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DEVELOPMENT OF ARMING YEAST WITH AMYLASE ENZYME FROM *ASPERGILLUS NIGER* AS A MODEL FOR DELIVERY OF ENZYME

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Abstract: Alpha amylase enzyme from fungal origin is of great importance in food and feed industry. The enzyme is widely accepted in fermentation, brewing, pharmaceutical and textile industry. The aim of the study was to surface design yeast for anchoring of amylase enzyme on to the cell wall from *Aspergillus niger* using membrane protein and their characterization. The pYD1 vector was constructed enclosing amylase cDNA from *A. niger* and transformed into *Saccharomyces cerevisiae* EBY100 cells. The expression was induced by addition of galactose in the medium and assessed by immunofluorescence and plate assay methods. The amylase enzyme was successfully surface displayed using yeast as host, yielding an enzyme activity of 8.12 ± 0.8 µmol/min/mL. The yeast with surface-displayed amylase exhibited amylolytic activity and produced glucose and maltose as major hydrolysis products using starch as substrate. Further, the displayed enzyme was visualized by higher activity and immunofluorescence assay. Releasing glucose and maltose from starch, the surface display of amylase may help yeast with its survival and metabolic activity after ingestion by animals, enhancing its positive effects on animals. The surface-displayed amylase showed maximum activity at temperature of 50°C and optimal pH of 7.

Keywords: α-Amylase, *Aspergillus niger*, surface display, immunofluorescence, paper chromatography, yeast

Introduction

Alpha-Amylase (EC.3.2.1.1), is an endo-amylolytic enzyme that effectively catalyzes the hydrolysis of α-1,4 glycosidic linkage of starch, glycogen and other polysaccharides, releasing

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glucose, maltose, oligosaccharide and dextrin's. This type of enzyme is commonly used in various industrial bioprocesses, like paper making, food and feed fermentation, and starch processing. Fungal amylases are of greater interest than bacterial amylases because of their increased activity. Although the fermentation and purification of amylases are conventional technique but purification of amylases from fermentation media increases production cost. In many cases, *S. cerevisiae*, particularly preferred for fermentation of amylolytic substrate to desired products, such as bioethanol (Aydemir *et al.*, 2014). Because *S. cerevisiae* has no or little amylase activity, introduction of amylases into yeast can streamline and expand its application.

S. cerevisiae is of GRAS status, and both live yeast and its fermentation products are commonly used as food and feed supplements for animal and human consumption. When used as directly fed microbial in ruminant animals, live yeast can improve forage digestion and microbial protein synthesis, resulting in improved feed efficiency and productivity (Desnoyers *et al.*, 2009; Poppy *et al.*, 2012), whereas non-viable yeast or yeast fermentation products have little effect (Hristov *et al.*, 2010). However, yeast does not persist in the rumen, only remaining to be detectable in the rumen for approximately 30 h (Durand-Chaucheyras *et al.*, 1998). The primary constituents in the diets of ruminant animals are forage and starch. Cellulolytic and amylolytic bacteria, fungi, and protozoa can hydrolyse these polysaccharides, but they release little free sugars to the rumen fluid because they use membrane-associated glucoside hydrolases (GH) and most of the hydrolysis products are taken up immediately by the hydrolysing microorganisms (Miron *et al.*, 2001; Ljungdahl, 2008). Therefore, the glucose concentration in the rumen is very low, less than 0.7 mM (Kajikawa *et al.*, 1997). The lack of available sugars may be the main reason for the short survival of yeast in the rumen after consumption by ruminants. Such lack of sugars may also limit the metabolic activity of yeast. We hypothesize that yeast with amylase activity may survive longer and be more active in the rumen, enhancing its positive effects on feed digestion and microbial protein synthesis.

