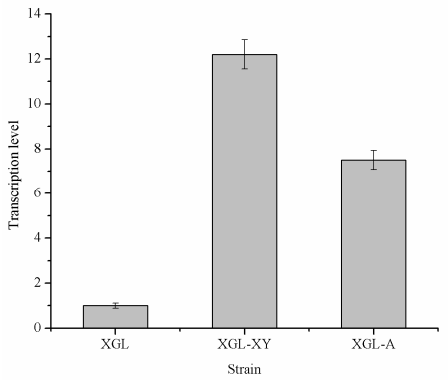

Fig. 2 Transcription level of *purA* in *B. subtilis* XGL, *B. subtilis* XGL-XY and *B. subtilis* XGL-A

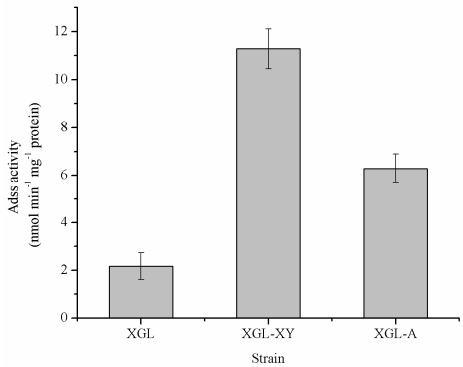

Fig. 3 AdSS activity in *B. subtilis* XGL, XGL-XY, and XGL-A

To assess the effect of *purA* overexpression on adenosine production, shake-flask fermentations were carried out. As shown in Table 3, *B. subtilis* XGL-XY exhibited 25.5 % and 29.2% higher adenosine production and yield than *B.*

subtilis XGL, respectively, indication that overexpression of *purA* could effectively enhance the adenosine production. However, it was notable that the cell growth had decreased, which could possibly be explained by metabolic burden caused by plasmid replication.

Effect of integrating additional *purA* to genome on adenosine production by *B. subtilis* XGL-A

Considering the metabolic burden caused by pBEA and further engineering modification, *B. subtilis* XGL-A was constructed (Fig. 4), which carried an additional copy of *purA* under control of the P43 promoter inserted at the *purA* locus by a Campbell-like mechanism [19]. PCR with the primer DJH-S and DJH-A together with sequencing further confirmed the integration of the *purA* gene in *purA* locus (data not shown). As expected, the transcription level of the *purA* gene in *B. subtilis* XGL-A was about 7.5-fold higher than that in *B. subtilis* XGL, but lower than that in *B. subtilis* XGL-XY, in addition (Fig. 2), the AdSS activity in *B. subtilis* XGL-A was significantly increased by up to 2.9-fold (Fig. 3) , indicating the successful molecular manipulation.

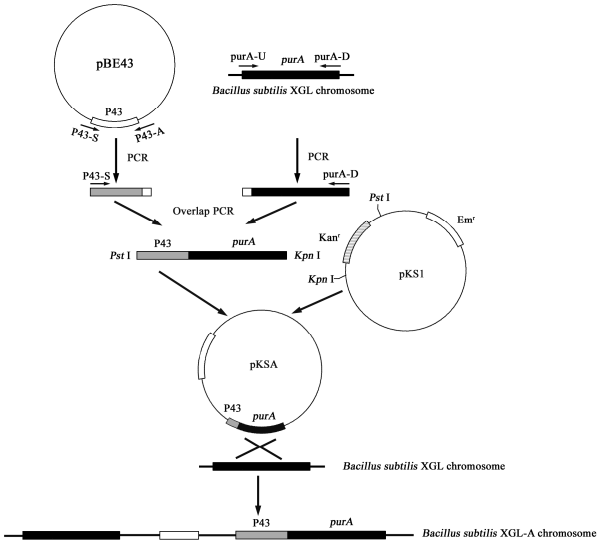

Fig. 4 Construction of *B. subtilis* XGL-A

Shake-flask fermentations were performed to assess the effects of integration additional *purA* on adenosine production. As shown in Table 3, the adenosine production and yield by *B. subtilis* XGL-A was 37.5% and 48.5% higher than that by *B. subtilis* XGL, respectively, indicating that glucose was more efficiently converted into adenosine. It was interesting to note that although the AdSS activity of *B. subtilis* XGL-XY was higher than that of *B. subtilis* XGL-A, its adenosine production was lower. Shimaoka (2007) observed a similar phenomenon, however, the reason still remains unknown[20]. Moreover, biomass of *B. subtilis* XGL-A was remarkably enhanced and was closed to that of *B. subtilis* XGL.

