EVALUATION OF RECOMBINANT GLUCOAMYLASE EXPRESSION BY A NATIVE AND α-MATING FACTOR SECRETION SIGNAL IN PICHIA PASTORIS

Kazi Muhammad Rezaul Karim1* and Tasmia Tasmim2

Address(es): Kazi Muhammad Rezaul Karim, Ph.D.,
1 Institute of Nutrition and Food Science, University of Dhaka, Dhaka-1000, Bangladesh.
2 Department of Nutrition and Food Engineering, Faculty of Allied Health Science, Daffodil International University, Dhaka-1207, Bangladesh.

*Corresponding author: ekarim98@gmail.com, rezaul.infvs@du.ac.bd

INTRODUCTION

Raw starch degrading glucoamylase (RSDEs) is a vital industrial enzyme that yields β-D glucose from the non-reduced ends of soluble or raw starch by hydrolyzing α-1,4 glycosidic linkages successively (Bhatti et al., 2007; Norouzian et al., 2006). Raw starch-degrading enzymes (RSDEs) can break down the uncooked starch particles below its gelatinization temperature and represent the opportunity in their promising application in the starch business (Robertson et al., 2006). Glucoamylase is additionally employed in, confectionery, beverage, juice, baking, p...
1 M sorbitol, 1% [w/v] yeast extract and 150µg ml⁻¹ zeocin). Buffered Glycerol-complex Medium (BMGY) (2 % peptone, 1 % yeast extract, 1 % [v/v] glycerol, 4 x 10⁻² [w/v] biotin, 1.34 % [w/v] YNB and phosphate buffer 100mM (pH 6.0)), and Buffered Methanol-complex Medium (BMMY) (0.5% methanol, 1% yeast extract, 1.54% YNB, 2% peptone, 10-5% biotin and potassium phosphate 100mM (pH 6.0)).

Construction of plasmids for Glucoamylase (GA2) expression

The raw starch degrading glucoamylase gene sequence (Gene accession number KJU936058) was taken from the previous study (Karim et al., 2019), and was preserved in the pGEMT-Easy vector in the laboratory. This glucoamylase (GA2) gene comprised of 1839 nucleotides in its sequence, representing 612 amino acids residues (Karim et al., 2019). Although the first 19 amino acids of its N-terminus were considered as signal peptide sequence, but the primers were designed after 20 amino acids and identified as without signal peptide sequences for the purpose of securing secretion. The pPICZαC and pPICZB (Invitrogen) were chosen as a yeast expression vector (due to the availability of vector and restriction enzymes) and used for the cloning of GA2 cDNA (used template as cloned pGEMT-Easy vector) without and with signal peptide sequences, respectively. GA2 gene without the signal sequence was amplified by PCR using the following list of expression primers:

5’ GCCGAATTCAGACTCAGAAAGCAGCAGGATAAGCAATCTGGCTTA

and 5’ TATATATGCCGCCCTCATACCTGCTGCTTGCT

(reverse for cloning of pPICZB). The designed primers contained an EcoR1 and NotI restriction site in the forward and reverse primers (underline and bold) respectively. In addition, the forward primer was designed to include FLAG tag containing 24 nucleotides (DYKDDDDK), and the reverse primer lacking in the native stop codon. The primers were designed to be in frame with the C-terminal 6x His tag located in the pPICZB. So that the recommended enzyme can be easily purified either at N-terminal FLAG tag or C-terminal His tag. PCR was performed with taq DNA polymerase as described in the previous study (Karim et al., 2019). The purified PCR product was digested with EcoR1 and NotI and ligated into pPICZαC (also digested with EcoR1 and NotI), producing the recombinant expression plasmid pPICZαC-GA2.

At the same time, glucoamylase (GA2) cDNA (cloned pGEMT-Easy vector as a template) with native signal sequences was also amplified by PCR using another pair of primers (5’- GCCGAATTCAGACTCAGAAAGCAGCAGGATAAGCAATCTGGCTTA- and 5’- TATATATGCCGCCCTCATACCTGCTGCTTGCTTG- for cloning of pPICZB). The designed second set of primers contained an EcoR1 and KpnI restriction site in the forward and reverse primers respectively; and the reverse primer having the native stop codon. The amplification was carried out according to a previous study (Karim et al., 2019). The purified PCR product was cutdown with EcoR1 and KpnI and ligated into pPICZαC (also cutdown with EcoR1 and KpnI), producing the P. pastoris secretion recombinant plasmid pPICZαC-GA2. The recombinant expression plasmid containing raw starch degrading glucoamylase cDNA (pPICZαC-GA2 and pPICZB-GA2) were then transformed into E. coli XL1-Blue. The recombinant plasmid pPICZαC-GA2 and pPICZB-GA2 were then confirmed by colony PCR (by AXO1 primers).