Surface display technology entails the expression and anchoring of enzymes or peptides on the surface of yeast cells. In the last decade, this technology has been used in arming yeast with foreign enzymes, like glucoamylase, xylanase (Yeasmin *et al.*, 2011), lipase (Shiraga et al., 2005), cyclodextrin glucanotransferase (Wang *et al.*, 2006), laccase (Bleve *et al.*, 2014) and cellulase (Fujita *et al.*, 2002). Zeng *et al.* (2011) and Li *et al.* (2011) have successfully cloned and expressed, but not surface displayed, α-amylase in *Pichia pastoris*, whereas Khaw *et al.*,

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(2007) anchored and displayed both amylase and glucoamylase in yeast for ethanol production. Similarly, Apiwatanapiwat *et al*. (2011) developed yeast with simultaneously surface-displayed glucoamylase, cellobiohydrase, endoglucanase, and β-glucosidase for production of biofuel using cassava pulp. So, the present study was undertaken to develop a system for heterologous expression and display of amylase enzyme from *A. niger* on the surface of *S. cerevisiae* cells exploiting α-agglutinin as the membrane anchor. The yeast with the surface-displayed amylase may help improve the positive effects on host when fed to animals.

Materials and Methods

Strains and Media

S. cerevisiae EBY100 (MAT**a** *ura*3-52*trp*1 *leu2*Δ1 *his*3 Δ200 *pep*4:*HIS3prb*1Δ1.6R *can*1*GAL*) (Boder and Wittrup, 1997) was used as the host strain for expression and surface display of amylase enzyme, while *Escherichia coli* DH5α was used as the host for routine DNA manipulation procedures. Both strains and plasmid pYD1 was purchased from Invitrogen, Carsland, USA. Luria Bertani medium (1% tryptone, 0.5 % yeast extract, 0.5% sodium chloride) supplemented with appropriate antibiotics was used for growth of *E. coli* at 37˚C whereas YPD medium comprising yeast extract, peptone and dextrose was used to cultivate yeast cells at 30˚C. Selection of yeast transformants was achieved using YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.01% leucine).

Construction and transformation of vector containing amylase cDNA

Total RNA of *A. niger* NRRL334 was extracted with a Qiagen RNase Easy Plant Mini Kit (Maryland, USA) and the amplification of putative amylase cDNA was performed with a Single Step RT-PCR Kit (Invitrogen, USA) using degenerate primers for *A. niger* amylases. Polymerase Chain Reaction was performed for the amplification of amylase cDNA using forward primer 5´-ATCAGGATCCATGATGGTBGCNTGGTGGTCG-3´ (underlined, *Bam*HI restriction site) and the reverse primer 5´-GTTACTCGAGCGAVSWACTRCARATYTTGCTACC-3´ (underlined, *Xho*I restriction site). The PCR was performed with initial denaturation for 5 min at 95ºC followed by 35 cycles of denaturation at 95ºC for 45 s, annealing at 58ºC for 45 s and extension at 72ºC for 2.30 min with a final extension for 10 min at 72ºC. The resultant amplicon was purified using a Qiagen Gel Extraction kit (QIAGEN, Inc., Valencia, CA, USA) and double digested with both *Bam*HI and *Xho*I (NEB, USA). Then, purified *amy* cDNA was ligated into the

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*Bam*HI-*Xho*I sites of pYD1 vector using T4 DNA ligase enzyme (Invitrogen Corporation, Carlsbad, CA, USA) resulting in formation of chimeric plasmid, known as pYD1-*amy*, followed by transformation into *E. coli* DH5α competent cells using heat shock treatment as per manufacturer instructions. The cloned pYD1-*amy* cDNA was confirmed by PCR using 5´-GTCGATGTGGTTGCTAACC-3´ as forward and 5´-CTTACACCAACGACATAGCC-3´ as reverse primers and then by sequencing of the insert.

Transformation, induced expression, and surface display of amylase on S. cerevisiae EBY100

The transformation of pYD1-*amy* plasmid into *S. cerevisiae* was achieved using the lithium acetate method as per instruction manual (Clontech, USA). For induction and surface display of amylase, recombinant *S. cerevisiae* EBY100 harbouring pYD1-*amy* plasmid was pre-cultivated in 10 ml of YNB-CAA medium consisting of 0.67% YNB, 0.5% casamino acids and 2% glucose with overnight shaking at 30ºC till the OD_{600} attained 2-5. *S. cerevisiae* EBY100 carrying pYD1 and *S. cerevisiae* EBY100 were included as controls. The yeast cells harvested through centrifugation (4000 rpm for 10 min at room temperature) were resuspended in YNB-CAA medium comprising 2% galactose to achieve an OD_{600} between 0.5 and 1, followed by cultivation at room temperature (22°C) for 48 h with shaking. Then, the cells were harvested by centrifugation (5000 rpm for 10 min at 4°C) and resuspended in 1× PBS buffer with 2 OD (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM L KH_2PO_4, pH 7.4) for immunofluorescence test and amylase activity assay.

