J Ind Microbiol Biotechnol (2010) 37:401–406
DOI 10.1007/s10295-009-0686-9

ORIGINAL PAPER

Expression of *Thermobifida fusca* thermostable raw starch digesting alpha-amylase in *Pichia pastoris* and its application in raw sago starch hydrolysis

Chao-Hsun Yang · Yu-Chun Huang · Cheng-Yu Chen · Chia-Ying Wen

Received: 30 October 2009 / Accepted: 14 December 2009 / Published online: 29 December 2009

Abstract A gene encoding the thermostable raw starch digesting α-amylase in *Thermobifida fusca* NTU22 was amplified by PCR, sequenced and cloned into *Pichia pastoris* X-33 host strain using the vector pGAPZαA, allowing constitutive expression and secretion of the protein. Recombinant expression resulted in high levels of extracellular amylase production, as high as 510 U/l in the Hinton flask culture broth. The purified amylase showed a single band at about 65 kDa by SDS-polyacrylamide gel electrophoresis after being treated with endo-*β*-*N*-acetylglycosaminidase H, and this agrees with the predicted size based on the nucleotide sequence. About 75% of the original activity remained after heat treatment at 60°C for 3 h. The optimal pH and temperature of the purified amylase were 7.0 and 60°C, respectively. The purified amylase exhibited a high level of activity with raw sago starch. After 48-h treatment, the DPw of raw sago starch obviously decreased from 830,945 to 378,732. The surface of starch granules was rough, and some granules displayed deep cavities.

Keywords Raw starch digesting α-amylase · *Pichia pastoris* · *Thermobifida fusca* · Sago starch

Introduction

Alpha-amylases (E.C. 3.2.1.1) are glycoside hydrolases that have been classified within the family GH13 [1]. They play an important role in starch degradation and represent about 25–33% of the enzyme world market, in second place after proteases [2].

Recently, the importance of enzymatic hydrolysis of raw starch without heating has become well recognized, mainly from the viewpoints of energy savings and effective utilization of the biomass, thereby reducing the cost of starch processing [3]. This has generated a worldwide interest in the discovery of several raw starch-digesting amylases that do not require gelatinization and can directly hydrolyze the raw starch in a single step below the gelatinization temperature [4].

Sago starch, extracted from sago palm, is annually produced in large quantities in southeast Asia. Sago starch represents an alternative cheap carbon source for fermentation processes that is attractive for both economic and geographical considerations [5]. The use of sago starch for bioconversion is limited by high paste viscosity and resistance of the raw granule to be digested by the commercial α-amylase from *Bacillus* and *Aspergillus* [6].

The importance and development of industrial biotechnology processing has led to the utilization of microbial enzymes in various applications. To produce enzymes for the development of enzymatic degradation of renewable lignocellulose, we have isolated a potent extracellular lignocellulolytic enzyme-producing thermophilic actinomycete, *Thermobifida fusca* NTU22, from compost soils collected in Taiwan [7]. Interestingly, the *T. fusca* NTU22 strain studied here also produces an extracellular amylase that releases maltotriose as the major end product from either soluble starch or from raw starch granules. Since amylases that produce maltotriose as their major end product from raw starch granules are relatively rare, optimization of the cultivation conditions for the production of this extracellular amylase by *T. fusca* NTU22 was investigated [8]. We recently reported the purification and some properties of

C.-H. Yang (✉) · Y.-C. Huang · C.-Y. Chen · C.-Y. Wen
Department of Cosmetic Science, Providence University, Taichung 43301, Taiwan
e-mail: chyang@pu.edu.tw

α-amylase from *T fusca* NTU22. The molecular weight of the purified enzyme was estimated by SDS-PAGE and gel filtration on Sepharose CL-6B to be 64 and 60 kDa, respectively [9]. The gene (*tfa*), encoding a maltotriose-producing α-amylase from *Thermobifida fusca* NTU22, was cloned, sequenced and expressed in *Escherichia coli* (Accession No: DQ473479). The gene consists of 1,815 base pairs and encodes a protein of 605 amino acids. The base composition of the *tfa* coding sequence is 69% G + C, and the protein has a predicted pI value of 5.5. The deduced amino acid sequence of the *tfa* amylase exhibited a high degree of similarity with amylases from *Thermomonospora curvata* and *Streptomyces* amylases [10].

