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ORIGINAL PAPER

Heterologous expression of *Thermobifida fusca* thermostable alpha-amylase in *Yarrowia lipolytica* and its application in boiling stable resistant sago starch preparation

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Abstract A gene encoding the thermostable α-amylase in *Thermobifida fusca* NTU22 was amplified by PCR, sequenced, and cloned into *Yarrowia lipolytica* P01g host strain using the vector pYLSC1 allowing constitutive expression and secretion of the protein. Recombinant expression resulted in high levels of extracellular amylase production, as high as 730 U/l in the Hinton flask culture broth. It is higher than that observed in *P. pastoris* expression system and *E. coli* expression system. The purified amylase showed a single band at about 65 kDa by SDS-polyacrylamide gel electrophoresis and this agrees with the predicted size based on the nucleotide sequence. About 70% 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 72-h treatment, the DP_w of raw sago starch obviously decreased from 830,945 to 237,092. The boiling stable resistant starch content of the sago starch increased from 8.3 to 18.1%. The starch recovery rate was 71%.

Keywords *Yarrowia lipolytica* · *Thermobifida fusca* · α-Amylase · Sago starch · Resistant starch

Introduction

Alpha-Amylases (E.C. 3.2.1.1) are glycoside hydrolases that have been classified within 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].

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 [3]. Interestingly, *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 [4]. We reported the purification and some properties of the 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 [5]. 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 [6].

In the light of economic benefits, several thermostable enzyme genes from thermophilic microorganisms have been cloned and expressed in mesophilic microorganisms to reduce the energy needed for cultivation [7, 8]. Recently, the *tfa* gene was amplified by PCR, sequenced, and cloned into *Pichia pastoris* X-33 host strain using the vector pGAPZαA allowing constitutive expression and secretion

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of the amylase. Recombinant expression resulted in the extracellular amylase production, as high as 510 U/l in the Hinton flask culture broth. The amylase activity was higher than that expressed in *E. coli* DH5α (pAMY13H8) [9], but the activity was still lower than that produced by the original strain, *T. fusca* NTU22.

Yarrowia lipolytica is wildly used in the industrial production of citric acid, peach flavor, and single-cell protein [10]. It is one of the most promising nonconventional and GRAS (generally regarded as safe) yeasts available as hosts for heterologous protein production [11], with a large number of molecular tools available for heterologous protein expression [12] and a high secreting capacity [13].

Resistant starch (RS) is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals [14]. RS can be utilized by microorganisms present in the human large intestine. Consequently, the metabolites formed during the fermentation process, i.e., short-chain fatty acids, may serve as a main energy source for the colonocytes [15] and thus help to maintain colon health. The slow hydrolysis of RS makes it useful for the slow release of glucose, which can be especially useful in controlling glycemic plasma responses [16]. Some other benefits include increased fecal bulk, lowered fecal pH, and increased excretion of butyrate and acetate [17]. RS results from the highly retrograded amylose fraction, the quality formed being directly proportional to the content and the length of amylose of starch. Lin et al. [18] treated corn starch with acid-methanol treatment and the RS content increased from 19.2 to 56.2%. Eerlingen et al. [19] hydrolyzed potato starch amylose with barley *β*-amylase and suggested that RS may be formed by aggregation of amylose helices in a crystalline B-type structure over a particular region of the chain (about 24 glucose units). Leong et al. [20] treated the sago starch with commercial pullulanase and found that RS content increased from 5.2 to 7.0% after 8 h of treatment. The boiling stable RS is a type of RS that can resist boiling water bath for 35 min [21].

The aim of this study was the constitutive expression of the α-amylase gene (*tfa*) from thermophilic actinomycetes, *T. fusca* NTU22, in *Y. lipolytic* P01g. Some applications of the enzyme on boiling stable resistant sago starch preparation 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 [3]. *Yarrowia lipolytica* Po1g (MatA, leu2-270, ura3-302:URA3, xpr2-332, axp-2) and pYLSC1 (Fig. 1) were purchased from Yeastern Biotech Co., Ltd (Taipei, Taiwan). The plasmid propagation in the expression work was accomplished with *E. coli* Top 10 F′ (Invitrogen, San Diego, CA, USA). The plasmid pAMY13H8 was constructed with pUC118 and a 3.0-kb *Bam*HI-*Hind*III inserted fragment, which possessed an amylase gene *tfa* [6].

