Efficient Extracellular Production of α-Amylase in *Bacillus subtilis* under the Influence of Xylose-Inducible Promoter

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This study focuses on the development of a xylose-inducible system to produce recombinant α-amylase from the bacterium *Thermoactinomyces vulgaris* 94-2A (*amyTV*) in the *Bacillus* expression system. To achieve this, a gene cassette was constructed using pWH1520 shuttle vector, containing a *PxylA* promoter comprising glucose box and *amyTV* gene. The cassette was initially cloned in *Escherichia coli* for replication and subsequently, introduced into the *Bacillus subtilis* (GSB26) for expression. To optimize the secretion of α-amylase, different concentrations of xylose were used as inducers. It was found that the maximum amylase activity was achieved when 2% xylose was used as the inducer. Furthermore, using glucose alone and in combination with xylose as inducers indicated that glucose acted as a catabolic repressor for *amyTV* expression. Moreover, protein was efficiently secreted and did not accumulate in the cellular fractions, even at high expression levels.

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INTRODUCTION

Bacteria have emerged as valuable cell factories for producing recombinant proteins, offering a versatile platform for various biopolymer applications (Rygus and Hillen 1992). The production of recombinant enzymes, specifically pure amylases, is of great significance in the biochemical processing of starch (Khlestkin *et al*. 2018; Aroob *et al*. 2021). Suitable host organisms, capable of producing recombinant enzymes in substantial quantities, are crucial for biochemical engineering. By harnessing the potential of bacteria as efficient cell factories (Ferrer-Miralles and Villaverde 2013) and employing advanced cloning and expression techniques, researchers have made remarkable strides in producing a wide range of recombinant proteins, including enzymes, with various industrial, medical, and scientific applications (Terpe 2006).

Escherichia coli has been extensively used for recombinant protein production (Tempe 2006; Makino *et al*. 2011). Recently, *Bacilli* have garnered significant attention as ideal host expression systems (Kumar *et al*. 2013; van Dijl and Hecker 2013) because the extracellular proteins synthesized by *Bacillus* are directly secreted into the culture

media (Liu *et al*. 2022). Thus, inducible expression systems in *B. subtilis* provide advantages especially for the expression of foreign genes that might encode lethal or detrimental gene products. Under repression conditions, the foreign gene can be stably maintained without selection pressure. After the induction of the gene of interest, regulated promoters allow the production of even toxic proteins for a limited period of growth. Moreover, the regulated expression also minimizes the exposure of the foreign gene products to proteases that are usually co-synthesized in *B. subtilis*, either as intracellular or secreted enzymes.

Several inducible promoter systems have been developed for use in a *Bacillus* expression system. One of the first expression systems used the IPTG inducible lac repressor/operator elements of the *E. coli* lac operon in *B. subtilis* (Yansura and Henner 1984; Terpe 2006). In addition, various other sugar inducible systems were established in *B. subtilis*. They are derived from the glucanate (glt) operon (Reizer *et al*. 1991), the sucrose inducible sacB operon (Pereira *et al*. 2001), or the inducible xylose isomerase gene of different *Bacillus* species as those from *B. megaterium* (Rygus and Hillen 1991a) or *B. subtilis* (Kim *et al*. 1996). For quite distinct reasons, secretion of heterologous proteins into the culture media by *B. subtilis* is also highly recommended (Wong 1995). Keeping into consideration the advantages of *Bacillus* expression system and importance of amylases, the present study aims at developing a xylose-inducible system to produce recombinant α-amylase derived from bacterium *Thermoactinomyces vulgaris* 94-2A (*amyTV*) within a *Bacillus* expression system.