In the light of the economic benefits, several thermostable enzyme genes from thermophilic microorganisms have been cloned and expressed in mesophilic microorganisms to reduce the energy needed for cultivation [11, 12]. Recently, among many mesophilic host systems, *Pichia pastoris*, methylotrophic yeast with the capability to perform many eukaryotic posttranslational modifications, has been considered as an excellent host system for heterologous protein expression. It is well known that the expression can be driven by strong alcohol oxidase I (*AOX1*) promoter under methanol induction [13]. In addition, the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter has been used for constitutive expression of several heterologous proteins without methanol induction in *P. pastoris* [14].

The aim of this study was the constitutive expression of the α-amylase gene (*tfa*) from thermophilic actinomycetes, *T. fusca* NTU22, in *P. pastoris* X-33. Some properties of the enzyme on raw sago starch hydrolysis were also investigated.

Materials and methods

Microorganisms and vectors

Thermophilic actinomycetes, *Thermobifida fusca* NTU22, which was isolated from compost soils collected in Taiwan, was used in this study [7]. *Pichia pastoris* X-33 and pGAPZαA were purchased from Invitrogen (San Diego, CA). The plasmid propagation in the expression work was accomplished with *E. coli* Top 10 (Invitrogen, San Diego, CA). The plasmid pAMY13H8 was constructed with pUC118 and a 3.0-kb *Bam*HI-*Hind*III inserted fragment, which possessed an amylase gene *tfa* [10].

Materials

Yeast extract, peptone, tryptone and agar were purchased from BD (Sparks, MD). Zeocin was obtained from Cayla (Toulouse, France). The restriction endonucleases and T4 DNA ligation kit were purchased from Roche (Mannheim, Germany). For polymerase chain reactions, the Vio Twin Pack Kits comprising VioTag DNA polymerase, polymerase chain reaction buffer and deoxynucleotides were obtained from Viogene (Sunnyvale, CA). HisPur Cobalt resin was purchased from Pierce (Rockford, IL). PiNK Prestained Protein Ladder was purchased from GeneDirex (Las Vegas, NV). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Endo-*β*-*N*-acetylcosaminidase H was purchased from New England Biolabs Inc. (Beverly, MA). Inorganic salts and all other chemicals were purchased from Sigma (St. Louis, MO).

Construction of the amylase expression plasmid

The *T. fusca* NTU22 α-amylase gene was amplified with primers of 5′-AAACTCGAGAAACGTGAGGCTGAAG CAATGGGAGTGCGCAGATCC-3′ (*Xho*I site is underlined) and 5′-AATTCTAGACCGCGCCAGGAGTCGTAG AAGTTCTGGGAAC-3′ (*Xba*I site is underlined) using pAMY13H8 as the template. The amplification was performed using a DNA thermal cycler (Perkin Elmer, Salem, MA) under the following conditions: the first step was initiated at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 67°C for 30 s and 72°C for 90 s, and the final extension was carried out at 72°C for 10 min. A 1.8-kb PCR product was recovered from the agarose gel and cloned into *Xho*I and *Xba*I digested pGAPZαA. After being transformed into *E. coli* Top 10, one recombinant plasmid designated as pGAPZ*α*-*tfa* was selected on low salt LB agar plates (5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl, 15 g/l agar, and adjusted pH to 7.5) containing 25 μg/ml Zeocin. The proper insert orientation was checked by restriction analysis and sequencing as described above.

Transformation and screening of *P. pastoris*

Ten micrograms of recombinant plasmid (pGAPZ*α*-*tfa*) was linearized with *Bgl*II and electroporated into *P. pastoris* X-33 under the following conditions: 1.5 kV, 25 μF, 200 ohm and 5 ms, using a GenePulser (Bio-Rad, CA). The transformants were selected at 28°C on the YPDS agar plates (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, 20 g/l agar and 1 M sorbitol) containing 100 μg/ml Zeocin for 4–6 days. Screening for high level expression transformants was done by replicating the colonies obtained from the YPDS agar plates containing 100 μg/ml Zeocin onto YPDS agar plates with a higher Zeocin concentration (2,000 μg/ml). Transformants with higher Zeocin-resistance were obtained and checked for the integration of the construct into the *P. pastoris* X-33 genome by genomic PCR [15].