Table 3 Physiological parameters of *B. subtilis* strains during exponential phase.

Parameters	Strains		
	XGL	XGL-XY	XGL-A
Adenosine production (g/L)	5.191±0.011	6.514±0.012	7.137±0.021
Yield (g adenosine/g glucose)	0.171±0.002	0.221±0.002	0.254±0.003
Biomass (g CDW/L)	2.814±0.013	2.437±0.018	2.705±0.011

Intracellular IMP in *B. subtilis* cells

As Fig. 1 showed, IMP is the substrate of AdSS. Since *B. subtilis* XGL is deficient in xanthine, it meant that metabolic flux from IMP to GMP is interrupted and the change of IMP concentration relates to adenosine production directly. Therefore the intracellular IMP concentration was measured in the three strains to detect the effect of *purA* overexpression on metabolite flux of adenosine biosynthesis. As shown in Fig. 5, higher IMP concentration (3.651 nmol mg^{-1} DCW (cell dry weight)) was detected in *B. subtilis* XGL, which might be the metabolic bottleneck for improvement of adenosine production. As expected, a lower IMP concentration was found in XGL-A and XGL-XY (2.762 and 2.314 nmol mg^{-1} DCW, respectively), indicating that more IMP was transformed to adenosine biosynthesis by overexpressed AdSS.

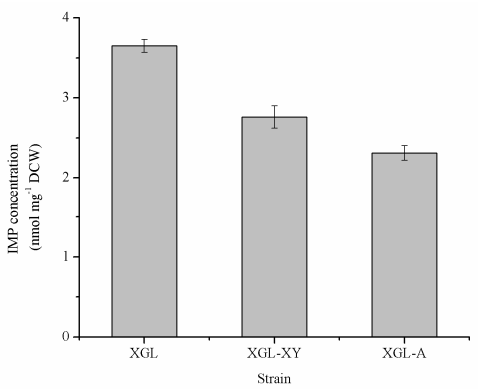

Fig. 5 Concentration of intracellular IMP in *B. subtilis* XGL, XGL-XY, and XGL-A

Adenosine production in fed-batch fermentation

To investigate the potential of the strain *B. subtilis* XGL-A in a larger scale fermentor, fed-batch fermentation was performed in 5-L fermentor. Compared to *B. subtilis* XGL, 29.4 % and 18.3 % increase in adenosine production and yield, and up to 17.3 g/L and 0.207 g/g glucose was achieved by *B. subtilis* XGL-A (Fig. 6). And it is worth note that similar biomass but lower glucose consumption rate was observed in *subtilis* XGL-A.

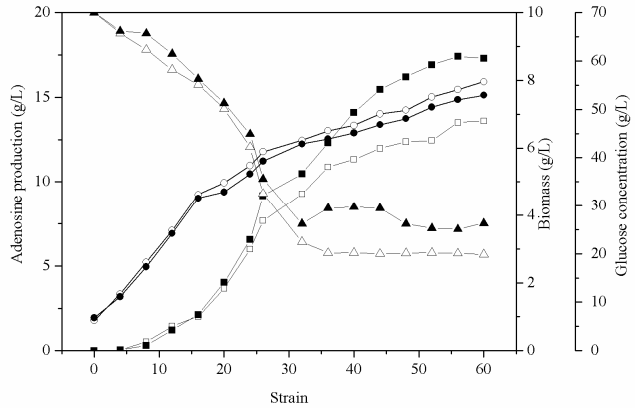

Fig. 6 Adenosine production (squares), glucose consumption (triangles) and biomass (circles) by *B. subtilis* XGL (open symbols) and *B. subtilis* XGL-A (solid symbols) during fed-batch fermentation