Transformation of Pichia pastoris GS115 and Expression of rGA2

Both GA2 construct (recombinant plasmids pPICZαC-GA2 and pPICZB-GA2) were linearized one by one with Sac1 and then transformed into P. pastoris GS115 by employing the Picha EasyComp Transformation kit (Invitrogen). The corresponding linearized blank plasmids were also transformed into P. pastoris (GS115) as a control. All transformants were cultured on YPDS zeocin plates at 30°C. Few colonies for each plate were selected after four days at random for Mut+ screening by colony PCR using AXO1 primers, according to previous studies (Karim et al., 2016; Karim et al., 2019). The Mut+ single colony from both glucoamylase transformants and control were then cultured separately into Buffered Glycerol-complex Medium (BMGY) at 28°C and 220 rpm for one day. Subsequently, Picha pastoris were collected by centrifugation at 3000 x g for 5 min at room temperature and resuspended to an OD600 of 1.3 with Buffered Methanol-complex Medium (BMMY) to promote the secretion of recombinant protein for seven days at 28°C and 220 rpm (Karim et al., 2019). 1 mL of each culture were withdrawn at 24-h intervals till 7 days, and absolute methanol were also added into the culture in every sampling point to make the final concentration of 0.5 % (v/v) of methanol (Karim et al., 2016).

Glucoamylase assays

Glucoamylase activity was estimated according to the technique used in the previous study (Karim et al., 2016), and the amount of glucose released was measured using 3, 5-dinitrosalicylic acid (DNS) method.

Statistical analysis

Descriptive analyses were presented using means and standard deviations (SD).

RESULTS

Construction of recombinant plasmids

The cDNA fragment of GA2 with and without the native signal sequence was amplified (respective primers) by PCR and cloned into pPICZB and pPICZαC vector respectively. The full length of the constructed plasmid of pPICZB_GA2 and pPICZαC_GA2 were 5144 and 5356 bp, respectively (Figure 1a and 1b), but without inset pPICZB and pPICZαC plasmid are in 3328 bp and 3598 bp in length, respectively. Constructed recombinant plasmids pPICZB_GA2 and pPICZαC_GA2 were linearized by Sac1, and the linearized plasmids were about 5.1 kb and 5.35 kb in sizes in gel electrophoresis, respectively (Figure 2).

Figure 1a.

Figure 1b.

Figure 1. The constructed recombinant plasmid pPICZαC_GA2 was in 5356 bp in size, containing Eco R1 (1213) and Not I (3020) (Figure 1a) in the frame. It also contained N-terminal FLAG tag in the open reading frame. The full length of the recombinant plasmid pPICZB_GA2 was in 5144 bp in size, having Eco R1 (943) and kpnI (2792) in the frame (Figure 1b).

Transformation, Expression of Glucoamylases (GA2) cDNA

To determine the heterologous expression level with and without native signal peptide of glucoamylase gene, its cDNA was introduced to Picha pastoris. Linearized recombinant plasmids (pPICZαC_GA2 and pPICZB_GA2) were transformed into Picha pastoris by the Picha EasyComp Transformation kit. The transformation efficiency was low in this method, only a few colonies were found in both YPDS agar plates after 4 days of incubation. Colony PCR was performed on selected transformed colonies from both plates using AXO1 primers. The
amplification using AXO1 primers resulted in two closed bands for Mut+ in GS115 having pPICZαC_GA2 insert with sizes of 2.4 kb and 2.2 kb (Figure 3); whereas Mut- in GS115 transformed colony with pPICZB_GA2 produced two close bands at 2.2 kb and 2.1 kb (Figure 3). Meanwhile, the control yeasts transformed with pPICZαC and pPICZB produced a 600 bp and 300bp band, respectively (data not shown). These results demonstrated that pPICZB_GA2 and pPICZαC_GA2 were transformed into the yeast chromosome.

**Figure 2** Electrophoresis of linearized recombinant plasmids by SacI restriction enzyme; L1= pPICZB_GA2 and L2= pPICZαC_GA2 (1% agarose pre stained with Ethidium Bromide); Marker: 1kb DNA ladder, Vivantis.

**Figure 3** Electrophoresis of PCR analysis for recombinant glucoamylase expression strain. Primers: 5’ AOX1 (5’-GACTGGTTCAATGGACAAGC-3’) and 3’AOX1 (5’-GCAATGGCATTCTGAC ATCC-3’), Invitrogen. L1= selected transformants with pPICZαC_GA2 and L2= selected transformants with pPICZB_GA2 (1% agarose pre stained with Ethidium Bromide); Marker: 1kb DNA ladder, Genebiotech.

