JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Production of α-Cyclodextrin Glycosyltransferase in *Bacillus megaterium* MS941 by Systematic Codon Usage Optimization

Jingwen Zhou,*[,†,‡,§] Hua Liu,[†,‡,§] Guocheng Du,[†] Jianghua Li,[†] and Jian Chen*[,†]

[†]School of Biotechnology and Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

[‡]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

ABSTRACT: α-Cyclodextrin glycosyltransferase is a key enzyme in the cyclodextrin industry. The Gram-positive bacterium *Bacillus megaterium* was chosen for production of recombinant α-CGTase for safety concerns. Successful production of heterologous α-CGTase was achieved by adapting the original *α-cgt* gene to the codon usage of *B. megaterium* by systematic codon optimization. This balanced the tRNA pool and reduced ribosomal traffic jams. Protein expression and secretion was ensured by using the strong inducible promoter P_{xyl} and the signal peptide SP_{LipA}. The impact of culture medium composition and induction strategies on α-CGTase production was systematically analyzed. Production and secretion at 32 °C for 24 h using modified culture medium was optimal for α-CGTase yield. Batch- and simple fed-batch fermentation was applied to achieve a high yield of 48.9 U·mL^{-1}, which was the highest activity reported for a *Bacillus* species, making this production system a reasonable alternative to *Escherichia coli*.

KEYWORDS: *Bacillus megaterium MS941, codon usage optimization, α-cyclodextrin glycosyltransferase, process optimization*

1. INTRODUCTION

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) is a key extracellular enzyme in cyclodextrin (CD) production, capable of converting starch or starch derivates to CD via a cyclization reaction.[1] CGTase also catalyzes three other reactions: coupling, disproportionation, and starch hydrolysis.[1] CGTases are classified as α-, β-, and γ-CGTases according to the major CDs produced, which differ in the number of glucose units (α-CD, β-CD, and γ-CD).[2] Because of its small internal cavity, high water solubility, and nonbioavailability, α-CD has special applications in molecular recognition, nanomaterials, and the food industry.[3] In addition, many carbohydrate derivatives and other compounds such as sugar alcohols, vitamins, glycosides, and polyols can be used as acceptors in CGTase-catalyzed reactions, improving the properties and application of the products.[4]

As α-CGTase has gained increasing industrial interest in recent years, its production has drawn extensive attention. Current research mainly focuses on the selection and construction of α-CGTase-producing strains. *Escherichia coli* has been the main host for the heterologous expression of α-CGTase. However, *E. coli* does not normally secrete proteins extracellularly. Complicated process control technologies are needed to avoid the inefficient secretory systems and inactive inclusion bodies.[5] A number of studies have attempted to improve α-CGTase production using methods that enhance secretion. However, the yields of α-CGTase in most of these studies were below 30 U·mL^{-1}.[3,6] In one study, a yield of 273.5 U·mL^{-1} was achieved with fed-fermentation, but the fermentation process was complicated.[7]

In addition, the cell envelope of *E. coli* contains lipopolysaccharides that are pyrogenic endotoxins in humans and other mammals.[8] This presents safety concerns because most applications of α-CGTase catalytic products are in pharmacy, agriculture, food, and cosmetics. Therefore, a Gram-positive recombinant expression system is needed to achieve efficient production of α-CGTase.

Bacillus megaterium is interesting for industrial heterologous protein production because of its ability to naturally secrete several proteins into the culture medium.[9] Secretion of recombinant proteins into the growth medium reduces the effort and cost of protein purification. *B. megaterium* was evaluated as Generally Recognized As Safe (GRAS) by the US Food and Drug Administration. It possesses efficient secretory systems that allow proteins to be released into the cultivation medium, facilitating their isolation and purification. *B. megaterium* also shows high plasmid stability and a lack of alkaline proteases.[10] Recently, considerable efforts were undertaken to significantly enhance the levels of heterologous production in *B. megaterium* using the plasmidless *B. megaterium* strain MS941, which is derived from the wild-type strain DSM319 by knocking out the major extracellular protease gene *nprM* and using a strong xylose-inducible promoter system.[9] In this system, most exoproteins are secreted via the Sec pathway using N-terminal leader sequences. The signal peptides SP_{LipA} and SP_{Pac} are the most widely used in this system.[10] High secretion levels of many heterologous proteins including dextransucrase, levansucrase, and antibody fragment have been achieved using this expression system.[11,12] The conventional approach to improving protein productivity is to raise the cell concentration as well

Received: June 30, 2012
Revised: September 22, 2012
Accepted: September 26, 2012
Published: September 26, 2012

dx.doi.org/10.1021/jf302819h | *J. Agric. Food Chem.* 2012, 60, 10285−10292

to optimize the process. However, protein productivity can also be enhanced by optimizing factors that have an impact on gene expression.

Codon optimization adjusts codons in a target sequence to high-frequency codons preferred by the expression host. Codon optimization involves making synonymous mutations that change the nucleotide sequence of a gene without changing the amino acid sequence it encodes. Increasing evidence indicates that the choice of synonymous codons can influence protein production by changing the mRNA secondary structure and improving correlation with tRNA levels in the host cell.[13] Codon usage bias evolved to regulate expression levels. Codons used at high frequency have high levels of corresponding tRNAs in the host, optimizing translational efficiency.[14] In addition, synonymous codon substitution of genes, especially at the 5′-end, can affect mRNA structure and stability and thus affect translation initiation and elongation.[15] Various strategies have been used to minimize bias to increase heterologous expression. For example, (1) supplying extra copies of rare tRNA genes to host cells.[16] However, this method can cause significant stress to host−cell metabolic processes and resolves only some, but not necessarily all, codon bias issues. (2) "Codon optimization" substitutes codons in a target sequence for preferred high-frequency codons used by the expression host. This is also called the "one amino acid-one codon" approach and has been successful for heterologous production in some host expression systems including *B. megaterium*.[9] However, this method can lead to tRNA traffic jams. In some cases, high levels of expressed proteins lead to the formation of insoluble products. (3) "Codon harmonization" recodes a target gene sequence to match the codon usage bias inherent in the native host more closely when expressed in the heterologous host. This method has been successfully applied to the expression of several antigens in *E. coli*.[17]

In this study, the importance of genetic construction design on the extracellular recombinant α-CGTase production in *B. megaterium* MS941 was assessed. First, the systematic codon optimization method, which is based on the codon optimization method, was used. However, although the most preferred codons were used during optimization, the second-most preferred codons were alternatively used to prevent the depletion of tRNAs from consecutively choosing the same codon for frequently used amino acids. Second, based on a previously established protein production system, the strong, inducible xylose promoter of the pSTOP1622 vector was used. Finally, the signal peptide SP_{LipA} was applied. The influence of culture media and induction strategies were also followed to enhance the extracellular α-CGTase yield in *B. megaterium* MS941. Finally, batch- and simple fed-batch-fermentation were applied to achieve a high yields of 48.9 $U{\cdot}mL^{-1}$. This is highest activity reported in a *Bacillus* species.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids. All strains and plasmids used in this study are in Table 1. *E. coli* JM109 was used as a parental strain to clone and maintain plasmids. All bacterial strains were stored at −80 °C.