Biomass and amylase activity assay

Biomass production of the *P. pastoris* transformant was evaluated by optical density at 600 nm (OD_{600} value). Amylase activity was determined by measuring the release of reducing sugar from soluble starch [9]. The reaction mixture contained 0.1 ml of appropriately diluted crude enzyme and 0.9 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) of soluble starch. After incubating at 60°C for 15 min, the amount of reducing sugar released in the mixture was determined by the dinitrosalicylic acid method. The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol glucose per minute under the assay condition.

Cultivation and expression of amylase in Hinton flask

The high Zeocin-resistant transformant was incubated in 50 ml YPD broth (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose) in 500-ml Hinton flasks and shaken (150 rpm) at 28°C. After the cultivation was carried out for several days, the culture broth was centrifuged at 10,000*g* for 30 min at 4°C, and finally the supernatant was tested for amylase activity.

Enzyme purification

All purification procedures were done at 4°C in 50 mM MES buffer (pH 7.0) unless otherwise stated. After 72-h cultivation of the *P. pastoris* transformant (pGAPZ*α*-*tfa*) in a 500-ml Hinton flask, the fermentation broth was centrifuged by 3,000*g* for 30 min to remove cells. The supernatant was then applied to a HisPur^{TM} Cobalt column (1 cm × 5 cm) pre-equilibrated with the MES buffer. After it was washed with wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole; pH 7.4) to remove the unbinding protein, the enzyme was eluted with elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4). The eluted enzymatically active fractions were pooled and used as purified enzyme.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass of the purified enzyme was determined by using SDS-PAGE (10% polyacrylamide). PiNK Prestained Protein Ladders were used as molecular mass standards. The electrophoresis was carried out at 150 V for 1 h. The gel was stained with 0.27% Coomassie Brilliant Blue R-250 and destained by washing overnight with a mixture of acetic acid–methanol–water (10:20:70, V/V).

Deglycosylation of amylase from *P. pastoris* transformant

The purified amylase from *P. pastoris* transformant was deglycosylated by denaturing the glycoprotein at 100°C for 10 min, and then endo-*β*-*N*-acetylglycos-aminidase H was added to perform deglycosylation at 37°C for 1 h. All manipulations followed the manufacturer's instructions.

Average molecular weight

The weight-average degree of polymerization (DPw) of starch was determined by high-performance size-exclusion chromatography (HPSEC) [16]. The solution of starch was prepared by precipitating from an aliquot of starch-DMSO solution (2.1 ml, 5 mg starch per mL DMSO) with excess absolute ethyl alcohol and centrifuged at 4,000*g* for 10 min. The precipitated amorphous starch pellet was dissolved in deionized water (15 ml, 95°C) and stirred with a magnetic stirrer in a boiling water bath for 30 min. The starch solution was filtered through a 5.0-mm syringe filter (Millipore, Billerica, MA), and then the filtrate (100 ml) was injected into an HPSEC system. The system consisted of an isocratic pump (G1310A series, Hewlett Packard, Wilmington, DE), a multi-angle laser light scattering (MALLS, model Dawn DSP, Wyatt Technology Co., Santa Barbara, CA) and refractive index (RI) detectors (HP1047A). The columns used were PWH (guard column), G5000PW and G4000PW (TSK-Gel, Tosoh, Tokyo, Japan) columns connected in series and kept at 70°C. The mobile phase was 100 mM $NaNO_3$ containing 0.02% NaN_3 at a flow rate of 0.5 ml/min.

Morphology

The morphology of starch was examined by using cold cathode field emission scanning electron microscopy (JEOL JSM-6700F, Tokyo, Japan). Starch samples were mounted on circular aluminum stubs with double sticky tape, coated with gold, and then examined and photographed at an accelerating potential of 3.0 kV [17].

