JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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

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https://doi.org/10.15414/jmbfs.3428

ARTICLE INFO

Received 11. 7. 2021
Revised 10. 11. 2021
Accepted 12. 11. 2021
Published xx.xx.201x

Regular article

OPEN ACCESS

ABSTRACT

Raw starch degrading enzyme specially glucoamylase with starch binding domain (SBD) has great values in the starch processing industry because it digests the starch particles below the gelatinization temperature by releasing glucose from the non-reducing ends sequentially. The purpose of the study was to measure the secretion levels of recombinant glucoamylase from *Pichia pastoris, by* using the α-mating factor secretion signal peptide (α-MF) and the native signal peptide of glucoamylase from *Aspergillus flavus* NSH9. The *Aspergillus flavus* NSH9 gene (with and without native signal sequences), encoding a pH and thermostable glucoamylase with an SBD, was successfully cloned and expressed in *Pichia pastoris* to produce recombinant glucoamylases. The constructed recombinant plasmids pPICZB_GA2 (having a native signal peptides) and pPICZαC_GA2 (having the α-MF) were 5144 and 5356 bp in length respectively. Recombinant *pichia* having α-MF signal sequence (plasmid, pPICZαC_GA2) gave the highest level of secretions of recombinant glucoamylase after 6 days of incubation period with 0.5% methanol. In conclusion, yeast expression vector signal peptide is more efficient for heterologous expression/secretions of recombinant glucoamylase compared to its native signal sequences.

Keywords: Raw starch degrading glucoamylase, signal peptide, *Pichia pastoris*, *Aspergillus flavus* NSH9

INTRODUCTION

Raw starch degrading Glucoamylase (RSDGs) 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 breakdown 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, prescription drugs, and many soured foods industries for manufacturing production (**Pandey *et al.*, 2000; Karim *et al.*, 2018**). It is also calculable that RSDEs may scale back the entire value of ethanol production by 10 to 20% (**Moshi *et al.*, 2016; Sun *et al.*, 2010**). RSDEs are derived from a variety of microorganisms, as well as bacterium, yeast, and fungi, like Bacilli sp., *Saccharomycopsis fibuligera*, and genus *Aspergillus* sp. (**Karim *et al.*, 2019; Sun *et al.*, 2010**). Alpha amylases with raw starch digesting capacity account for the bulk of raw starch degrading enzymes (**Bozic *et al.*, 2017**), but previous studies also reported that RSDGs are capable of directly digesting the uncooked starch to provide glucose as the sole product in a single step (**Lin *et al.*, 2011**). A novel raw starch digesting glucoamylase (GA2) was isolated from *A. flavus* NSH9 in the previous study and expressed in *Pichia pastoris* which exhibited high pH and temperature stability, indicating its great possibility for starch processing (**Karim *et al.*, 2019; Karim *et al.*, 2017**). This glucoamylase (GA2) gene comprised of 1839 nucleotides in its sequence, representing 612 amino acids residues (**Karim *et al.*, 2019**). This GA2 has a signal peptide sequence at its N-terminus consisting of first 19 amino acids and having the starch-binding domain (SBD) at the C-terminus (**Karim *et al.*, 2019**). N-terminal signal peptide results in the robust stimulation of the expression of recombinant proteins (**Sletta *et al.*, 2007**). On the other hand, several features such as expression host, vectors, promoters, signal peptide, gene copy number, site of integration of gene, the recombinant strain stability, and regulatory protein, etc. are important for the effective expression/overexpression (**Nevalainen & Teo, 2003; Kumar & Stayanarayana, 2009**). Currently *P. pastoris* is extensively used as an expression host for recombinant protein production (**Li *et al.*, 2007; Marx *et al.*, 2009; Byrne, 2015**). *P. pastoris* is selected as its usually known as–a safe organism, and its parallel characteristic to *Saccharomyces cerevisiae* permits the sharing of procedures and few genetic components such as secretion signals sequence (**Barrero *et al.*, 2018**). *Pichia pastoris* can express the heterologous protein either in an intracellular or secretory pathway depending on the existence of a signal peptide or sequence. Yeast expression vector such as pPICZB is for intracellular expression while the pPICZαC vector is for extracellular expression., but in this study pPICZB vector was used as a secretary expression vector due to addition of signal sequence in the constructed plasmid. In previous studies, various secretion signal sequences along with native signal peptide were used effectively for the expression of recombinant protein, but achievement was variable (**Cregg *et al.*, 1993; Scorer *et al.*, 1993**). The existence of a signal sequence dose not continuously make sure the higher secretion of recombinant protein into the periplasmic area (**Chung *et al.*, 1998; Xu *et al.*, 2017; Sevillano *et al.*, 2016**) and the creation of soluble, active proteins that are properly folded (**Betton, 1996**). Thus, the choice of an ideal signal sequence is essential for the effective secretion of heterologous protein. Here in the study, we evaluated the level of secretion of recombinant raw starch degrading glucoamylase (GA2) with the α-mating factor secretion signal and the native signal peptide of glucoamylase (GA2) from *A. flavus* NSH9 in *Pichia pastoris*.

