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Engineering a repression-free catabolite-enhanced expression system for a thermophilic alpha-amylase from *Bacillus licheniformis* MSG

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ABSTRACT

Most expression systems used for the over production of many enzymes employ carbon catabolite repressible promoters and hence must use sugar free, rich complex media. Use of expression systems to overcome carbon repression opens an avenue for exploiting cheap carbon sources for the production of recombinant enzymes. A self-inducible, catabolite repression free and above all a glucose-activated expression system has been developed using an industrially suitable thermophilic alpha-amylase as a model. The alpha-amylase gene of *Bacillus licheniformis* MSG without the 5′ *cre* operator produced unimpeded glucose-enhanced expression when fused to the phosphate starvation-inducible strong *pst* promoter with optimum translation signals in a protease deficient *Bacillus subtilis*. A combination of high glucose with limited phosphate permitted sufficient biomass and fast transition to quiescent phase by phosphate starvation permitting 1250-fold induction for 70 h. A ~300-fold high productivity (9070 U mL^{-1}) and 131-fold increase in specific expression in 2% glucose and a 100-fold high yield in 4% molasses were obtained compared to the production by the parent strain. The yield was 18.5-fold higher than that from the native promoter in an isogenic clone. This strategy of catabolite enhanced enzyme expression uncoupled from biomass formation can be applied for cost effective high production of proteins using starch or molasses.

1. Introduction

Bacillus subtilis is the most characterized and developed host for the production of industrial enzymes and pharmaceutically important proteins. It has excellent fermentation capacities, expression of thiol disulfide oxidoreductase-like proteins involved in the folding pathway of disulfide-containing proteins and multiple secretion pathways to secrete large quantities of extracellular proteins directly into the medium thereby facilitating downstream processing (Pohl and Harwood, 2010; Schallmey et al., 2004; Tjalsma et al., 2004; Westers et al., 2004).

The expression of enzymes involved in the degradation and utilization of carbon sources as well as several other genes in *Bacillus* is subject to catabolite repression (CR), a problem that has impeded high-level production of enzymes. CR in *B. subtilis*, manifested in the presence of glucose, malate or other readily metabolized sugars (Meyer et al., 2011; Singh et al., 2008), is globally mediated by the transcription factor carbon catabolite protein A (CcpA). This in the presence of co-repressors Hpr (Ser-P) or Crh (Ser-P), binds to the *cis*-acting catabolite responsive element (*cre*) located within the promoter or the mRNA coding regions of catabolic operons and thereby represses transcription of a large set of about 300 genes (Fujita, 2009).

The accumulation of degradation products such as maltose and glucose in batch cultures by expression of enzymes such as amylase in media containing starch, starch hydrolysates etc. can lead to CR of enzyme synthesis. Consequently, strategies to abolish CR become an important consideration in large-scale commercial production of proteins. The α-amylase promoter of *Bacillus licheniformis* and *B. subtilis* is repressed 10- to 15-fold in the presence of glucose (Laoide et al., 1989). The use of CR-resistant mutants of *cis*-regulatory sequences, or trans-regulatory proteins such as CcpA, have yielded only a 3- to 10-fold increase in amylase expression (Henkin et al., 1991; Weickert and Chambliss, 1990). Further, the *cre* sequence of the cloned genes in multi copy plasmids do not titrate out the repressor (Sibakov, 1986).

The α-amylase promoters of *Bacillus* sp. have been employed for overexpression of several proteins (Kawabata et al., 2012; Xiao et al., 2004). Inducer-specific promoters have been a priority over constitutive promoters, auto inducible promoters, or growth-phase regulated promoters for improving protein expression. Most of the improvingly developed inducer- specific promoters such as the IPTG-inducible *spac* promoter (Nguyen et al., 2005), promoters induced by maltose (Ming-Ming et al., 2006), mannitol (Heravi et al., 2011), mannose (Wenzel et al., 2011), and xylose (Nguyen

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et al., 2005) are regulated by CR. Alternative expression systems not influenced by CR are desirable as a rational choice for continuous high expression of enzymes used in the breakdown of secondary carbon sources.

The phosphate regulated promoter of the *pstSCAB* operon (phosphate specific transport) in *B. subtilis* has been used for expressing recombinant phytase (Kerovuo et al., 2000). Genes in the *pho* regulon are self-inducible and are activated under P_i limitation by the two component PhoPR system in which the PhoP protein after phosphorylation by the PhoR binds the *pho* box sequences (TT(A/T/C)ACA) in the promoter. Efficient expression requires four pho box sequences with an 11-bp periodicity (Prágai et al., 2004).

The objective of this study was to engineer a catabolite repression-free over expression scheme from a strong heterologous promoter that is self-inducible for the cost-effective production of carbon repressible enzymes using α-amylase as a model. We combined the highly inducible, tightly regulated *B. subtilis pstSCAB* promoter (Qi et al., 1997) and optimum translational signals to a 5′*cre* operator-free α-amylase gene and established strategies for attaining maximum expression in quiescent (non-proliferating) cells of 'protease-deficient' *B. subtilis*. The scheme in fact resulted in glucose-enhanced unrestrained high expression of an industrially useful thermophilic α-amylase with optimum activity at 90 °C and a wide pH range of 5–10, from *B. licheniformis* MSG, isolated from a hot spring in Gujarat, India. The strategy was successfully extended for production in batch fermentations using molasses or potato starch and corn steep liquor (CSL).