Yeast immunofluorescence

The visualization of yeast cells displaying amylase was performed by immunostaining with fluorescent antibody Anti V5-FITC (Invitrogen Corporation, USA) and microscopy as per procedure of Wang *et al.* (2015) using 200 fold diluted antibody. In brief, anchored yeast cells after washing twice with 2 ml of PBS buffer resuspended in 200 µL of 1x PBS buffer with 10% Fetal Bovine Serum (FBS) and incubated for 20 min at room temperature. The diluted antibody was then added after washing the yeast cells thrice with PBS buffer without FBS and incubated at room temperature for 1 h in dark. The cells were observed under florescent microscope (Axio A1, New York, USA) to visualize binding of antibody on cell surface.

Determination of enzymatic activity of surface-displayed amylase

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The functional activity of the amylase displayed yeast cells was evaluated by inoculating induced recombinant yeast cells, EBY100 and EBY100-pYD1 cells into YNB agar plates containing 1% starch (Sigma-Aldrich, St Louis, MO, USA). The amylase activity was indicated by a clear zone of starch hydrolysis surrounding yeast colonies.

The surface-displayed amylase activity was estimated using the DNS method of Miller, (1959) with slight modification. The quantity of reducing sugar released was measured using glucose standard curve. The specific amylase activity was defined as the amount (µmol) of glucose produced per min per ml of yeast cells at 2.0 OD.

Characterization of surface displayed amylase

Different ranges of pH from 3.0-10.0 were tested to evaluate the effect of pH on the displayed amylase activity at 50°C. The optimum temperature was determined at optimized pH at 30, 40, 50, 60, 70 and 80°C. The amylase activity was measured as stated above.

Paper chromatography

The hydrolysis products of the displayed amylase were determined using starch as the substrate and the method of Mohamad *et al*. (2014) with some modifications. The paper chromatogram was developed using solvent system of acetic acid/n-butanol/water (45:180:75) and silver oxide reagent as staining agent. Glucose and maltose were used as reference saccharides.

Results and discussion

Construction of Vector for displaying amylase

The insertion of the amylase cDNA into pYD1 vector from *A. niger* NRRL 334 was confirmed with correct open reading frame and orientation using PCR and DNA sequencing. The *amy* cDNA was fused with Aga2 protein subunit protruding outside the cell wall, which in turn was linked to Aga1 subunit (embedded in the wall) for surface display and secretion of amylase protein on to the yeast cell wall (Fig. 1).

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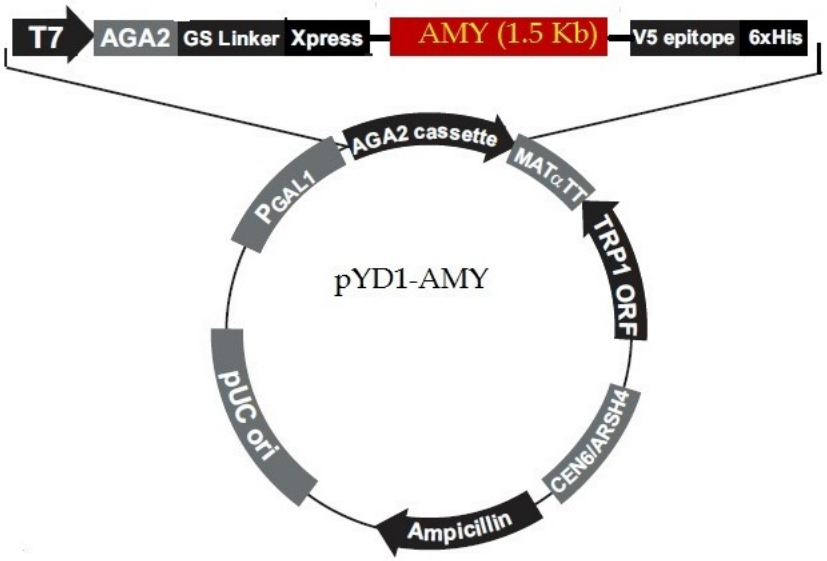

Figure 1. Schematic representation of the pYD1-*amy* plasmid containing the amylase cDNA of *A. niger*

The 3´end of the *amy* cDNA was linked to V5 epitope and a polyhistidine tag which can be exploited for possible detection and separation of the displayed protein. The resultant plasmid termed as pYD1-*amy* was transformed into yeast, and the cloned *amy* cDNA was deposited in GenBank (GenBank Accession Number KU668705). When compared with various amylase genes from different fungi, the 1.5 kb cloned cDNA showed no mutation encoding a protein of 499 amino acids.