Result

Expression of amylase gene (*tfa*) in *P. pastoris*

The *tfa* coding sequence was cloned into the pGAPZαA vector at the *Xho*I/*Xba*I restriction sites as described earlier. This construction allowed the *tfa* coding sequence to be theoretically in-frame with the α-factor secretion signal in pGAPZαA. Following a sequence check, the construct denoted as pGAPZ-*tfa* was *Bgl*II-linearized and electroporated into *P. pastoris*

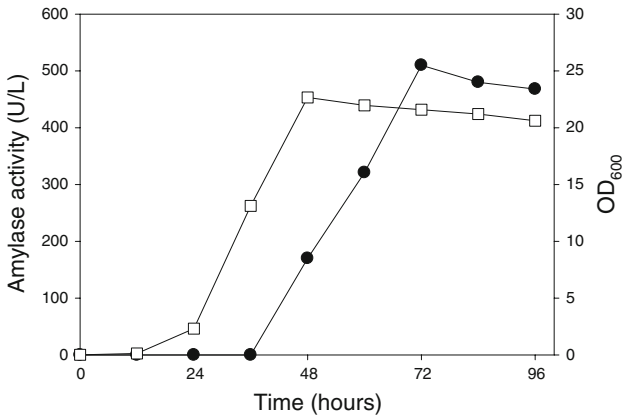

Fig. 1 Time course for the expression of amylase activity by *P. pastoris* transformant (pGAPZ-*tfa*). Cells were grown aerobically in a 500-ml Hinton flask loaded with 50 ml of medium consisted of YPD broth, and were incubated at 28°C, 150 rpm for 96 h

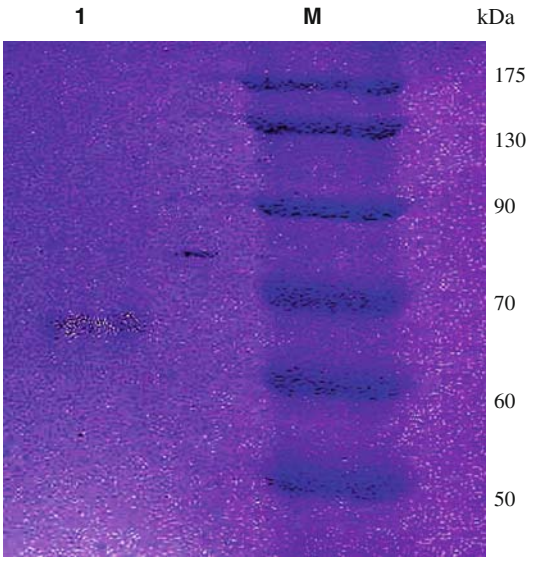

Fig. 2 SDS-PAGE of the purified amylase from *P. pastoris* transformant (pGAPZ-*tfa*). Lane M: PiNK Prestained Protein Ladder. Lane 1: Purified amylase from the *P. pastoris* transformant (pGAPZ-*tfa*)

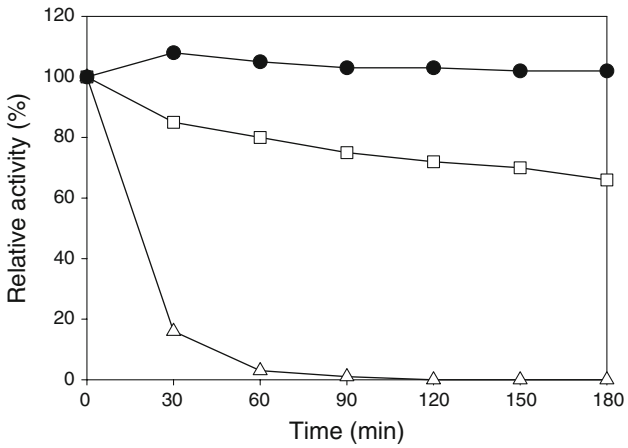

Fig. 3 Thermal stability of the amylase from the *P. pastoris* transformant (pGAPZ-*tfa*). The enzyme was incubated at various temperatures for 30–120 min, and the residual enzyme activities were determined: *dark circle*, 50°C; *open square*, 60°C; *open triangle*, 70°C

X-33. Nine transformants were selected for high resistance to Zeocin (2,000 μg/ml). The genomic PCR assay revealed that all the transformants contained an integrated *tfa* coding sequence in genomic DNA. Among these nine transformants, the transformant (pGAPZ-*tfa*) that could produce the highest amylase activity was selected for further experiments.