2. Methods

2.1. Bacterial strains, media, growth conditions and expression studies

The bacterial strains and plasmids used in this study (Table 1) were maintained at 37 °C on Luria agar slants. *B. licheniformis* MSG, isolated from a hot spring in Unai, South Gujarat was cultured at 50 °C and used as the source of the thermophilic α-amylase.

Minimal medium used was Hulett's defined medium (Müller et al., 1997) containing Hulett's salt solution (HSS, modified with 0.304 mM $FeCl_3$), 0.5% glucose, 2 mM $CaCl_2.2H_2O$, 0.05% casamino acids and phosphate added as KH_2PO_4 to obtain different desired P_i levels. The high phosphate added medium (HPAM) contained 2 mM KH_2PO_4. In addition to the added P_i, casamino acids (0.05%) contributed to ~0.20 mM phosphate (0.13–0.3 mM, varying with the manufacturer and batch) (Table S1, Appendices A and B). Thus the moderately low phosphate medium (MLPM) had 0.62 mM phosphate of which 0.42 mM was KH_2PO_4; very low phosphate medium (VLPM) contained 0.25 mM phosphate (0.05 mM as KH_2PO_4). NPAM had no added phosphate, and contained phosphate only from the casamino acids. Low cost production medium was formulated by substituting glucose with 2–4% molasses or 1% potato starch, and casamino acids with corn steep liquor (CSL) up to 0.3%.

Antibiotics and amino acids were used at the following concentrations: ampicillin, 100 μg mL^{-1} for *E. coli*; chloramphenicol, 8 μg mL^{-1} for *E. coli*, 6 μg mL^{-1} for *Bacillus*; erythromycin, 10 μg mL^{-1} for *E. coli*, 3 μg mL^{-1} for *Bacillus*; leucine, methionine and tryptophan, each 20 μg mL^{-1}

Cultures were inoculated to an OD_{600} of ~0.2 and grown overnight serially in 15 mL each of LB, HPAM, MLPM, VLPM and NPAM, and then used to seed 100 mL similar media to an OD_{600} of ~0.2 for induction of the *pst* promoter. Catabolite repression was studied in the presence of glucose at concentrations of 0.02%, 0.5%, 2% or in 1% glycerol. Expression in low cost media was carried out with corresponding substitutions as described above. Growth was measured as optical density at 600 nm. One OD_{600} unit corresponded to ~0.95 mg protein.

2.2. DNA manipulations, delivery and detection

Protocols for DNA purifications, modifications and transformation were as described (Sambrook and Russell, 2001). Electroporation of *B. subtilis* was carried out in a ECM 630 electroporator with 0.5–2 μg of DNA as described (Schäffer et al., 2002), except that the cells after harvesting were washed thrice in cold 0.1 M Mannitol, 1 mM HEPES, pH 7. Polymerase chain reaction (PCR) was performed in an Eppendorf Mastercycler Personal

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype and features[a]	Source
Strains		
B. licheniformis MSG	Wildtype, from a hot spring in Gujarat, India	This study
B. subtilis 1A447	*hsrM1 leuB8 metB5 SP10(R)*	BGSC[b]
B. subtilis 1A748	*glgB:lacZα*ΔM15 *leu met* hsd_{R1} R^- M^+, Km^r	BGSC
B. subtilis 1A297	*amyE aspT1 trpC2*	BGSC
B. subtilis WB800	*trpC2; nprE nprB aprE epr mpr, bpf vpr wprA*; Cm^r, Hyg^r	Dr. Roy Doi, Univ. of California
E. coli DH5α	F^- Φ80d *lacZ*ΔM15 *Δ* (*lacZYA-argF*), U169, *endA1, recA1, hsdR17*, (*rk, mk*$^+$), deoR, *thi-1, supE44,* λ$^-$ *gyrA96, relA1*,	Lab stock
Plasmids		
pNEB206A	*E. coli* vector (2706 bp), *lac* promoter, Ap^r.	New England Biolabs (Cat.No. E5500S)
pSNamy	pNEB206A carrying the gene encoding BLA.MSG (4302 bp), Ap^r	This study
pHPS9	*E. coli–B. subtilis* shuttle vector; p59 promoter, 5660 bp; Cmr, Emr	BGSC
pHPst	pHPS9 with *p59* promoter replaced by *pst* promoter; 5406 bp; Cm^r, Em^r	This study
pSTamy641	pHPst carrying BLA.MSG gene. 7026 bp; RBS & start codon spacing: 641 bp; Cm^r, Em^r	This study
pSTamy13	pHPst carrying BLA.MSG gene. 6399 bp; RBS & start codon spacing:13 bp; Cmr, Emr	This study
pSTamy9	pHPst carrying BLA.MSG gene. 6395 bp; RBS & start codon spacing:9 bp; Cmr, Emr	This study
pMSGamy	Derivative of pSTamy13 carrying the native promoter in place of *pst*	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphanicol resistance; Em^r, erythromycin resistance; Ap^r, ampicillin resistance; Kmr, kanamycin resistance; Hyg^r, hygromycin resistance; RBS, ribosome binding sequence.
[b] BGSC, Bcillus Genetic Stock Centre, Ohio.

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