Materials

Yeast extract, yeast nitrogen base w/o amino acid, peptone, tryptone, and agar were purchased from BD (Sparks, MD, USA). 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, USA). DEAE-Sepharose CL-6B and Sepharose CL-6B were purchased from GE Healthcare (Little Chalfont, UK). The protein assay kit and the SDS-PAGE molecular weight standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Inorganic salts and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Construction of the amylase expression plasmid

The *T. fusca* NTU22 α-amylase gene was amplified with primers of 5′-CACGGCCGTTCTGGCCATGGGAGTGC GCAGATCCC-3′ (*Sfi*I site is underlined) and 5′-GGAGGT ACCTCAGCGCCAGGAGTCGTAGAAGTTCTC-3′ (*Kpn*I site is underlined) using pAMY13H8 as the template. The amplification was performed using a DNA thermal cycler (ABI 2720 Thermal Cycler, MA, USA) under the following conditions: the first step was initiated at 95°C for 2 min,

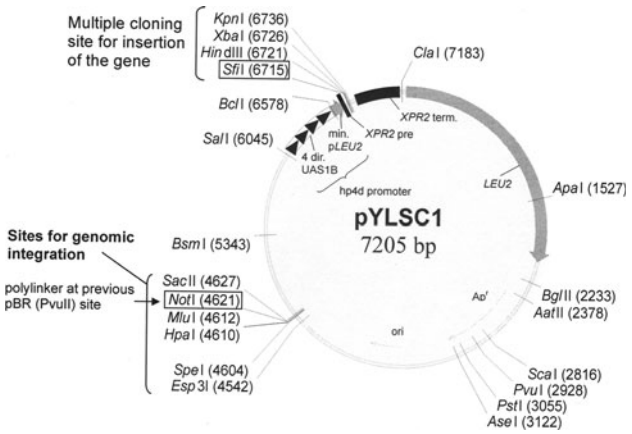

Fig. 1 The gene map of pYLSC1

followed by 30 cycles of 95°C for 20 s, 64°C for 20 s, and 72°C for 30 s, and the final extension was carried out at 72°C for 2 min. A 1.8-kb PCR product was recovered from the agarose gel and cloned into *Sfi*I and *Kpn*I digested pYLSC1. After being transformed into *E. coli* Top 10′, one recombinant plasmid designated as pYLSC1-*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 100 μg/ml ampicillin. The proper insert orientation was checked by restriction analysis and sequencing as described above.

Transformation and screening of *Y. lipolytica*

The recombinant plasmid pYLSC1-*tfa* was linearized with *Not*I, and transformed into *Y. lipolytica* Po1g under the method of Xuan et al. [22]. The transformants were selected at 28°C on the YNB agar plates (20 g/l glucose, 6.7 g/l yeast nitrogen base w/o amino acid) for 2–4 days. The transformants were tested for amylase activity by replicating the colonies onto the YNB agar plates, which contained 10 mg/ml soluble starch. After incubation at 28°C for 48 h, the plates were stained with iodine solution. Colonies with a clear zone resulting from hydrolysis of starch were selected for further analysis. Transformants with higher amylase activity were obtained and checked for the integration of the construct into the *Y. lipolytica* Po1g genome by genomic PCR.

Biomass and amylase activity assay

Biomass production of the *Y. lipolytica* 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 [5]. 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 a Hinton flask

The high amylase activity transformant was incubated in 50 ml YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, pH 4.0) in 500-ml Hinton flasks and shaken (200 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. After 60-h cultivation of *Y. lipolytica* transformant, the fermentation broth was centrifuged at 10,000 × *g* for 30 min to remove cells. The supernatant was then concentrated by ultrafiltration (Pellicon XL, Biomax 10 K, Millipore) and applied to a DEAE-Sepharose CL-6B column (2.6 × 10 cm) previously equilibrated with 20 mM Tris buffer (pH 6.0). After washing with the same buffer to remove inactive protein, the enzyme was eluted with a linear gradient of the buffer containing NaCl from 0.0 to 0.6 M (flow rate: 60 ml/h). The enzyme activity was detected within the range 0.1–0.2 M NaCl. The eluted enzymatically active fractions were pooled and applied to a Sepharose CL-6B column (1.6 × 100 cm) previously equilibrated with 20 mM Tris buffer (pH 6.0). Proteins were eluted at a flow rate of 30 ml/h. The eluted enzymatically active fractions were pooled and used as the purified enzyme.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass of the purified enzyme was determined by using SDS-PAGE (10% polyacrylamide). Low-molecular-weight standards were used as molecular mass markers. 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).