Genomic stability of *B. subtilis* XGL-A

Genomic stability in a recombinant strain is significantly important for its utilization in industry, and instability is frequently observed during cultivation processes. Since *purA* was integrated to genomic DNA by single crossover, reverse mutations may take place. So genomic stability of *B. subtilis* XGL-A was detected at the end of fermentation process, which showed that the genomic stability decreased only 2.6% (data not shown), indicating the strain is stable and suitable for large-scale fermentation[21]. Further study will focus on replacing promoter of *purA* in *B. subtilis* XGL genome with P43 to enhance its transcription and to make the strain more suitable for industrial production.

CONCLUSION

Overexpression of *purA* could increase metabolic flux of IMP to AMP and thus enhance adenosine production. Moreover, integrating additional *purA* to genome caused less burden to the host and lead to higher adenosine production. Taken together, results in this study suggest that the modification have potential applications in the industrial production of adenosine.

Acknowledgments

We are grateful to South China University of Technology for providing the plasmid pKS1 used in this study. This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1166) and by the Tianjin Municipal Education Commission (No. 20120630).

REFERENCES

[1] RD Lasley; PJ Konyn; JO Hegge; et al. *Amm. J. Physiol.*, **1995**, 269(4 Pt 2), H1460-H1466.

[2] J Linden. *Mol. Pharmacol.*, **2005**, 67(5), 1385-1387.
[3] Q Lu; J Newton; EQ Harrington; et al. *Am. J. Resp. Crit. Care.*, **2011**, 183(1), A1949
[4] JP DiMarco; TD Sellers; RM Berne; et al. *Circulation*, **1983**, 68(6), 1265-1263.
[5] JP DiMarco; W Miles; M Akhtar; et al. *Ann. Intern. Med.*, **1990**, 113(2), 104-110.
[6] M Hori; M Kitakaze. *Hypertension*, **1991**, 18(5), 565-574.
[7] Kunst F; Ogasawara N; Moszer I; et al. *Nature*, **1997**, 390(20), 249-256.
[8] U Sauer; DC Cameron; JE Baily. *Biotechnol. Bioeng.*, **1998**, 59(2), 227-238.
[9] M Schallmey; A Singh; OP Ward. *Can. J. Microbiol.*, **2004**, 50(1), 1-17.
[10] HH Saxild; P Nygaard;. *J. Gen. Microbiol.*, **1991**, 137(10), 2387-2394.
[11] JC Qian; XP Cai; J Chu; et al. *Biotechnol. Lett.*, **2006**, 28(12), 937-941.
[12] T Asahara; Y Mori; NP Zakataeva; et al. *Appl. Microbiol. Biotechnol.*, **2010**, 87(6), 2195-2207.
[13] HJ Li; GQ Zhang; AH Deng; et al. *Biotechnol. Lett.*, **2011**, 33(8), 1575-1580.
[14] YS Konstantin; AN Alex. *FEMS. Microbiol. Lett.*, **2005**, 245(2), 315-319.
[15] JL Kenneth; DS Thomas. *Methods*, **2001**, 25(4), 402-408.
[16] SH Fisher; B Mangasanik. *J. Bacteriol*., **1984**, 158(1), 55-62.
[17] S Mehrotra; H Balaram. *Biochim. Biophys. Acta.*, **2008**, 1784(12), 2019-2028.
[18] RH Rüllerr; N Loffhagen; W Babel. *J. Microbiol. Meth.*, **1996**, 25(1), 29-35.
[19] B Vosman; J Kooistra; J Olijve; et al. *Mol. Gen. Genet.*, **1986**, 204(3), 524-531.
[20] M Shimaoka; Y Takenaka; O Kurahashi; et al. *J. Biosci. Bioeng*., **2007**, 103(3), 255-261.
[21] N Lange; A Steinbüchel. *Appl. Microbiol. Biotechnol*., **2011**, 91(6), 1611-1622.