MATERIALS AND METHODS

Strains, plasmid, and enzyme

The Raw starch degrading glucoamylase gene was used in this study that was previously isolated from *A. flavus* NSH9 (**Karim *et al.*, 2019**), and was preserved in the laboratory. The yeast expression vectors pPICZαC, pPICZB, and *P. pastoris* GS115 yeast strain were purchased from Invitrogen (Carlsbad CA, USA). *Escherichia coli* XL1-Blue collected from laboratory, pGEMT-Easy vector (Promega, US), DNA polymerase (EURx Gdansk Poland), and restriction endonucleases were purchased from Fermentas (Germany). Zeocin was obtained from Invitrogen. Other reagents were all analytical grade and were obtained from Sigma-Aldrich.

Cell cultures media

The different cultures media were used for *Pichia pastoris* cultivation depending on the objective of the study. The different media used were YPD (1% yeast extract, 2% glucose , 2% peptone), YPDS (2% [w/v] dextrose, 2% [w/v] peptone,

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^{-5} % (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, 1.34% 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 KU936058) 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´GGC**GAATTC**A*GACTACAAAGACGACGACGATAAG*CAACCTGTCCTTA GACAG 3´forwaed) and 5´
TATATAT**GCGGCCGC**CCGCCAAACATCGCTCTG 3´reverse for cloning of pPICZαC. The designed primers contained an *Eco*R1 and *Not*1 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 pPICZ*α*C. 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 *Eco*R1 and *Not*1 and ligated into pPICZαC (also digested with *Eco*R1 and *Not*1), 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´- GCG**GAATTC**AACGATGGTGTCTTTCTCCTC-3´ and 5´-ATA**GGTACC**TCACCGCCAAACATCGCTCTG- 3) for cloning of pPICZB. The designed second set of primers contained an *Eco*R1 and *Kpn*1 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 *Eco*R1 and *Kpn*1 and ligated into pPICZB (also cutdown with *Eco*R1 and *Kpn*1), producing the *P. pastoris* secretion recombinant plasmid pPICZB_GA2. The recombinant expression plasmids 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 *Sac*I and then transformed into *P. pastoris* GS115 by employing the Pichia EasyComp Transformation kit ~~as~~ procedures (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 *AOX1* 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, *Pichia 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-dinitrosalicyclic 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 *Sac*1, and the linearized plasmids were about 5.1 kb and 5.35 kb in sizes in gel electrophoresis, respectively (Figure 2).

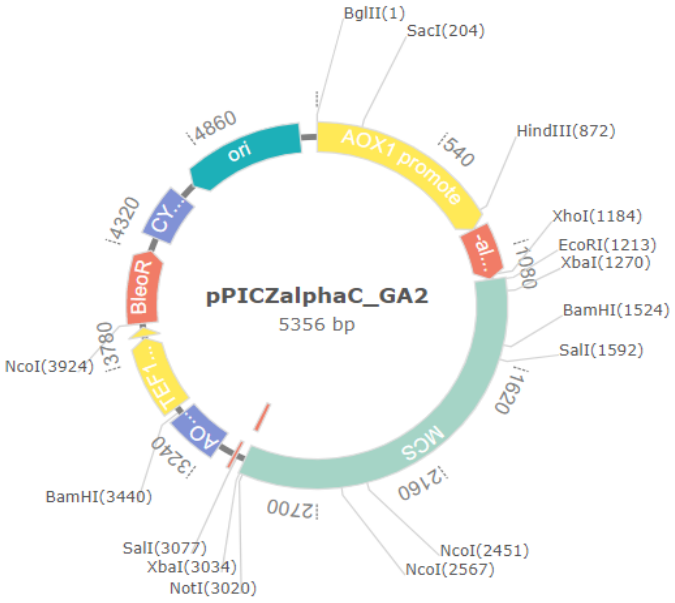

Figure 1a.

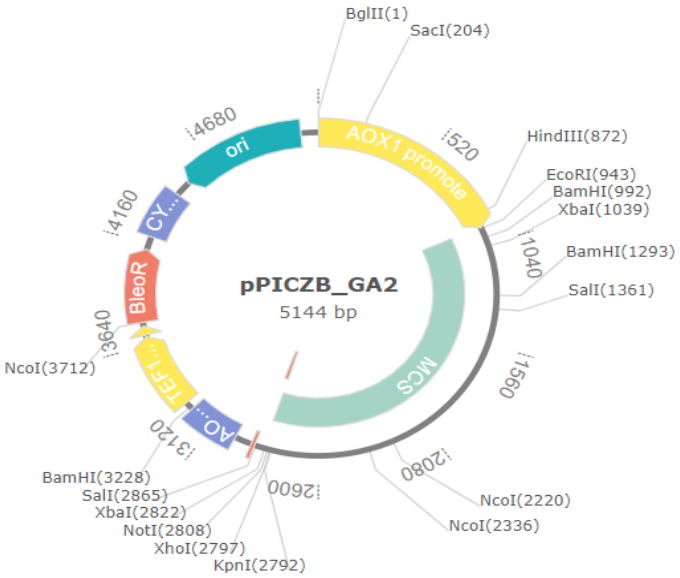

Figure 1b.