EXPERIMENTAL

Bacterial Strains, Plasmids, Media, and Chemicals

E. coli DH5α (Stratagene, LaJolla, CA) was utilized as host for transformation. The target protein was expressed and secreted using the *Bacillus subtilis* (GSB26) strain. For direct PCR cloning and nucleotide sequencing, pGEM™-T vector (Thermo Fisher Scientific) was employed. The growth of *B. subtilis* was achieved in low phosphate culture media (2X TBY having composition as: tryptone 1.6%, yeast extract 1%, and NaCl 0.5%). To ensure proper selection and maintenance of transformed cells, antibiotics (obtained from Sigma-Aldrich) were added to the growth medium at specific concentrations: ampicillin (100 µg/mL) and tetracycline (10 mg/mL). Starch used in the study was purchased from Sigma-Aldrich (Steinheim, Germany) while the amylase assay kit was obtained from Sigma, USA.

Construction of Gene Cassette Encoding α-Amylase from *T. vulgaris*

The gene encoding α-amylase from *T. vulgaris* was cloned following protocol by Hofemeister *et al*. (1994), followed by subcloning in pGEM™-T vector. The sequence of the genes was determined using standard dideoxy chain termination method using the T7 promoter and T7 terminator primers in T7 DNA sequenase kit. The gene was identified through sequencing and subsequently cloned into *Sma*I linearized *B. megaterium* pWH1520 vector having xylose inducible promoter (*PxylA*) and xylose repressor gene (*xylR*). The resultant plasmid was named *xylA-amy(TV)*. Successful cloning was verified by restriction digestion analysis using *Mst*I and *Hind*III.

Expression Analysis and Protein Quantification

To obtain a heterologous expression of *amyTV* gene, *Bacillus subtilis* (GSB26) cells were transformed using *xylA-amy(TV)* recombinant plasmid. After induction with xylose as inducer, the bacterial cultures were cultivated in 2X TBY medium for different durations to optimize the suitable time for protein secretion. The cells and extracellular fractions were separated by centrifugation at 8000×*g*. The obtained cell pellets were washed with sterile water and subjected to sonication for cell lysis. Proteins obtained through cell lysis (comprising both soluble and insoluble fractions) were separated using centrifugation at 16000×*g*. All the fractions obtained (including extracellular, soluble and insoluble fractions) were subjected to protein analysis using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining the gel with G-250 dye. Protein quantification was done using the Bradford assay (Bradford 1976) using BSA as a standard.

Optimization of Induction Conditions and Inducer Concentration

The *xylA-amy(TV)* cassette was used to optimize the conditions for inducing *amyTV* secretion in *B. subtilis*. The experiments aimed to determine the induction conditions, ideal amount of inducer and the optimal induction time. To conduct the experiments, overnight cultures of *B. subtilis* having *xylA-amy(TV)* cassette present in them were diluted 100-fold in fresh growth medium, and 10 mg/mL tetracycline was added. The cultures were then grown at 37 °C. The inducer, 1% xylose, was added at various optical density (OD_{540}) values during the growth of the cultures, spanning from 0.1 to 1.0. Cells were allowed to grow for period of 5-6 h and expression level was analyzed on SDS PAGE.

Further experiments were conducted to optimize the inducer concentration. The 100-fold diluted cultures were divided into equal portions and supplemented with different amounts of xylose, ranging from 0.01% to 2.5%. These cultures were grown for 5 h, and the amount of secreted *amyTV* was measured using amylase activity assay.

Analysis of Catabolite Repression

In order to analyze how glucose affects the reversal of xylose induction in the synthesis of *amyTV*, an overnight grown culture of *xylA-amy(TV)* was diluted 100-fold. This culture was then divided into three portions: one without any sugar supplementation, and the other two with the addition of xylose (2%) and glucose (2%), respectively. After 1, 2, 3, and 5 h, small samples (aliquots) were taken from each portion. Then, 2% glucose was added to each portion, and the cultures were incubated for an additional 5 h. Amylase activity of each fraction was analyzed using amylase activity assay.