Enzymatic resistance

Enzymatic resistance of starch was determined using the method of Englyst et al. [14]. A starch sample was enzymatic hydrolyzed by using pancreatin and amyloglucosidase at 37°C up to 120 min. For evaluating the enzymatic resistance of starch granules, the rapid digestible starch (RDS), slow digestible starch (SDS), resistant starch (RS), and boiling stable resistant starch (BSRS) contents were obtained by measuring the released glucose units using a glucose oxidase–peroxidase assay. RDS was measured after incubation with enzymatic hydrolysis for 20 min and RS is the starch not being hydrolyzed after 120 min incubation. SDS is the starch being hydrolyzed within 20–120 min incubation, which was calculated from total starch content minus RDS and RS values. The starch sample was first placed in boiling water bath for 35 min, and immediately cooled in an ice-water bath for 5 min. Then the RS content was measured as BSRS [21].

Average molecular weight

The weight-average degree of polymerization (DP_w) of starch was determined by high-performance size-exclusion chromatography (HPSEC) [23]. 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 magnetic stirrer in 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 Tech. Co., Santa Barbara, CA) and a 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 [24].

Result

Expression of amylase gene (*tfa*) in *Y. lipolytica*

The *tfa* coding sequence was cloned into the pYLSC1 vector at the *SFi*I/*Kpn*I restriction sites as described earlier. This construction allowed *tfa* coding sequence to be theoretically in-frame in pYLSC1. Following a sequence check, the construct denoted as pYLSC1-*tfa* was *Not*I-linearized and transformated into *Y. lipolytica* Polg. Many transformants were selected on YNB plate. The genomic PCR assay revealed that most of the transformants contained an integrated *tfa* coding sequence in genomic DNA. Among these transformants, the transformant (pYLSC1-*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 YPD broth at 28°C. Transformant (pYLSC1-*tfa*) grew logarithmically from 12 to 36 h and then entered a stationary phase (Fig. 2). The biomass reached approximately 46 of the OD_{600} value after 36-h incubation. The rapid accumulation of the extracellular amylase was in parallel with an increase of biomass. The maximum activity (730 U/l) was reached in the culture broth after 60-h incubation. No amylase activity was detected in the culture broth of the control strain, *Y. lipolytica* (pYLSC1), under the same culture conditions (data not shown).

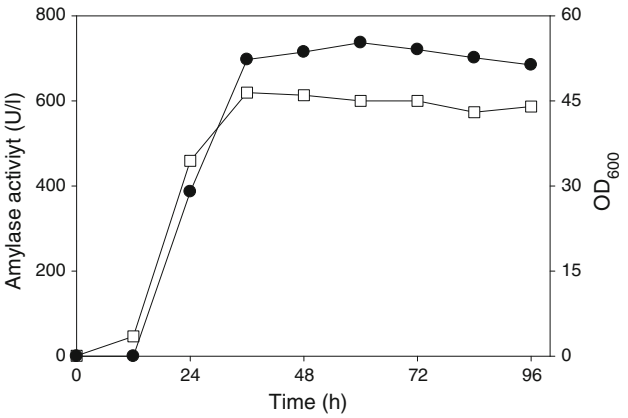

Fig. 2 Time course for the expression of amylase activity by *Y. lipolytica* transformant (pYLSC1-*tfa*). Cells were grown aerobically in a 500-ml Hinton flask loaded with 50 ml of medium consisting of YPD broth and were incubated at 28°C, 150 rpm for 96 h. The *dark circle* indicates amylase activity and the *open square* OD_{600}

Purification and properties of amylase from *Y. lipolytica* transformant

Purification of the amylase was described previously in the section Materials and methods. The results of the purification are summarized in Table 1. The purified enzyme obtained exhibited 21.87% of the total initial activity and there was a 10.76-fold increase in specific activity compared with the cell-free extract.

As shown in Fig. 3, 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 *Y. lipolytica* transformant (pYLSC1-*tfa*) were 7.0 and 60°C, respectively. About 70% of the original amylase activity remained after heat treatment at 60°C for 3 h (Fig. 4). It is similar with 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.

Effect of enzymatic treatment times on sago starch hydrolysis

Table 2 summarizes the weight-average degree of polymerization (DP_w) of sago starch before and after treated at

Table 1 Summary of the purification of amylase from *Y. lipolytica* transformant (pYLSC1-*tfa*)

Step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Purification (fold)	Yield (%)
Culture filtrate	77.68	445.43	5.73	1	100.0
Ultrafiltration	37.22	303.92	8.17	1.43	68.23
DEAE-sepharose CL-6B	5.17	189.07	36.57	6.38	42.45
Sepharose CL-6B	1.58	97.40	61.65	10.76	21.87