Heterologous expression of recombinant glucoamylases was under the transcriptional regulation of the AOX1 promoter in *P. pastoris*, and the expressions were induced by the supplement of methanol. On day six, *Pichia pastoris* GS115 (pPICZαC_GA2) had the maximum recombinant enzyme activity of 8.57 U/ml at 0.5% methanol (Fig. 4) in the culture. It was observed that α-factor signal sequences in the pPICZαC more effective for extracellular secretions of recombinant glucoamylase compare to native signal peptide sequence of *Aspergillus flavus* NSH9 (Figure 4).

**Figure 4** Extracellular expression of recombinant glucoamylase with native and pPICZαC vector signal sequence at 0.5% methanol. Highest secretion of recombinant glucoamylase was observed after 6 day of incubation from α-factor signal sequence (pPICZαC_GA2). Assay condition was at pH (5.0) and temperature (55°C). Error bars show standard deviation among three independent observations.

**DISCUSSION**

Raw starch-digesting enzymes (RSDEs) can digest the uncooked starch particles directly below the temperature of starch gelatinization, which may significantly decrease the cost of starch-based bioethanol (Wang et al., 2018). However, few studies have been reported that raw starch-digesting glucoamylases are capable to breakdown the raw starch directly to produce glucose as the sole product in a single step (Lin et al., 2011). Due to the increasing high industrial benefit of raw starch degrading glucoamylase, its demand is also high day by day, and many researchers try to maximize its production by genetic engineering. The level of homologous/heterologous expression of protein depends on many factors, among them promoter and signal peptide are recognized to be vital factors for regulating the protein expression (Xu et al., 2014 & 2017). In this study, we try to compare the production of recombinant glucoamylase by using two different signal peptides, native, and expression vector signal peptide. The secretory expression by pPICZαC vector in *Pichia pastoris* makes use of the pre-pro MATα sequence from *S. cerevisiae* (Brake et al., 1984). This excretion signal sequence consists of two sections: a 19-amino acid N-terminal signal sequence that directs translocation into the endoplasmic reticulum (ER), followed by a 66-amino acid pro region (Otte & Barlowe, 2004; Duncourt & Barlowe, 2010).

On the other hand, the pPICZB vector was constructed as a secretory expression vector by the addition of a native signal peptide sequence of glucoamylase from *Aspergillus flavus* NSH9. In this experiment, the expression of rGA2 with native signal peptide gave a lower expression level, which was also previously observed in a few studies (Ohnishi et al., 1992). In one previous study, Li and his co-workers used four secretory signal sequences (α-MF, CRN, Suc20r or α-MF-op) to measure secretion competence of human CRP expressed in *P. pastoris* X-33; and the highest expression level recorded from the α-MF signal sequence (Li et al., 2017). The extracellular expression of CRP was 5 times more than by the native signal sequence of CRP (CRN) (Li et al., 2017), but in this study, it was about 1.5 folds higher in α-MF signal peptide. This α-MF secretion signal supports posttranslational modification into the endoplasmic reticulum (ER), so proteins that can bend in the cytosol may be incorrectly translocated and thus inadequately secreted (Barrero et al., 2018). Furthermore, if the protein is self-associated, the α-factor pro-region can potentially induce accumulation, thus hindering ER exports. Few studies have attempted to work on improving the α-factor secretion signal for higher secretary expression (Barrero et al., 2018; Joo et al., 2017; Massahi et al., 2015; Ahn et al., 2016). Though the higher extracellular expression of recombinant glucoamylase by α-MF secretion signal observed in this study, still has few limitations. The expression level of rGA2 could be improved either by optimization of expression media or maybe changing expression host, vector, and promoter; because the successfully hyper-expression of recombinant protein depends on few factors and not all cDNA equally expressed in *Pichia* (Bottner et al., 2007). At the same time, few recombinant proteins are easily breakdown in *P. pastoris* culture medium by protease due to high cell density and besides lysis of *Pichia* itself in the culture media (Li et al., 2007).

**CONCLUSION**

In this study, the raw starch degrading glucoamylase cDNA with and without signal peptide sequence from *Aspergillus flavus* NSH9 has been successfully...
cloned and expressed as a biologically active enzyme in Pichia pastoris. The study reported that α-mating factor secretion signal peptide is more efficient for the secretion of recombinant glucoamylase from Pichia pastoris, and it will help the starch industry for further modification or gene engineering.

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**Conflict of Interest:** The authors declare that there is no conflict of interest.

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