α-Amylase Secretion

To analyze the secretion of *amyTV* under the conditions of inducible synthesis, an overnight grown culture of *xylA-amy(TV)* in *B. subtilis* was 100-fold diluted and the culture was further grown to OD_{540} 0.5. At this time, xylose (2%) was added to the growth medium of one portion and the other portion was used as a non-induced control. One milliliter fraction were collected after 30, 60, and 90 min of growth. This was followed by centrifugation of the samples, and then cells and the supernatants (extracellular fraction) were collected. The cells were washed with tris-acetate buffer (10 mM tris-acetate, pH 7.0, 10 mM $CaCl_2$), resuspended in 0.5 mL tris-acetate buffer and sonicated for 5 min. The cell lysates were again centrifuged providing soluble (cytosolic)

and insoluble (cell wall) fractions. The insoluble pellet fraction was resuspended in 0.5 mL tris-acetate buffer. The amylase activity in the pellet, cytosolic fraction, and supernatant were then measured.

α-Amylase Activity Assay

The enzyme activity of recombinant α-amylase from *B. subtilis* was determined using standard dinitro-salicylic acid method (DNS assay) method (Miller 1959; Aroob *et al*. 2022). The amount of liberated sugars was quantified in terms of the amount of reducing sugars liberated upon incubation with the substrate. In a standard assay mixture, 100 µL of 1% (w/v) starch in suitable buffer was mixed with 100 µL of solution containing appropriate amount of enzyme followed by incubation at 60 °C for 20 min. The reaction was stopped by quenching in ice water, and the released reducing ends were determined by the DNS method. Activity unit was defined as µmoles/min/mg.

RESULTS

Sequence Analysis

The amino acid sequence of *amyTV* belonging to glycoside hydrolase family 13 is available on Uniprot under accession No. Q60051. The gene comprises of 1446 nucleotides encoding an amino acid chain of 482 amino acids. The amino acids from 1 to 29 are predicted as signal sequence while those from 30 to 482 encode α-amylase region having distinct GH13 catalytic region (42 to 393) and classical α-amylase C-terminus region (405 to 482) (Fig. 1B). The upstream and downstream regulatory elements of *amyTV* have previously been studied extensively by Hofemeister *et al*. (1994).

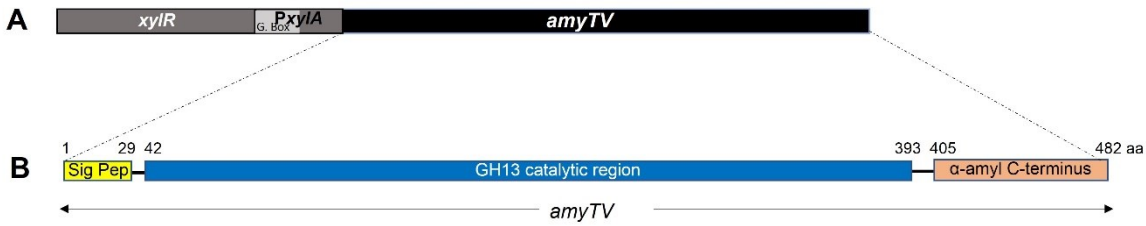

Fig. 1. (A) Structure of the *B. subtilis* expression construct *xyl-amy(TV)* containing the glucose box (G. box) for studying the inducible expression of α-amylase in *B. subtilis*; *amyTV*, the promoterless α-amylase from *T. vulgaris*; *xylR*, repressor protein; P*xylA*, The inducible xylose isomerase promoter of *B. megaterium*; (B) Graphical representation indicating presence of signal sequence region, GH13 catalytic region and α-amylase C-terminus region in *amyTV*

Construction of *Xyl-amy*(*TV*) Cassette and Expression Analysis

The DNA fragment of *amyTV* was ligated between *Mst*I and *Hin*dIII fragments into the *Sma* I linearized *B. megaterium* vector pWH1520. This DNA fragment laid downstream of the glucose box of the P*xylA*-promotor. The construct was named *xylA-amy*(*TV*) (Fig. 1A). The expression of the *xylA-amy*(*TV*) in *Bacillus* (GSB26) was obtained using 1% xylose as the inducer. The protein was found to be produced in the extracellular fraction (Fig. 2). Parallel analysis of uninduced samples, without the addition of xylose, indicated absence of amylase in the extracellular fraction.