Figure 1 The constructed recombinant plasmid pPICZαC_GA2 was in 5356 bp in size, containing *Eco R1* (1213) and *Not* 1(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 *kpn*1(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 *Pichia pastoris.* Linearized recombinant plasmids (pPICZαC_GA2 and pPICZB_GA2) were transformed into *Pichia pastoris* by the Pichia 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 *AXO*1 primers. The

amplification using *AXO*1 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.

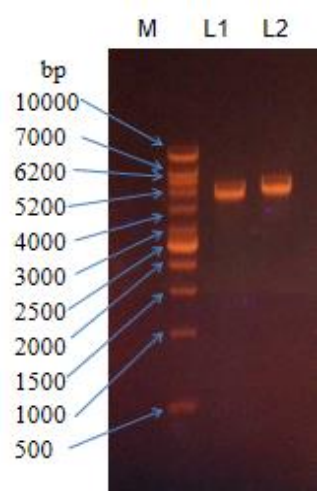

Figure 2 Electrophoresis of linearized recombinant plasmids by *Sac*I restriction enzyme; L1= pPICZB_GA2 and L2= pPICZαC_GA2 (1% agarose pre stained with Ethidium Bromide); Marker:1kb DNA ladder, Vivantis.

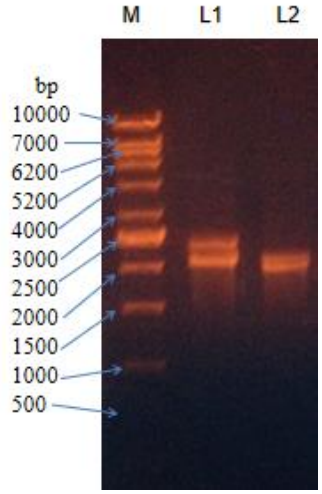

Figure 3 Electrophoresis of PCR analysis for recombinant glucoamylase expression strains. Primers: 5′ AOX1 (5′-GACTGGTTCCAATTGACAAGC-3′) and 3′AOX1 (5′-GCAAATGGCATTCTGAC ATCC-3′), Invitrogen. L1= selected transformants with pPICZαC_GA2 and L2= selected transformants with pPICZB_GA2 (1% agarose pre stained 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).

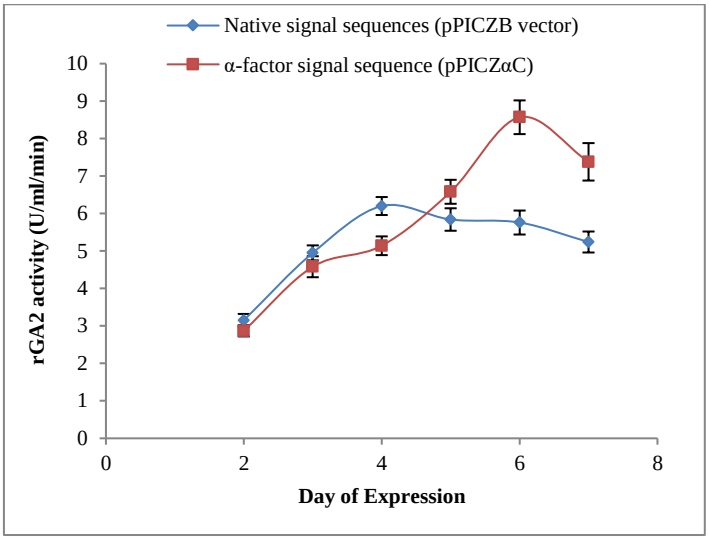

Figure 4 Extracellular expression of recombinant glucoamylase with native and pPICZαC vector signal sequence at 0.5% methanol. Highest secretion of recombinant glucoamylase was observed after 6 day of incubation from α-factor signal sequence (pPICZαC_GA2). Assay condition was at pH (5.0) and temperature (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 biorefining (**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; Dancourt & 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, SUC2or 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.

Acknowledgement: The authors would like to thank Dr. Ahmad Husaini, Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Malaysia for his laboratory support during the cloning part. The research project was partially supported financially by the University Grant Commission (UGC), Bangladesh, under the research grant scheme for University Teacher (2017-18).

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

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