2.2. Medium and Culture Conditions. For shake-flask cultivation, LB media consisted of (all in $g{\cdot}L^{-1}$): 10 peptone, 5 yeast extract, and 10 NaCl. Modified TB was employed for all shake-flask and fermenter cultures and contained (in g L^{-1}): 12 glycerol, 24 yeast extract, and 12 peptone, with 17 mM KH_2PO_4 and 72 mM K_2HPO_4. The feeding media contained (all in g L^{-1}): 500 glycerol, 15 peptone, 30 yeast extract. The 2×YT, SB, SOC, AS, MOPS media were as described.[3,9]

Table 1. Bacterial Strains and Plasmids Used in This Study

strains/ plasmids	relevant characteristics	reference
P. macerans JFB05-01	*α-cgt* gene template	3
B. megaterium MS941	*B. megaterium* DSM319, Δ*nprM*	33
E. coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *e14*$^-$(*mcrA*$^-$), *supE*44 *relA*1, Δ(*lac-proAB*)/ F′[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Takara
pMD18-T simple	Amp^r	Takara
pSTOP1622	P_{xyl}, Tet^r, Ap^r, *E. coli*−*B. megaterium* shuttle expression vector	34
pSTOP1622-SP_{lipA}-wt*α-cgt*	SP_{LipA}, wt*α-cgt* gene	This study
pSTOP1622 -wt*α-cgt*	*α-cgt* gene	This study
pSTOP1622-SP_{lipA}-co*α-cgt*	SP_{LipA}, co*α-cgt* gene	This study

2.3. Codon Usage Optimization. The codons of the *α-cgt* gene (2061 bp) were optimized based on the codon preference of *B. megaterium* using a codon algorithm in GeMS software[18] and a codon table containing a fractional preference for each codon equal to that found in the *B. megaterium* genome. Modifications were made throughout the sequence. A codon-optimized *α-cgt* gene (co*α-cgt*) was successfully assembled using overlapping PCR (Genscript, Nanjing, China).

2.4. Genetic Operations. Standard molecular techniques were used. Primers used in this study are in Table 2. The wild-type *cgt* (wt*α-cgt*) coding gene was cloned from *Paenibacillus macerans* JFB05-01[3] using primers P5 and P6, which introduced *Bsr*GI and *Bam*HI sites. The PCR product was purified by electrophoresis on 0.8% agarose with a gel extraction kit (TakaRa, Dalian) and digested with *Bsr*GI and *Bam*HI, then inserted into pSTOP1622. The resulting plasmid, called pSTOP1622-wt*α-cgt* (Figure 1), was checked by restriction analysis and sequencing.

SP_{LipA}-wt*α-cgt* was obtained in two steps by PCR amplification. The SP_{LipA} fragment was amplified from *B. megaterium* carrying the *lipA* gene open reading frame, using primers P1 and P2, which introduced a *Bsr*GI site at the 5′-end of the fragment. The wt*α-cgt* gene was obtained using the primers P3 and P4 and the genome of *P. macerans* JFB05-01[3] carrying the fragment as template. The resulting fragments (wt*α-cgt* and SP_{LipA}) were purified, pooled, and used as templates for the amplication of SP_{LipA}-wt*α-cgt* with the primers P1/P4. PCR products were purified and digested with *Bsr*GI and *Bam*HI as above and inserted into pSTOP1622. The resulting plasmid, called pSTOP1622-SP_{LipA}-wt*α-cgt* was checked by restriction analysis and sequencing.

The SP_{LipA}-co*α-cgt* construction had a similar molecular method as the pSTOP1622-SP_{LipA}-wt*α-cgt* construction. Using the primers P5/P6/P7/P8, the plasmid pSTOP1622-SP_{LipA}-co*α-cgt* was obtained. DNA sequencing confirmed the integrity of the vectors.

Transformation of *B. megaterium* strain MS941 used a protoplast transformation method as described.[19]

2.5. RNA Isolation and Real-Time Reverse Transcription PCR Analysis. Total RNA was isolated from *B. megaterium* MS941 3 h after xylose induction using the Total RNA Midi Kit (Qiagen, Hagen, Germany). Using the relevant PCR primers (Table 2) and a Real-Time One Step RNA PCR Kit (Takara, Dalian, China), cDNA was synthesized and amplified. P11/P12 were used for 16S, P13/P14 for wt*α-cgt*, and P15/P16 for co*α-cgt*. Reverse transcription (RT)-PCR experiments were performed on a Roche Light Cycler 480 II real-time PCR system (Roche, Indianapolis, IN) using SYBR Premix *Ex Taq*TM II (Takara, Dalian, China). PCR parameters were 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 55 °C for 7 s, and 72 °C for 10