Optimization of Inducer Concentration and α-Amylase Secretion

Optimization studies on inducer concentration showed that adding the inducer during the early logarithmic phase of the culture gave the most effective results for optimal induction. Furthermore, maximum α-amylase activity was observed when the cultures were induced with 2% xylose (Fig. 3). Therefore, for the subsequent induction experiments, a xylose concentration of 2% was considered optimal.

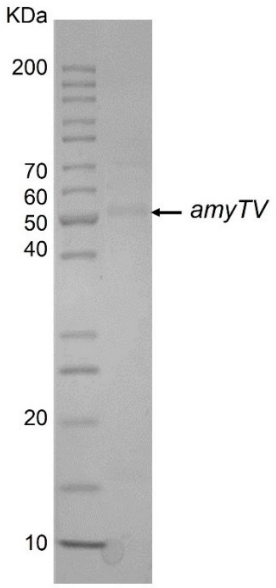

Fig. 2. Expression analysis of *amyTV* in extracellular fraction of *B. subtilis* (GSB26)

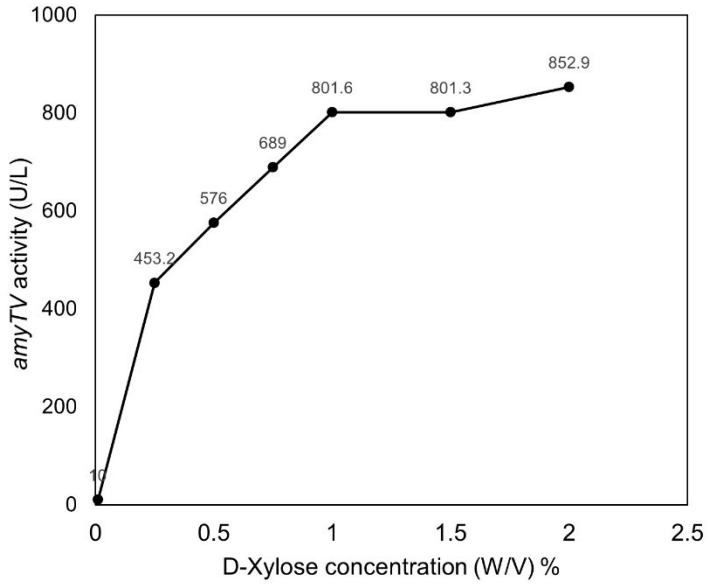

Fig. 3. Estimation of the optimum D-xylose (inducer) concentration for induction of *amyTV* secretion in *B. subtilis.* The concentration of D-xylose was 0.01% to 2.0% and *amyTV* activity was estimated after 5 h from adding the inducer.

Glucose Repression

Catabolite repression is the phenomenon during which the glucose binds to the operator or associated silencers to inhibit the synthesis of certain enzymes (Hueck and Hillen 1995). Utilizing catabolite repressors to fine tune the heterologous expression of genes of interest in heterologous systems is a strategy that has already been implemented in previous studies (Nair and Sarma 2021; Liu *et al*. 2022). In this study, it was found that the addition of 2% glucose resulted in complete repression of the *amyTV* gene expression. In addition, when glucose was added to a xylose induced culture, the expression and secretion of α-amylase were both suppressed (Fig. 4).