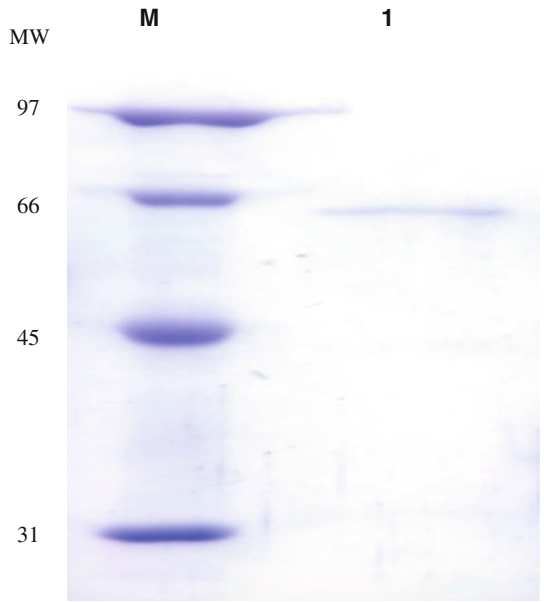

Fig. 3 SDS-PAGE of the purified amylase from *Y. lipolytica* transformant (pYLSC1-*tfa*). *Lane M* low-molecular-weight marker; *Lane 1* purified amylase from *Y. lipolytica* transformant (pYLSC1-*tfa*)

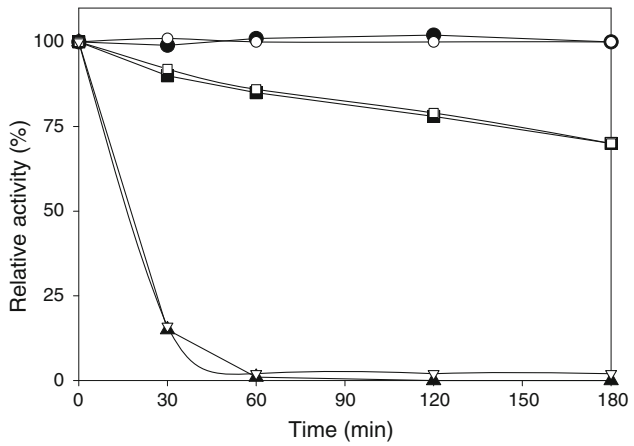

Fig. 4 Thermal stability of the amylases from *T. fusca* NTU22 and the *Y. lipolytica* transformant (pYLSC1-*tfa*). The enzyme was incubated at various temperatures for 30–120 min, and the residual enzyme activities were determined; *T. fusca* NTU22: *dark circle* 50°C; *dark square* 60°C; *dark triangle* 70°C; *Y. lipolytica* transformant: *open circle* 50°C; *open square* 60°C; *open triangle down* 70°C

50°C in the solution containing 4 U/ml amylase activity for different periods of time. Native sago starch had the highest DP_w among the starches studies. The DP_w of starch obviously decreased with increasing time of enzymatic treatment. In this study, the longest time of treatment was 72 h, and the recovery rate of starch granules after 72 h of treatment was above 70%. Results in Table 2 indicate that enzymatic treatment can be used to prepare starch with different molecular sizes, and the longer the treated time the lower molecular size of the starch prepared. The DP_w of sago starch used in enzymatic resistance observation ranged from 830,945 to 237,092.

The HPSEC profiles (Fig. 5) of enzyme-treated starches show bimodal distributions. Generally, the F1 fraction consists of amylose and the F2 fraction consists of amylopectin [18]. After enzymatic treatment, the F1 peak decreased and the F2 fraction increased. The weight percentages (%) of both fractions of starches before and after enzymatic treatment are also shown in Table 2. The weight percentage of F1 fraction gradually decreased with increasing time of enzymatic treatment. However, the weight percentage of F2 fraction showed a reverse tendency. The result indicates that the amylose chains (F1 fraction) of starch degrades to shorter chains during enzymatic treatment, and leads to the increase of the weight percentage of F2 fraction.

Scanning electron microscopy of the raw sago starch granules showed that the surface of the natural granules were smooth (Fig. 6a). After treatment of amylase for 24–72 h, the surface of starch granules was rough and some granules broke into pieces (Fig. 6b–d). 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.