The fermentation conditions for constitutive expression of the amylase were investigated in a 500-ml Hinton flask loaded with 50 ml of YPD broth at 28°C. Transformant (pGAPZ-*tfa*) grew logarithmically from 24 to 28 h and then entered a stationary phase (Fig. 1). The biomass reached approximately 22 of the OD_{600} value after 48-h incubation. The amylase production pattern in this transformant indicated that the amylase synthesis began in the middle logarithmic phase and continued to be produced during the stationary phase where the maximum activity (510 U/l) was reached in the culture broth. No amylase activity was detected in the culture broth of the control strain, *P. pastoris* (pGAPZαA), under the same culture conditions (data not shown).

Properties of amylase from *P. pastoris* transformant

As shown in Fig. 2, the purified enzyme showed an apparent single protein band on SDS–PAGE (10% gel). The subunit size of the single protein band was estimated to be 65 kDa from its mobility relative to standard proteins by SDS-PAGE. The optimal pH and temperature of the amylase from *P. pastoris* transformant (pGAPZ-*tfa*) were 7.0 and 60°C, respectively. About 75% of the original amylase activity remained after heat treatment at 60°C for 3 h (Fig. 3). It was slightly higher than the enzyme from *T. fusca* NTU22. The enzyme was stable over the pH range of 6.0–10.0 at 4°C for 24 h. The comparisons of amylase properties from *T. fusca* NTU22 and *P. pastoris* transformant (pGAPZ-*tfa*) were shown in Table 1.

Enzymatic hydrolysis of sago starch

After enzymatic hydrolysis of raw sago starch with recombinant amylase for 48 h, the degree of hydrolysis was 26%. Figure 4 showed the weight-average degree of polymerization (DPw) of raw sago starch and after being treated with amylase at 25°C for 24 and 48 h. Native sago starch had the highest DPw. The DPw obviously decreased with increasing time of amylase treatment.

Table 1 Comparison of the properties of α-amylases from *T. fusca* NTU22 and *P. pastoris* transformant (pGAPZ-*tfa*)

Source	*T. fusca* NTU22	*P. pastoris* transformant
Molecular mass (by SDS–PAGE) (kDa)	64	65
Optimum temperature (°C)	60	60
Optimum pH	7.0	7.0
Thermostability[a] (%)	70	75
pH stability range	6.0–10.0	6.0–10.0

[a] Enzyme activity remained after treatment at 60°C for 3 h

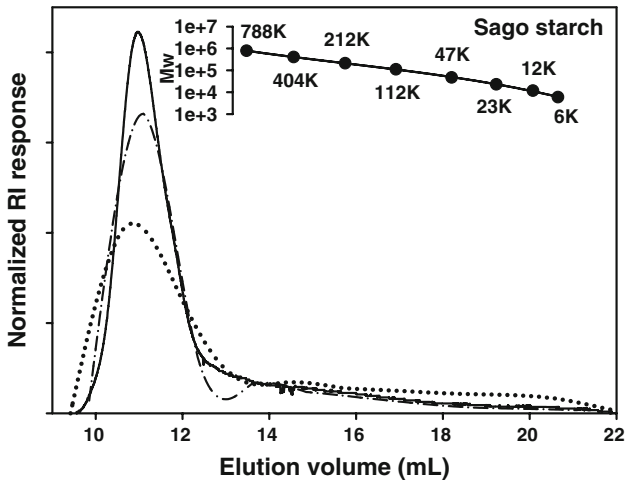

Fig. 4 HPSEC profile of native sago starch with amylase treated for 0, 24 and 48 h. The weight-average molecular weight (DPw) of the native sago starch treated for 0, 24 and 48 h was 830,945, 541,335 and 378,732, respectively. *Thick line*, 0 h; *thick line followed by a dot*, 24 h; *dotted line*, 48 h

After 24 and 48 h treatment, the DPw obviously decreased from 830,945 to 541,335 and 378,732, respectively. The scanning electron microscopy of the raw sago starch granules showed that the surface of the natural granules were smooth (Fig. 5a). After treatment of amylase for 48 h, the surfaces of starch granules were rough, and some granules displayed deep round cavities (Fig. 5b). Shell residues, resulting from total digestion of the inner parts of granules, could be observed for some of the materials. Some granules even lost their structure. However, the progress of degradation was not homogeneous. Both smooth granules and disrupted granules were observed in the same sample.