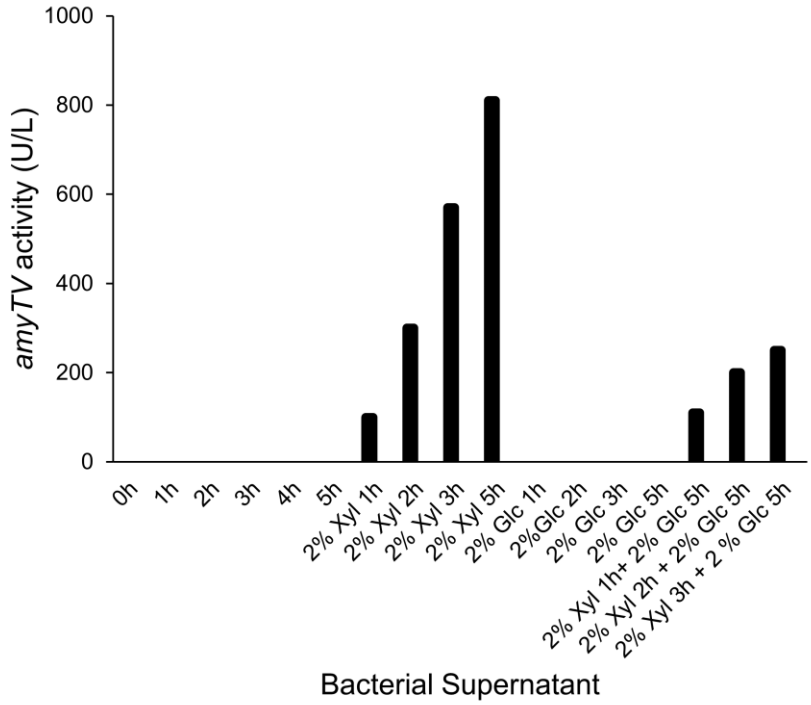

Fig. 4. Amylase activity was measured in the bacterial supernatant under different conditions: without an inducer, in the presence of either xylose or glucose as inducers, or with a combination of both as inducers. The activity assay was carried out at various time intervals ranging from 0 to 5 h.

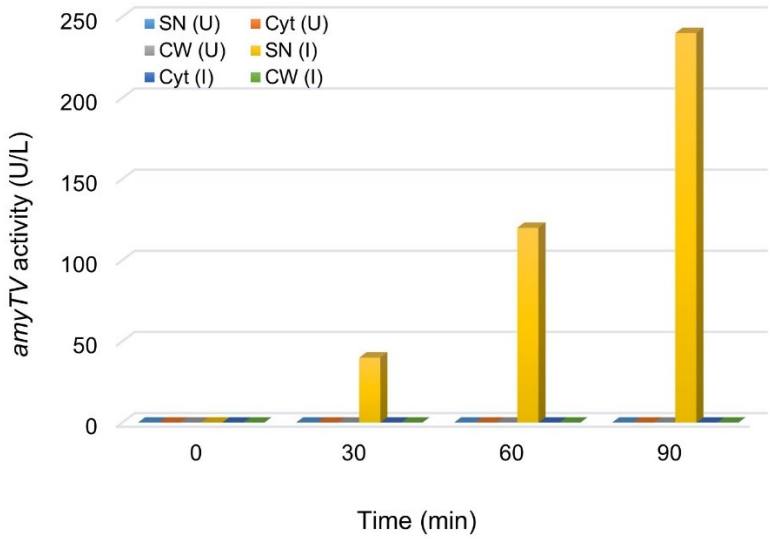

Fig. 5. Activity of recombinant *amyTV* in *B. subtilis*. Bars show α-amylase activity in three different fractions: the supernatant (SN), the cell wall (CW), and the cytosol (Cyt). "U" and "I" refer to uninduced and induced, respectively. The culture was grown in 2X TBY in the presence of 2% xylose as an inducer.

Secretion of the Induced *amyTV* protein

Analysis of secretion of *amyTV* in extracellular fraction showed that *amyTV* was completely secreted under the conditions of inducible synthesis. The *amyTV* residual activities in the pellet and cytosol fractions were 0.1% to 1% relative to activity in the supernatant. The assay also demonstrated *amyTV* secretion 30 min after the induction, which was followed by a sustained rise in amylase activity (Fig. 5).