Table 2. PCR Primers Used in This Study

number	primer	sequence (5′−3′)[a]
P1	SP_{lipA}-F-wt	GG*TGTACA*ATGAAAAAAGTACTTATGGC
P2	SP_{lipA}-R-wt	ACGCTCGTATCGGGTGAACTAGTGGCGCCTGCGCCAGACG
P3	wtCGT-F-Fusion	TCTGGCGCAGGCGCCACTAGTTCACCCGATACGAGC
P4	wtCGT-R-Fusion	CG*GGATCC*ATTTTGCCAGTCCACCGTCCACCGTCAC
P5	wtCGT-F	GG*TGTACA*TCACCCGATACGAGCGTGGACAAC
P6	wtCGT-R	CG*GGATCC*ATTTTGCCAGTCCACCGTCAC
P7	SP_{lipA}-F-co	GG*TGTACA*ATGAAAAAAGTACTTATGGC
P8	SP_{lipA}-R-co	CAGACGTATCAGGAGAGGCGCCTGCGCCAGACGG
P9	coCGT-F-Fusion	TCTGGCGCAGGCGCCACTAGTTCTCCTGATACGTCTGTG
P10	coCGT-R-Fusion	CG*GGATCC*TTAGTTTTGCCAATCCAC
P11	16S-S	AACGATGAGTGCTAAGTG
P12	16S-A	GAGTTGTCAGAGGATGTC
P13	WTCGT-S	CGATTGACGGGCGCGGCTTTG
P14	WTCGT-A	GCAGCAGGCCGTGGCGGTCGTTA
P15	COCGT-S	CGATTGATGGACGAGGTTTTGG
P16	COCGT-A	GTTAGATGCTGTTCCTGAAGACG

[a]Italic sequences indicate restriction sites used for gene construction; Underlined sequences indicate fused sequence.

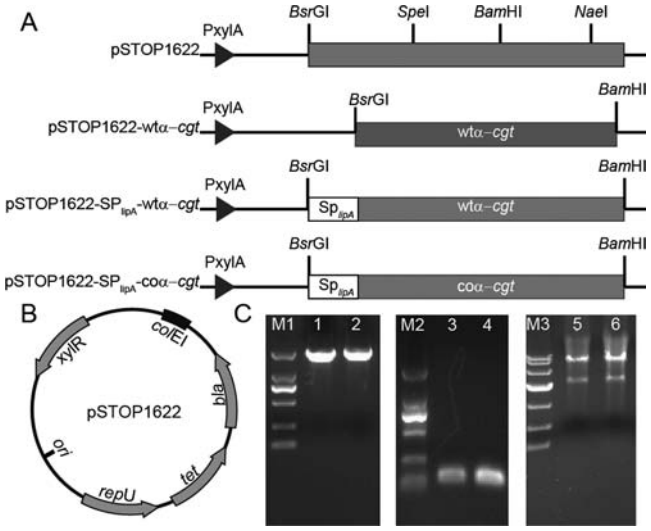

Figure 1. Construction of expression vectors. (A) Structure of the plasmid using the promoter P_{xylA}. Signal peptide (SP) is from *B. megaterium* extracellular lipase *LipA*. (B) Expression vector pSTOP1622. (C) 1, wt*α-cgt* gene (2061 bp); 2, co*α-cgt* gene (2061 bp); 3 and 4, SP_{LipA} gene (84 bp); 5, SP_{LipA}-wt*α-cgt* gene (2145 bp) and pSTOP1622 vector (6535 bp); 6, SP_{LipA}-co*α-cgt* gene (2145 bp) and pSTOP1622 vector (6535 bp). M1 and M2, DNA marker DL2000; M3, DNA marker DL 10 000.

s. Gene expression levels were determined relative to 16S gene expression. Three experiments were run for each condition analyzed.

2.6. Culture Conditions. For shake-flask cultures, transformants were grown for seed cultures in 20 mL of LB medium containing 10 μg mL^{-1} tetracycline at 37 °C and 200 rpm. A 10% (v/v) concentration of inoculum was added to modified TB medium containing 10 μg mL^{-1} tetracycline for shaking flask cultures. When the OD_{578} reached 0.4, 5 g·L^{-1} xylose was added into growth medium at 32 °C and 200 rpm to induce expression of the recombinant protein.

Batch culturing was performed in a 3 L fermentor (BioFlo 115, New Brunswick Scientific Co.) by adding 10% seed cultures into 1.5 L of modified TB medium. When the OD_{578} reached 1.0, xylose was added to a final concentration of 5 g L^{-1}. The temperature was controlled at 32 °C. Cell density and extracellular α-CGTase activity were measured every 3 h. To control excessive foaming, one or two drops of diluted antifoam were added to the fermentor. Agitation speed was kept at 400 rpm. Tetracycline was added to all media at a final concentration of 100 μg mL^{-1}. For simple fed-batch fermentation, at 15 h of cultivation, 36 mL of feeding media were intermittently fed into the cultivation culture, while other conditions were unchanged.

2.7. Assay of α-CGTase Activity. The α-CGTase activity was determined using a methyl orange method as described by Li et al.[20] All experiments were evaluated under the same conditions and performed in triplicate.

2.8. Miscellaneous Methods. Culture density was determined as OD_{578} using a spectrophotometer (BioPhotometer Plus, Eppendorf, Hamburg, Germany). Samples were diluted approximately with 0.9% (w/v) NaCl when the OD_{578} exceeded 0.8. To determine the dry cell weight (DCW), 10 mL of culture broth was centrifuged at 8000*g* for l5 min. The pellet was washed and resuspended with 0.9% (w/v) NaCl, centrifuged again, and dried at 105 °C. SDS-PAGE was performed as previously described[7] using a 5% stacking gel and 12% separating gel (Bio-Rad, Hercules, CA). Gels were visualized with 0.25% Coomassie Brilliant Blue R-250. Protein concentration was determined by the Bradford method. A fermentation supernatant was used for glycerol and xylose analysis using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with a differential refractive index detector (RID), with 5 μL of sample injected into a Shodex SUGAR SH1011 column (8.0 mm i.d. × 300 mm, Showa Denko, Japan) at a constant temperature of 50 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.8 mL min^{-1}. The detector temperature was maintained at 30 °C during analysis.