Discussion

In the native form, starch exists in relatively inert granular structures that are composed of macromolecules arranged in a polycrystalline state. Gelatinization, heating in the presence of water, enhances the chemical relatively toward hydrolytic enzymes [18]. Raw starch digesting amylases are those capable of digesting raw potato starch and are economically attractive as they can increase the range of starch sources for direct hydrolysis. The thermostable raw starch digesting amylases are expected to give better hydrolysis of raw starch at temperatures ranging between 60 and 70°C without losing activity during prolonged incubation [19]. Binding of the raw starch digesting amylases to the starch granules is usually affected by a C-terminal domain within the enzyme, which has been shown to be necessary for degradation of granular starch by mold glucoamylases [20]. Itkor et al. [21] observed that the amylase preparation was more than 98% adsorbed onto both raw corn and potato starches. Similar results were also observed by Saha et al. [22] and Gautam and Gupta [23] where the bacterial enzyme preparation could adsorb onto raw corn starches. Study on the affinities of the enzyme preparation from *Bacillus* sp. I-3 towards raw potato starch granules revealed that the enzyme had a very strong affinity for the same. The C-terminal part of the *T. fusca* amylase showed a homology to the C-terminal of the glucoamylase of *Aspergillus niger* and β-amylase of *Clostridium thermosulfurogenes* [24]. This domain encompasses approximately 100 amino acids

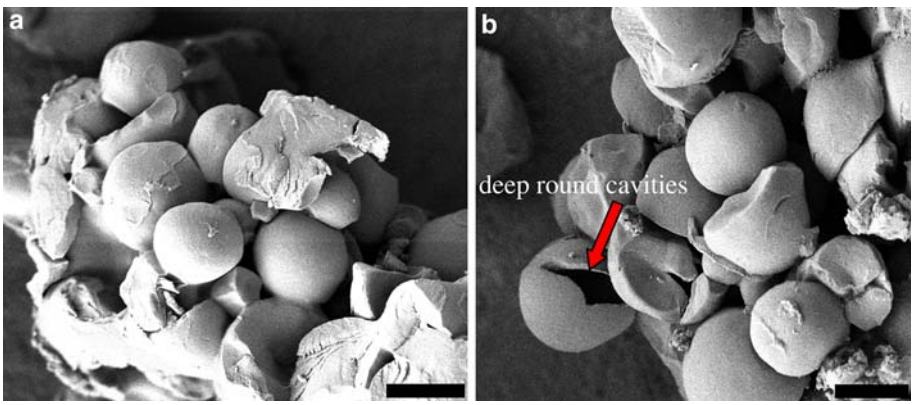

Fig. 5 Scanning electron micrographs (2,000×) of native and enzyme-treated sago starch. (**a**) Native sago starch granules; (**b**) sago starch granules were hydrolyzed by α-amylase for 48 h. Bar = 10 μm

and has been suggested to enable binding to granular starch. The presence of this domain in the *T. fusca* amylase may thus explain the ability of this enzyme to hydrolyze raw starch granules.

In this study, we expressed the α-amylases gene from *T. fusca* NTU22 in *P. pastoris* X-33 rather than in *E. coli* because of its potential application as a food supplement. In addition, the amount of extracellular proteins in the culture broth of the *P. pastoris* transformant was fewer than that in the cell-free extract of *E. coli* transformant. This will facilitate the application of amylase in an industrial process immediately without complicated purification procedures.

In the *GAP* promoter expression system, the cloned heterologous protein will be expressed along with cell growth if the protein is not toxic for the cell [25]. A similar result was observed in this study. As shown in Fig. 2, the rapid production of the extracellular amylase was in parallel with an increase in biomass accumulation of *P. pastoris* transformant. This system is more suitable for large-scale production because the hazard and cost associated with the storage and delivery of large volumes of methanol are eliminated [26]. Thus, the features of the *GAP* expression system may contribute significantly to the development of cost-effective methods for large-scale production of heterologous recombinant proteins.

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