Surface expression of amylase cDNA in yeast

After induction with galactose (2%), the expression of *amy* protein which is fused with Aga2p-subunit on the yeast cell surface were determined by amylase activity test and immunoblotting analysis at different time points. Immunofluorescence staining with Anti-V5-FITC Antibody showed the green fluorescent signal in yeast cells carrying pYD1-*amy* and confirmed the expression of the cloned pYD1-*amy* cDNA and surface anchoring of the amylase (Fig. 2a) whereas negligible florescence was observed on EBY100 cells carrying only pYD1 (2b).

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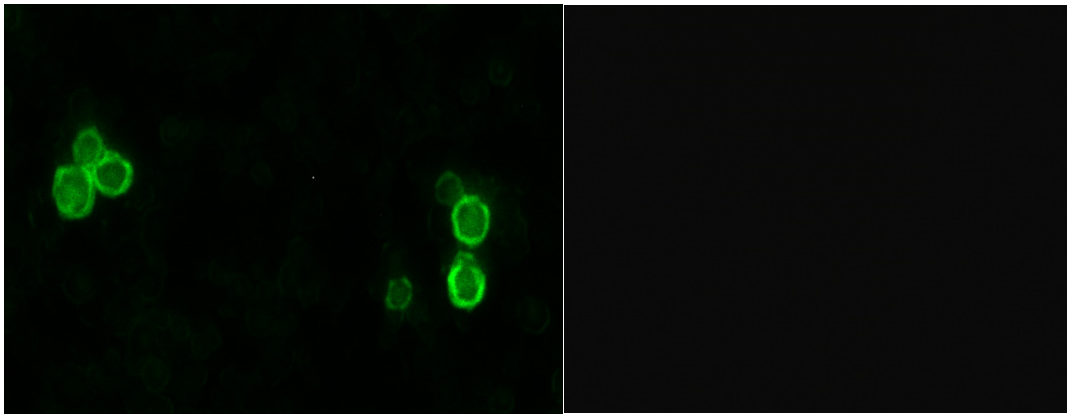

(A) **(B)**

Figure 2. Immunofluorescence images of *S. cerevisiae* EBY100 after staining with Anti-V5-FITC conjugated antibody. (A) *S. cerevisiae* EBY100 harboring pYD1-*amy*, (B) control cells (*S. cerevisiae* EBY100 only)

The result confirmed the expression of the cloned pYD1-*amy* cDNA and surface anchoring of the amylase. The surface-displayed amylase activity was confirmed by formation of a halo around the colonies of yeast harboring pYD1-*amy* on YNB-CAA plates containing starch (Fig. 3).

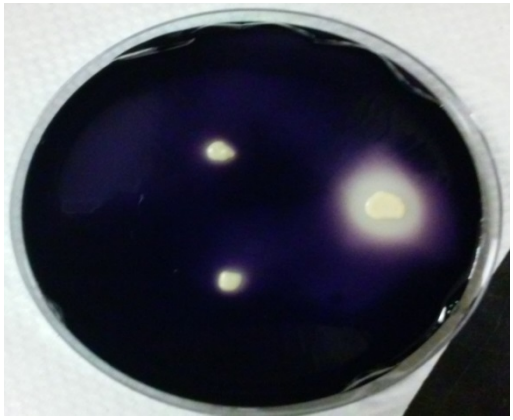

Figure 3. Test of amylase activity on YNB-CAA agar plate containing starch. A) *S. cerevisiae* EBY-100, B) EBY-100 carrying pYD1, C) EBY-100 containing pYD1-*amy*

No halo was observed with the yeast host EBY100 or the yeast that carried pYD1 vector only. These results clearly indicated that the surface-displayed amylase was functional in hydrolyzing starch.