3. RESULTS AND DISCUSSION

3.1. Wild-Type *α-cgt* Gene Expression in *B. megaterium* MS941. In previous work, a wild-type strain, *P. macerans* strain JFB05-01, capable of secreting α-CGTase, was isolated.[3] The *α-cgt* gene was 2061 bp and encoded a peptide of 687 amino acids with a molecular weight of 72 kDa. For the heterologous production and secretion of α-CGTase in *B. megaterium* MS941, the mature *α-cgt* gene with and without the SP_{LipA} signal peptide was cloned under the control of a xylose inducible promoter system into the *B. megaterium* expression vector pSTOP1622.[21] The resulting *α-cgt* gene-containing vectors were named pSTOP1622-wt*α-cgt* and pSTOP1622-SP_{LipA}-wt*α-cgt*. However, only 0.1 U·mL^{-1} and no obvious α-CGTase activity was seen in the LB and A5 media, although various xylose addition schemes were tested for the induction of *α-cgt* expression. Furthermore, no α-CGTase was detected in

Table 3. Codon Preference in *B. megaterium* and Codon Usage in the Wild-Type and the Optimized Synthetic *α-cgt* Gene

amino acid	codon	*B. megaterium*	*wtα-cgt*	*coα-cgt*	amino acid	codon	*B. megaterium*	*wtα-cgt*	*coα-cgt*
Ala	GCA	0.89	1	31	Leu	CUA	0.17	0	0
	GCC	0.03	22	0		CUC	0.01	8	0
	GCG	0.14	27	0		CUG	0.01	16	0
	GCU	1.00	10	29		CUU	0.41	4	23
Arg	AGA	0.07	0	7		UUA	1.00	1	14
	AGG	0.01	3	0		UUG	0.05	8	0
	CGA	0.01	2	5	Lys	AAA	1.00	13	27
	CGC	0.33	6	0		AAG	0.11	14	0
	CGG	0.02	4	0	Met	AUG	1.00	13	13
	CGU	1.00	3	6	Phe	UUC	1.00	20	0
Asn	AAC	1.00	33	39		UUU	0.84	13	33
	AAU	0.46	21	15	Pro	CCA	1.00	1	12
Asp	GAC	0.56	32	1		CCC	0.01	5	0
	GAU	1.00	19	50		CCG	0.15	16	0
Cys	UGC	0.49	0	0		CCU	0.76	2	12
	UGU	1.00	0	0	Ser	AGC	0.59	15	9
Gln	CAA	1.00	8	20		AGU	0.22	2	10
	CAG	0.05	12	0		UCA	1.00	1	11
Glu	GAA	1.00	11	19		UCC	0.04	0	0
	GAG	0.15	8	0		UCG	0.04	11	0
Gly	GGA	0.52	4	45		UCU	0.99	16	16
	GGC	0.49	43	0	Thr	ACA	1.00	5	39
	GGG	0.03	19	0		ACC	0.00	18	0
	GGU	1.00	11	32		ACG	0.14	38	25
	CAC	1.00	3	0		ACU	0.42	3	0
	CAU	0.72	8	11	Trp	UGG	1.00	13	13
Ile	AUA	0.00	3	0	Tyr	UAC	0.95	17	0
	AUC	0.58	15	0		UAU	1.00	16	33
	AUU	1.00	19	37	Val	GUA	1.00	7	18
						GUC	0.03	13	0
						GUG	0.16	24	16
						GUU	0.81	6	16

the culture supernatant, or in the intracellular soluble or insoluble protein fractions by SDS-PAGE (data not shown).

3.2. Design of *α-cgt* Gene for Expression in *B. megaterium* MS941. The codon bias of heterologous genes is often a limiting factor in expression in host systems. After careful examination of the coding sequence of the wild-type *α-cgt* gene, large differences were observed between the most frequently used codons in the wt-*α-cgt* gene and codon usage of *B. megaterium* (Table 3). For example, in the native sequence, 60% of the codons for Gln had a frequency below 0.05 in the *B. megaterium* genome and 44% of the codons for Ala had a frequency below 0.03. This might have led to the failed expression of the target protein in this host cell. To overcome this limitation, we hypothesized that codon optimization of the *α-cgt* gene would result in protein expression. The systematic codon optimization strategy used in this study was a preference table (http://www.kazusa.or.jp/e/index.html) with a probability based on the weight of each codon within the set encoding a given amino acid. Using this algorithm, a codon-optimized sequence was designed using the GeMS software package,[18] and named *coα-cgt*.

Codon preference in *B. megaterium* and codon usage in the wild-type and co*α-cgt* genes are in Table 3. The most significant changes were for Ala and Gln, with only small changes for Asn. The C+G content was significantly changed from 55.58% (wt*α-cgt*) to 37.59% (co*α-cgt*). To facilitate gene design, the systematic codon optimization strategy permits flexibility in codon selection by avoiding: (1) repetitive elements that may lead to gene deletions, (2) internal Shine-Dalagarno sequences, (3) secondary mRNA structures, and (4) restriction sites. After optimization, the codon usage was well adapted to the preferences of *B. megaterium*, as demonstrated by a CAI of 0.88. The expression of the codon optimized *α-cgt* gene was first tested with 20 mL LB shaking flask cultivation at 37 °C. Under the identical cultivation conditions, MS941 carrying wt*α-cgt* gene failed to produce or secrete *α*-CGTase. Previous studies using alternative expression hosts such as yeast and bacteria such as *Bacillus subtilis*, which are known for producing recombinant proteins, did not achieve CGTase production.[22] The common cause for failure in heterologous gene expression is primarily related to nonoptimal codon content. Preferred codons are correlated with the abundance of aminoacyl tRNAs in a species.[23] Different codon frequencies have been found to be translated at different rates, and rare codons can be a limiting factor in translational elongation because of the waiting time for an appropriate aminoacyl tRNA.[24] In addition, rare codons can stimulate mRNA degradation, and a slow elongation rate along an mRNA can cause a ribosomal pause.[25] A translational pause generates an unprotected region that could be exposed to endonuclease attack or other factors that influence mRNA stability.[26]

Well-adapted codons can have a metabolic advantage by selecting for translation efficiency and reducing the impact of misfolded proteins.[13] A successful synthetic gene design can

lead to dramatic improvements in heterologous expression. In this study, a systematic codon optimization method was used, based on a codon optimization method . Codons other than the most-preferred codons were used during optimization, which avoided rare codon usage in the host cell. Second-most-preferred codons were alternatively used to prevent tRNAs depletion from choosing the same codon for frequently used amino acids.[23]

To investigate whether differences in α-CGTase production among constructs could be explained by differences in mRNA levels, total mRNA was extracted and subjected to RT-PCR. The mRNA levels of the synthetic construct were approximately equal to that of the native construct (Figure 2A). Codon optimization did not appear to significantly influence gene transcription. However, at the protein level, the synthetic gene resulted in a level of α-CGTase protein production (251.5 μg mL^{-1}) that was 30-fold higher than that obtained using the native gene (Figure 2B). These data were consistent with order-of-magnitude increases reported for other codon optimization studies.[23,27] α-CGTase protein was observed by SDS-PAGE only for the synthetic gene (Figure 2C). Therefore, the codon optimization significantly influenced the translation stage. These data indicated that the positive effect of rare codon replacement in the target gene improved heterologous protein expression in *B. megaterium*.