DISCUSSION

An analysis of the regulatory promoter/repressor elements from the xylose utilizing operon of *B. megaterium* was performed in *B. subtilis* (GSB26) using *amyTV* amylase from *T. vulgaris* as heterologous protein. The P*xylA* promoter was found to be an efficient system for inducible secretion of *amyTV* in *B. subtilis*. Maximum expression was observed at inducer concentrations between 1% and 2% (Fig. 3). Addition of the inducer to growing cultures at OD_{540} 0.5 resulted in maximal α-amylase activity. After a period of 30 to 60 mins, *amyTV* was observed to accumulate in the culture medium, with the maximum accumulation achieved after approximately 16 h of growth. Upon comparing the data from the induction conditions in *Bacillus subtilis* with those in *Bacillus megaterium* (Rygus and Hillen 1991b), few differences were found. The optimal induction conditions for *Bacillus megaterium* required 0.5% xylose, while *Bacillus subtilis* needed at least twice that amount of inducer to achieve optimal results. Induction kinetics of β-galactosidase activity in *B. megaterium* have shown that maximum β-galactosidase activity [U] was reached in 180 mins (Rygus and Hillen 1991b), while the full level of secretion in *B. subtilis* was achieved in 12 to 16 h after induction. Between 30 and 60 min following induction, it was observed that *amyTV* was not found to be accumulated in either the cytosolic or membrane fractions of the cells. Instead, it was

completely secreted into the growth medium. The findings suggest that the secretory pathway of *B. subtilis* demonstrated remarkable potential in efficiently processing and releasing fully matured amylase, even after a significant increase in *amyTV* synthesis following xylose induction. The xylose inducible system could therefore efficiently be used for producing secretory proteins in *B. subtilis*. While xylose enhanced enzyme expression under the control of the xylose-inducible promoter, further experiments using non-metabolizable analogs would be required to confirm its role as a true inducer independent of its catabolic fate. Furthermore, to act as an inducer, the sugar must have a binding site for attaching to the repressor protein. This binding dissociates repressor from the promoter and hence starts transcription. Repressor proteins do not have binding sites for all sugars, and hence not all sugars can act as inducers. Therefore, this study successfully confirmed the hypothesis that xylose acts as a potential inducer. The xylose inducible systems can avoid proteolytic degradation of heterologous proteins in *B. subtilis* by selecting the phase of expression as controlled by addition of the inducer. When the regulation and strength of the P*xylA* promoter in *B. megaterium* was studied, the expression was found 150-fold inducible by xylose (Rygus and Hillen 1992). In this study, comparable results were obtained for secretory expression in *B. subtilis*. The expression of *amyTV* in *B. subtilis* (GSB26) was enhanced 500-fold after xylose induction.

The mechanism of catabolite repression in *B. subtilis* and gram-positive bacteria is complex and is still a matter of investigation (Hueck and Hillen 1995; Liu *et al*. 2022). In this study, it was interesting to observe that the activation of the xylose-inducible system could be promptly halted at any point during the induction period by simply adding glucose, the catabolite repressor (Fig. 4). This finding suggested that the potential target, likely the repressor *xylR*, possesses a balanced affinity for both xylose and glucose. Additionally, it appeared that *xylR* could be inactivated and then reactivated in response to consecutive changes in the presence of these sugars.

CONCLUSIONS

1. An expression cassette constructed using *B. megaterium* pWH1520 vector having P*xylA* promoter containing a glucose box region, a xylose repressor gene *xylR* was successfully used for amylase secretion studies in *B. subtilis* (GSB26).
2. When synthesizing the *amyTV* protein, it was found that the highest amount of amylase production was achieved using 2% xylose as the inducer.
3. Experiments where glucose or a combination of glucose and xylose were used as inducers demonstrated that glucose functioned as a repressor of *amyTV* expression.
4. The protein was efficiently secreted and did not accumulate in the cellular fractions, even at high expression levels.

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