Determination of starch hydrolysis products

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Based on paper chromatography, EBY100 cells surface-displaying the amylase produced glucose and maltose as the major products of starch hydrolysis (Fig. 4). Other oligosaccharides were minor hydrolysis products.

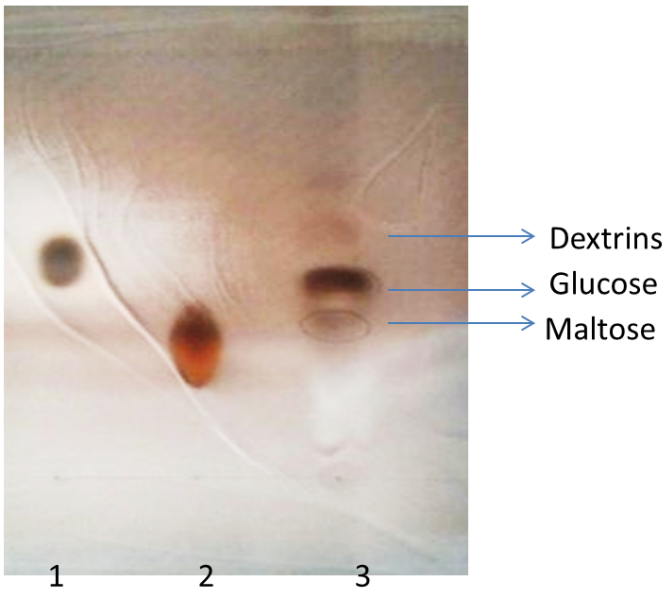

Figure 4. Paper chromatography of the starch hydrolysis products produced by yeast EBY100 carrying pYD1-amy. Lane 1, glucose standard: lane 2, maltose standard; lane 3, hydrolysis products of yeast carrying pYD1-*amy*

Alpha-amylase hydrolyses starch into glucose and simple oligosaccharides, primarily maltose. The amylase cDNA of *A. niger* was cloned and heterologously expressed, and the amylase protein was surface displayed successfully on to *S. cerevisiae* surface. Because yeast cells can be harvested readily by filtration of centrifugation, yeast surface-displayed amylase can be harvested more efficiently than amylases released into fermentation media, which often requires further downstream purification processes (Kuroda and Ueda, 2011; Tanaka *et al*., 2012). Although amylases displayed on yeast cell surface may not be used as purified amylases in some processes, it can be used as feed enzymes, and the presence of yeast cells provides additional benefits. Therefore, yeast with surface-displayed amylases and other glycoside hydrolases (Wang *et al*., 2015) can be used as a dual-purpose delivery agent of feed enzymes and live yeast. Indeed, Wang *et al*. (2015) recently demonstrated *in vitro* that yeast with surface-displayed xylanase

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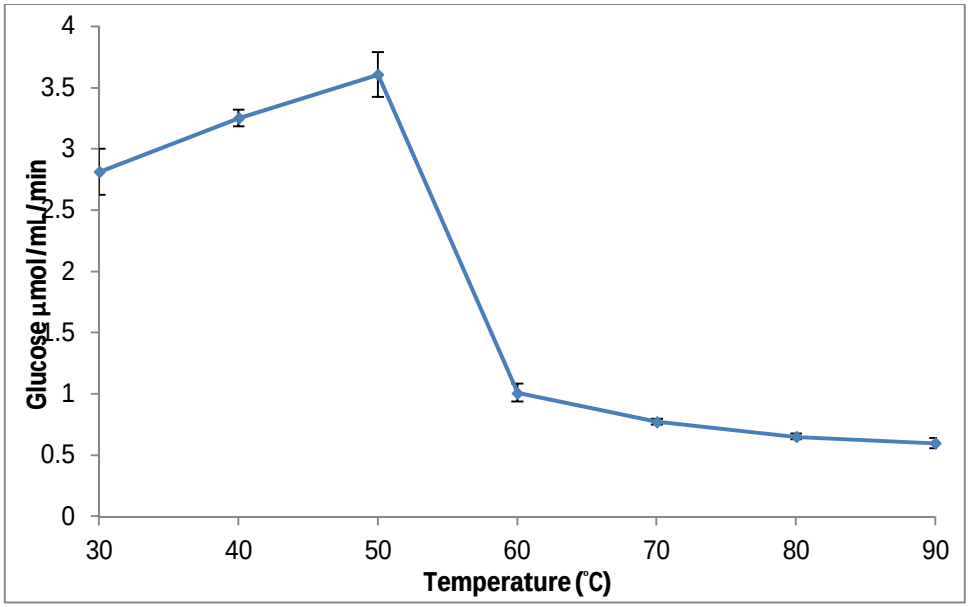
increased dry matter degradation, fungal and bacterial population and volatile fatty acid production. Although starch digestion does not need to be enhanced in the rumen, the surface-displayed amylase produces glucose and maltose as the major hydrolysis products. These sugars may improve the survival and metabolic activities of yeast, thus enhancing its positive effects on rumen function. Future research is needed to assess how the surface display of amylase improves yeast survival and metabolic activities.