3.3. Glycerol and co-α-CGTase Production in *B. megaterium*. Fermentation optimization is important for heterologous expression. Significant discrepancies have been observed when cultivating *B. megaterium* in LB or A5 medium.[9] To enhance the production of α-CGTase by *B. megaterium*, medium optimization was performed with flask experiments. *B. megaterium* MS941 (pSTOP1622-SP_{LipA}-co*α-cgt*) was grown in complex media (LB, TB, 2×YT, SB, SOC), semidefined media (A5), and minimal media (MOPS) supplemented with tetracycline until the OD_{578} reached 0.4. Induction was with xylose at a final concentration of 5 g L^{-1} at 37 °C for 24 h. The maximum extracellular activity (7.1 U·mL^{-1}) and the maximum specific productivity (extracellular activity/DCW 1.14 U·g^{-1}) were seen in TB media. TB media is considered to be more favorable because it provides an abundance of amino acids, vitamins, and trace elements to support higher biomass and product expression in *B. megaterium*.[28] TB media also has a relatively high pH-buffering capacity, which has beneficial effects on cell growth and enzyme stability.[29] Therefore, TB was chosen for to enhance the extracellular production of α-CGTase in *B. megaterium*.

The influence of carbon sources on the cultivation of *B. megaterium* with enhanced α-CGTase production was also assessed. Figure 3B shows that biomass was 6.49 g·L^{-1} with glycerol as the carbon source and 10.8 g·L^{-1} with dextrin. Extracellular α-CGTase yield reached 7.1 with glycerol and 7.5 U·mL^{-1} with dextrin after 24 h of cultivation. Specific productivity of α-CGTase was higher (1.14 U·g^{-1}) when glycerol was used; therefore, glycerol was the optimal carbon source for α-CGTase production. The influence of the glycerol concentration was further investigated, and the optimal glycerol concentration was 12 g·L^{-1}, at which the extracellular yield reached 9.2 U·mL^{-1} after 24 h of cultivation (Figure 3C).

3.4. Induction Temperature and co-α-CGTase Production in *B. megaterium*. Induction temperature is an important parameter for recombinant protein production in *B. megaterium*. However, the effect depends on several factors such as the nature of the recombinant proteins, the expression

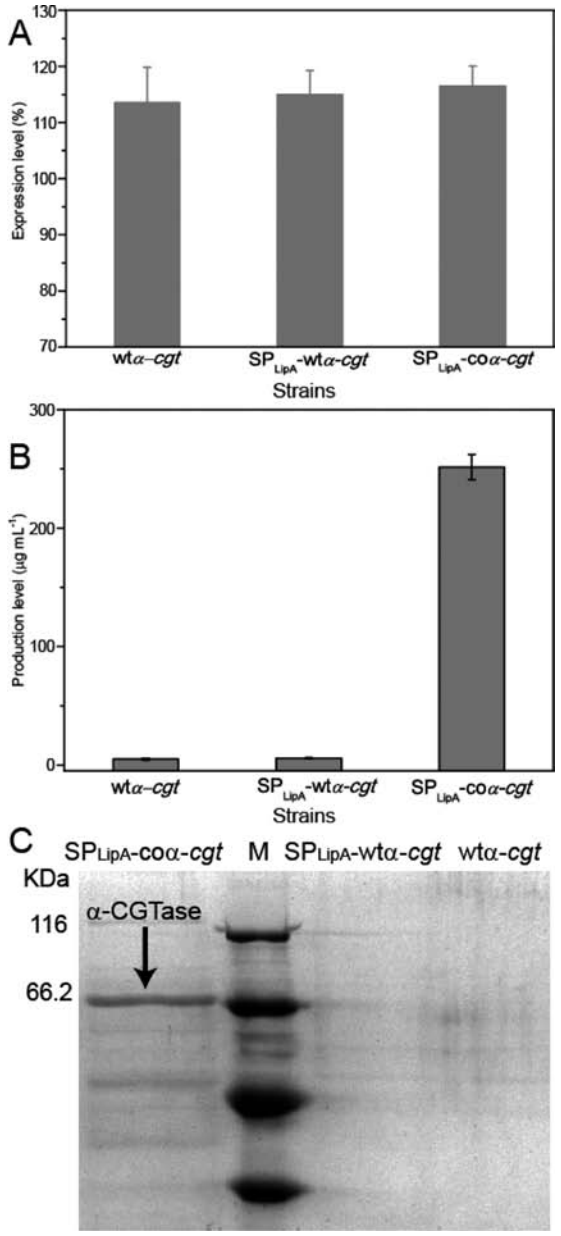

Figure 2. Transcription and expression level depends on *α-cgt* constructs. (A) Transcriptional level depends on *α-cgt* constructs. Transformants were grown on modified TB media for 4 h after induction, tRNA was extracted, and mRNA levels were quantified by RT-PCR and expressed as percentage of the mRNA of the constitutively expressed 16S gene. (B) Expression level depends on *α-cgt* gene constructs. (C) SDS-PAGE analysis of co*α-cgt* gene expression in culture supernatant fractions 24 h after induction in LB medium.

system, and medium composition. In previous work, for the production of other recombinant proteins in *B. megaterium* such as dextransucrase, 37 °C was the optimal induction temperature;[30] for antibody production, 41 °C was found to be optimal.[31] For production of α-CGTase in *E. coli*, a low-temperature strategy was applied to reduce protein aggregation and inclusion body formation; 25 °C was the optimal induction temperature.[3] Therefore, induction temperatures differ significantly for different proteins and different expression hosts. The influence of different induction temperatures on α-CGTase production in *B. megaterium* was investigated. As depicted in

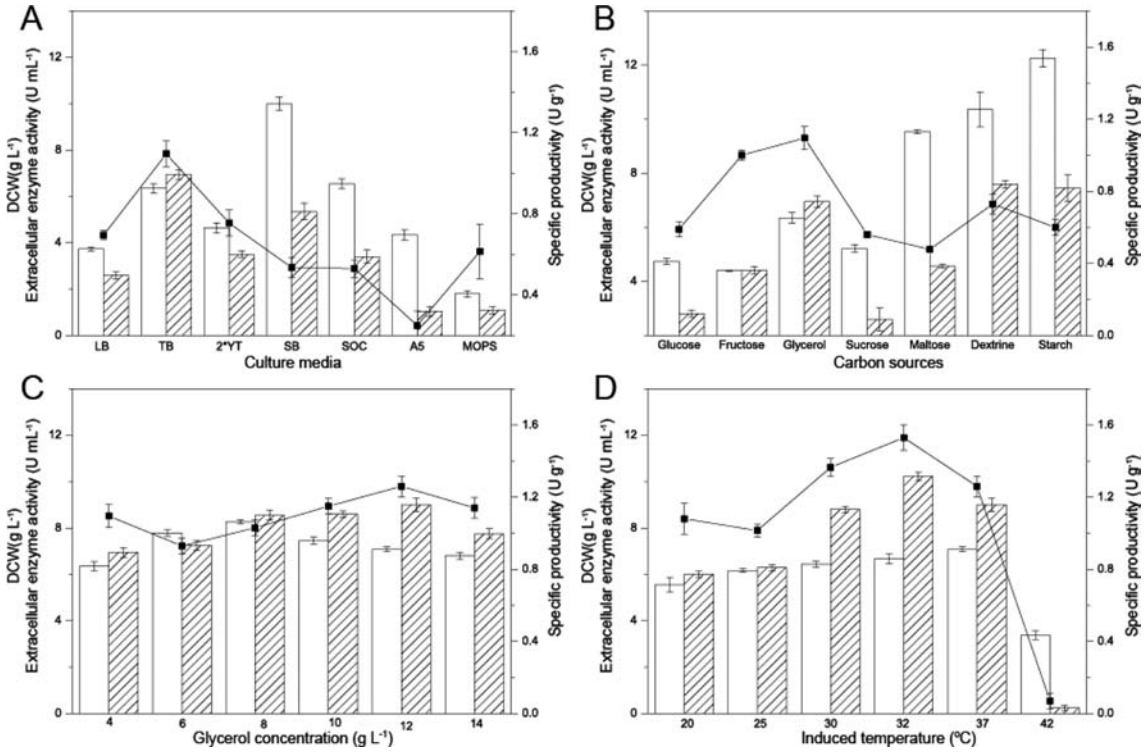

Figure 3. Comparison of α-CGTase production and cell growth during process optimization in flask levels. Comparison of α-CGTase production and cell growth in different culture media (A), carbon sources (B), glycerol concentration (C), and induced temperature (D). Extracellular α-CGTase activity (slash), DCW (white), and specific productivity (black squares).

Figure 3D, higher induction temperature had an inhibitory effect on α-CGTase production. When the induction temperature reached 42 °C, almost no α-CGTase activity was seen. The high temperature might have affected protein folding or spore-forming. At an induction temperature of 37 °C, the biomass reached 7.16 g·L^{-1}, which was the highest of all induction temperatures tested. However, a high level of biomass did not lead to a high α-CGTase yield. The extracellular α-CGTase yield was 9.2 U·mL^{-1} at 37 °C. Induction at 32 °C led a better balance of protein synthesis and translocation and attained the highest extracellular α-CGTase yield of 10.4 U·mL^{-1}. In addition to induction temperature, other induction strategies were considered. Different xylose concentrations (5, 10, 15, 20, 25 g L^{-1}) were compared, but showed almost no difference in cell growth or α-CGTase yield (data not shown). Finally, early (when OD_{578} reached 0.4) and late induction (when OD_{578} reached 4) of gene expression was compared by altering the addition of xylose. However, only half the α-CGTase production was obtained with late induction compared to early induction (data not shown). Hence, 5 g·L^{-1} xylose was added when the OD_{578} reached 0.4 for recombinant α-CGTase production in subsequent experiments.

3.5. Fed-Batch Fermentation for co-α-CGTase Yield in *B. megaterium* MS941. As the α-CGTase activity increased significantly in *B. megaterium* MS941 after codon optimization, and after medium and induction optimization in shaking flask experiments, batch fermentation was tried in a 3 L fermentor using the optimized medium and optimized induction strategies. After 40 h of cultivation, the results in Figure 4A were obtained. The α-CGTase activity in *B. megaterium* MS941 increased significantly with batch fermentation (16.5 U·mL^{-1}). After 15 h of cultivation, the concentration of glycerol was below 3 g L^{-1} in the culture medium (Figure 4B). The bacterial concentration indicated that a shortage of carbon source restricted growth. The concentration of the xylose also began to decrease (Figure 4B). Fed-batch operation allows higher cell densities than batch mode and is often applied to obtain high yields and productivities by controlling nutrient feeding.[32] A simple feeding strategy was applied. At 15 h of cultivation, 36 mL feeding media was intermittently fed into the cultivation culture, while other conditions were unchanged. When the fermentation period was extended to 40 h, the concentration of bacteria increased significantly to 22.45 g·L^{-1} and α-CGTase activity increased significantly to 48.9 U·ml^{-1} (Figure 4C). The concentration of xylose was kept to almost 5 g L^{-1} during the entire fermentation time. Glycerol concentration is shown in Figure 4D.

In this study, systematic codon usage optimization of the *α-cgt* gene makes expression of this gene in *B. megaterium* possible. Data from RT-PCR showed that the expression of α-CGTase by codon optimization was mainly due to the enhanced efficiency of translation elongation. This is the first time this gene has been expressed in *B. megaterium*. Furthermore, the production of α-CGTase yield was enhanced by medium optimization and induction strategy in flask cultivation, with final production of 10.4 U·mL^{-1}. Batch and simple fed-batch fermentation were performed to enhance α-CGTase yield to 48.9 U·mL^{-1}, the highest activity reported in a *Bacillus* species. However, the yield by simple fed-batch fermentation was still relatively low for industrial applications compared to α-CGTase expressed by *E. coli* using a combined feeding strategy.[7] Therefore, further studies should focus on genetic modification and processing technologies.