Effect of temperature and pH on enzyme activity

The yeast surface-displayed amylase was characterized with respect to its activity at different pH and temperature. Our results demonstrated that the surface-displayed amylase had an optimal temperature of 50˚C (Fig. 5a), and beyond that the enzyme lost its activity rapidly. The optimal pH value was 7, at the optimal temperature of 50 ˚C, however the amylase exhibited its activity from pH 4 to pH 10 also (Fig. 5b).



(a)

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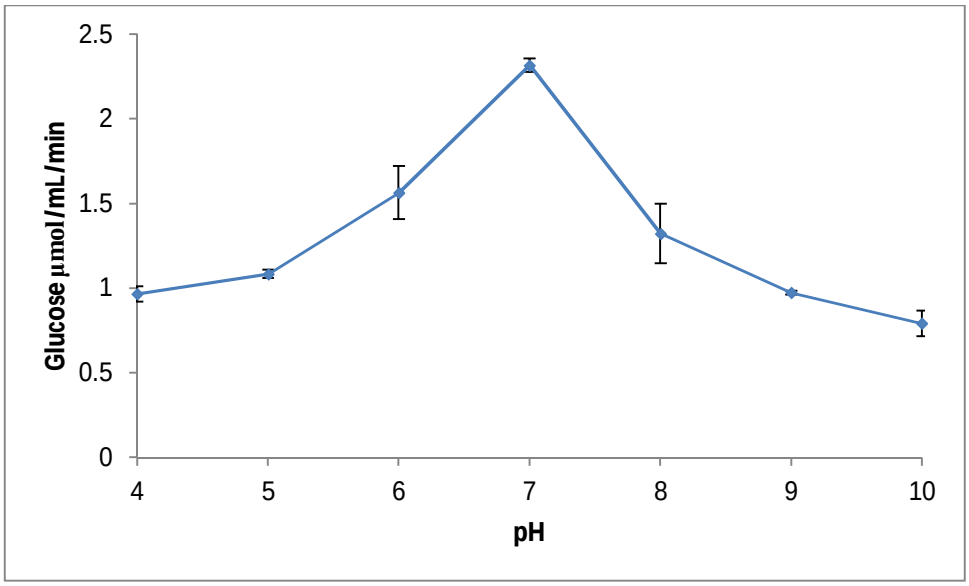

(b)

Figure 5. Effect of temperature (a) and pH (b) on activity of the amylase displayed on yeast. The data were the means of three replicates and error bars indicate standard deviation of the three replicates. A unit of enzymatic activity was defined as the amount (µmol) of glucose produced per min per ml (OD=2.0) of cells of yeast under assay conditions.

The temperature in the rumen is about 39°C and the pH fluctuates between 5.6 and 6.7, which is lower than the optimal temperature (50°C) and the optimal pH (7.0) of the amylase. The optimal temperature and pH of the amylase may be changed through mutagenesis so that the enzymatic activity of the surface-displayed amylase would correspond to the natural conditions. Additional research can insert both the fusion of the amylase and the anchor into the yeast chromosome (Inokuma *et al*., 2014) so it can be stably maintained.

Conclusions

Successful amylase display on the yeast cell surface using Aga2 protein as membrane anchor was successfully demonstrated and established by immunofluorescence microscopy. The yeast with the surface-displayed amylase exhibited amylolytic activity, producing glucose and maltose as the major products. The surface displayed amylase may enhance the benefits of feeding live yeast to animals.

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Conflicts of Interest

No conflict of interest declared.

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