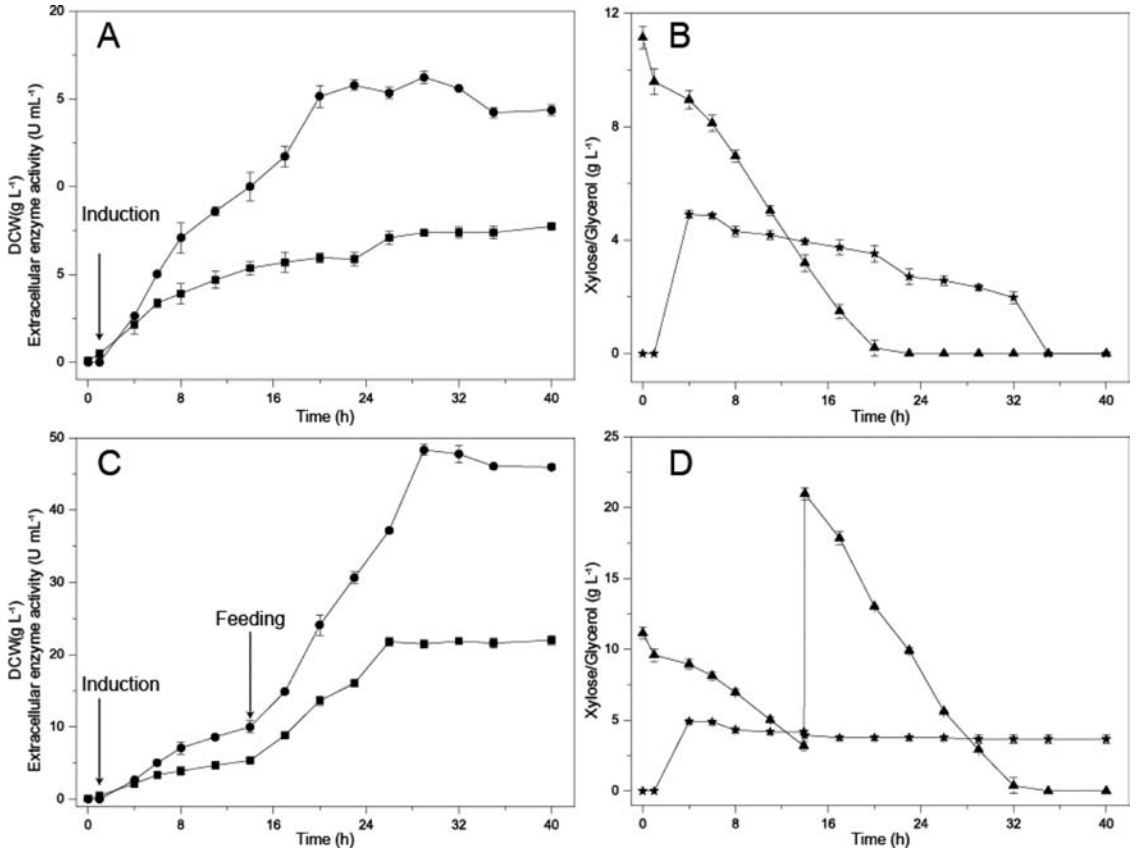

Figure 4. Batch and fed-batch culture of *B. megaterium* MS941 (pSTOP1622-SP_{LipA}-co*α-cgt*). A total of 5 g L^{-1} xylose was added at 1 h. (A) Batch culture: DCW (squares) and extracellular enzyme activity (circles). (B) Batch culture: glycerol (triangles) and xylose (stars). (C) Fed-batch culture: DCW (squares) and extracellular enzyme activity (circles). (D) Fed-batch culture: glycerol (triangles) and xylose (stars). Arrows indicate the point of induction and feeding.

AUTHOR INFORMATION

Corresponding Author

*Mailing address: School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China. Phone: +86-510-85329031. Fax: +86-510-85918309. E-mail: zhoujw1982@jiangnan.edu.cn (J.Z.), jchen@jiangnan.edu.cn (J.C.).

Author Contributions

§The first two authors contributed equally to this paper.

Funding

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program, 2012CB720806), the National High Technology Research and Development Program of China (863 Program, 2012AA023403, 2011AA100905), the Open Project Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (No. KLIB-KF201006), the Open Funding Project of the State Key Laboratory of Bioreactor Engineering, the Key Technologies R & D Program of Jiangsu Province, China (BE2011624) and the Fundamental Research Funds for the Central Universities (JUSRP211A29).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Thanks to Dr. Dieter Jahn for the kindly donation of strain *B. megaterium* MS941 and plasmid pSTOP1622.

REFERENCES

(1) Li, Z. F.; Wang, M.; Wang, F.; Gu, Z. B.; Du, G. C.; Wu, J.; Chen, J. *γ*-Cyclodextrin: a review on enzymatic production and applications. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 245−255.

(2) Ong, R. M.; Goh, K. M.; Mahadi, N. M.; Hassan, O. H.; Rahman, R. N. Z. R. A.; Illias, R. M. Cloning, extracellular expression and characterization of a predominant *β*-CGTase from *Bacillus sp.* G1 in *E. coli*. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 1705−1714.

(3) Li, Z. F.; Li, B.; Gu, Z. B.; Du, G. C.; Wu, J.; Chen, J. Extracellular expression and biochemical characterization of *α*-cyclodextrin glycosyltransferase from *Paenibacillus macerans*. *Carbohydr. Res.* **2010**, *345*, 886−892.

(4) Han, R.; Liu, L.; Li, J.; Du, G.; Chen, J. Functions, applications and production of 2-O-D-glucopyranosyl-l-ascorbic acid. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 313−320.

(5) Ma, N.; Wei, L.; Fan, Y.; Hua, Q. Heterologous expression and characterization of soluble recombinant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Actinosynnema pretiosum ssp auranticum* ATCC31565 through co-expression with Chaperones in *Escherichia coli*. *Protein Expression Purif.* **2012**, *82*, 263−269.

(6) Ding, R. R.; Li, Z. F.; Chen, S.; Wu, D.; Wu, J.; Chen, J. Enhanced secretion of recombinant *α*-cyclodextrin glucosyltransferase from *E. coli* by medium additives. *Process Biochem.* **2010**, *45*, 880−886.

(7) Cheng, J.; Wu, D.; Chen, S.; Chen, J.; Wu, J. High-level extracellular production of *α*-cyclodextrin glycosyltransferase with recombinant *Escherichia coli* BL21 (DE3). *J. Agric. Food. Chem.* **2011**, *59*, 3797−3802.

(8) Vavrová, L.; Muchová, K.; Barák, I. Comparison of different *Bacillus subtilis* expression systems. *Res. Microbiol.* **2010**, *161*, 791−797.

(9) Yang, Y.; Malten, M.; Grote, A.; Jahn, D.; Deckwer, W. D. Codon optimized *Thermobifida fusca* hydrolase secreted by *Bacillus megaterium*. *Biotechnol. Bioeng.* **2007**, *96*, 780−794.
(10) Stammen, S.; Mueller, B. K.; Korneli, C.; Biedendieck, R.; Gamer, M.; Franco-Lara, E.; Jahn, D. High-yield intra- and extracellular protein production using *Bacillus megaterium*. *Appl. Environ. Microbiol.* **2010**, *76*, 4037−4046.
(11) David, F.; Westphal, R.; Bunk, B.; Jahn, D.; Franco-Lara, E. Optimization of antibody fragment production in *Bacillus megaterium*: The role of metal ions on protein secretion. *J. Biotechnol.* **2010**, *150*, 115−124.
(12) Malten, M.; Hollmann, R.; Deckwer, W. D.; Jahn, D. Production and secretion of recombinant *Leuconostoc mesenteroides* dextransucrase DsrS in *Bacillus megaterium*. *Biotechnol. Bioeng.* **2005**, *89*, 206−218.
(13) Angov, E. Codon usage: Nature's roadmap to expression and folding of proteins. *Biotechnol. J.* **2011**, *6*, 650−659.
(14) Lithwick, G.; Margalit, H. Hierarchy of sequence-dependent features associated with prokaryotic translation. *Genome Res.* **2003**, *13*, 2665−2673.
(15) Tuller, T.; Carmi, A.; Vestsigian, K.; Navon, S.; Dorfan, Y.; Zaborske, J.; Pan, T.; Dahan, O.; Furman, I.; Pilpel, Y. An evolutionarily conserved mechanism for controlling the efficiency of protein translation. *Cell* **2010**, *141*, 344−354.
(16) Gvritishvili, A. G.; Leung, K. W.; Tombran-Tink, J. Codon preference optimization increases heterologous PEDF expression. *PLoS One* **2010**, *5*, e15056.
(17) Chowdhury, D. R.; Angov, E.; Kariuki, T.; Kumar, N. A potent malaria transmission blocking vaccine based on codon harmonized full length Pfs48/45 expressed in *Escherichia coli*. *PLoS One* **2009**, *4*, e6352.
(18) Jayaraj, S.; Reid, R.; Santi, D. V. GeMS: an advanced software package for designing synthetic genes. *Nucleic Acids Res.* **2005**, *33*, 3011−3016.
(19) Gamer, M.; Frode, D.; Biedendieck, R.; Stammen, S.; Jahn, D. A T7 RNA polymerase-dependent gene expression system for *Bacillus megaterium*. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 1195−1203.
(20) Li, Z. F.; Li, B.; Liu, Z. G.; Wang, M.; Gu, Z. B.; Du, G. C.; Wu, J.; Chen, J. Calcium leads to further increase in glycine-enhanced extracellular secretion of recombinant α-cyclodextrin glycosyltransferase in *Escherichia coli*. *J. Agric. Food. Chem.* **2009**, *57*, 6231−6237.
(21) Rygus, T.; Hillen, W. Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Appl. Microbiol. Biotechnol.* **1991**, *35*, 594−599.
(22) Tesfai, B.; Wu, D.; Chen, S.; Chen, J.; Wu, J. Strategies for enhancing extracellular secretion of recombinant cyclodextrin glucanotransferase in *E. coli*. *Appl. Biochem. Biotechnol.* **2012**, *167*, 897−908.
(23) Gasmi, N.; Fudalej, F.; Kallel, H.; Nicaud, J. M. A molecular approach to optimize hIFN alpha2b expression and secretion in *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 109−119.
(24) Scorer, C. A.; Carrier, M. J.; Rosenberger, R. F. Amino acid misincorporation during high-level expression of mouse epidermal growth factor in Escherichia coli. *Nucleic Acids Res.* **1991**, *19*, 3511−3516.
(25) Komar, A. A.; Lesnik, T.; Reiss, C. Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation. *FEBS Lett.* **1999**, *462*, 387−391.
(26) Koda, A.; Bogaki, T.; Minetoki, T.; Hirotsune, M. High expression of a synthetic gene encoding potato alpha-glucan phosphorylase in Aspergillus niger. *J. Biosci. Bioeng.* **2005**, *100*, 531−537.
(27) Huang, Y. Attenuated secretion of the thermostable xylanase xynB from *Pichia pastoris* using synthesized sequences optimized from the preferred codon usage in Yeast. *J. Microbiol. Biotechnol.* **2012**, *22*, 316−325.
(28) Pinotti, L. M.; de Souza, V. R.; Giordano, R. D.; Giordano, R. D. C. The penicillin G acylase production by *B. megaterium* is amino acid consumption dependent. *Biotechnol. Bioeng.* **2007**, *97*, 346−353.
(29) Fang, S.; Li, J.; Liu, L.; Du, G.; Chen, J. Overproduction of alkaline polygalacturonate lyase in recombinant *Escherichia coli* by a two-stage glycerol feeding approach. *Bioresour. Technol.* **2011**, *102*, 10671−10678.
(30) Malten, M.; Hollmann, R.; Deckwer, W.-D.; Jahn, D. Production and secretion of recombinant *Leuconostoc mesenteroides* dextransucrase DsrS in *Bacillus megaterium*. *Biotechnol. Bioeng.* **2005**, *89*, 206−218.
(31) Jordan, E.; Hust, M.; Roth, A.; Biedendieck, R.; Schirrmann, T.; Jahn, D.; Duebel, S., Production of recombinant antibody fragments in *Bacillus megaterium*. *Microb. Cell Fact.* **2007**, *6*.
(32) Lee, J.; Lee, S. Y.; Park, S.; Middelberg, A. P. J. Control of fed-batch fermentations. *Biotechnol. Adv.* **1999**, *17*, 29−48.
(33) Biedendieck, R.; Yang, Y.; Deckwer, W. D.; Malten, M.; Jahn, D. Plasmid system for the intracellular production and purification of affinity-tagged proteins in *Bacillus megaterium*. *Biotechnol. Bioeng.* **2007**, *96*, 525−537.
(34) Biedendieck, R.; Yang, Y.; Deckwer, W.-D.; Malten, M.; Jahn, D. Plasmid system for the intracellular production and purification of affinity-tagged proteins in *Bacillus megaterium*. *Biotechnol. Bioeng.* **2007**, *96*, 525−537.