Enzymatic resistance

Table 3 shows the RDS, SDS, RS, and BSRS contents of sago starch before and after enzymatic treatment. The RS, RDS, and SDS contents before and after enzymatic treatment were not significantly different. The BSRS content

Table 2 Weight-average molecular weight (DP_w) of HPSEC fractions and recovery rate of sago starch treated with amylase

Treatment	DP_w	F1		F2		Recovery
		%	DP_w	%	DP_w	Rate[c] (%)
Sago starch						
Native	830,945 ± 18,900	84.0 ± 1.1	989,198 ± 10,039	16.0 ± 1.1	1,857 ± 57	–
E[a]-12h[b]	823,901 ± 12,487	80.5 ± 0.5	950,309 ± 2,376	19.5 ± 0.5	1,856 ± 47	80
E-24h	541,335 ± 17,228	69.8 ± 0.3	775,309 ± 9,759	30.2 ± 0.3	1,273 ± 9	77
E-48h	378,732 ± 14,245	44.2 ± 0.0	524,691 ± 7,459	55.8 ± 0.0	1,823 ± 11	73
E-72h	237,092 ± 33,119	39.1 ± 0.3	385,391 ± 2,902	60.9 ± 0.3	2,315 ± 7	71

[a] E stands for enzymatic treatment

[b] Time of enzymatic treatment

[c] Recovery (%) = (weight of starch after treatment)/(weight of native starch used) × 100%

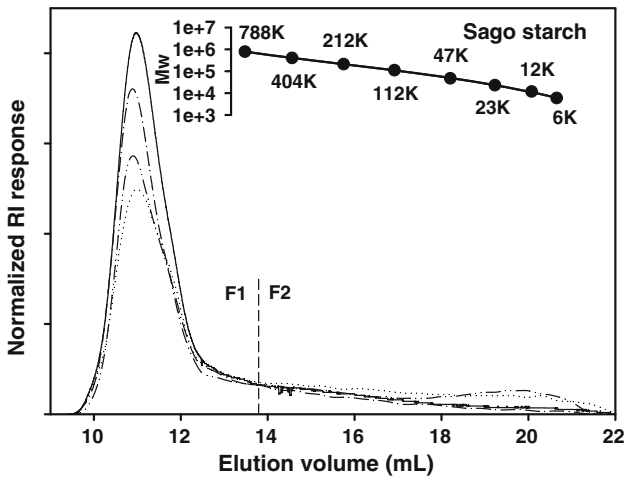

Fig. 5 HPSEC profile of native sago starch with amylase treated for 0, 24, 48, and 72 h. *Thick line* 0 h; *thick line followed by a dot* 24 h; *thick line followed by two dots* 48 h; *dotted line* 72 h

increased significantly as the enzyme-treated time increased from 0 to 72 h. The maximum BSRS content was achieved at 72 h (18.1%).

Discussion

A precise comparison between expression systems is difficult, due to variety of available data. Each protein is rarely produced in several hosts. In the case of the α-amylases from *T. fusca* NTU22, we had the opportunity to compare the production of a same protein in three different host systems: *E. coli* [6], *P. pastoris* [9], and *Y. lipolytica*. The *E. coli* is the first choice for heterologous protein expression due to the ease of genetic manipulation, availability of efficient genetic tools, high transformation efficiency, and rapid growth rates. However, the misfolding and intracellular production often presents the risk of inclusion body formation [25]. Recently, we attempted to use *P. pastoris* as host for heterologous expression. However, *P. pastoris* displayed low transformation efficiency and high occurrence of negative transformants. In this study, we successfully expressed the α-amylases gene from *T. fusca* NTU22 in *Y. lipolytica*. The amylase activity accumulated in the culture broth of *Y. lipolytica* transformant (pYLSC1-*tfa*) (730 U/l) is higher then that accumulated in the culture broth of *P. pastoris* transformant (pGAPZ-*tfa*) (510 U/l) and *E. coli* transformant (pAMY13H8) (350 U/l). Madzak et al. [26] heterologous expressed the laccase gene from basidiomycete *Pyxnoporus cinnabarinus* in the *P. pastoris* and *Y. lipolytica*. The maximum laccase activity of *P. pastoris* transformant in shake-flask culture was lower than that observed in *Yarrowia* system. It is similar with our result in this study.

The RSDS is important because it not only appears as dietary fiber but is also relatively thermally stable, remaining after thermal processing at 100°C. Several manufacturing processes have been developed to increase BSRS from various starches. Brumovsky and Thompson [22] produced BSRS by partial acid hydrolysis and hydrothermal treatments of high-amylose maize starch. The decrease of the molecular weight would allow a greater freedom of polymer motion. As shown in Table 2, the DP_w of sago starch decreased from 830,945 to 237,092. For this reason, we anticipated an enhanced ability to form more boiling-stable structure.

Amylopectin interferes with amylose retrogradation. The degree of polymerization (DP) of amylose also affects the yield of RS, which rises with DP up to 100 and remains level above that [27]. This indicates that amylopectin is unfavorable to the formation of RS. In our result, the DP_w decreased and the F2 fraction increased after enzymatic treatment for 72 h. The RS contents of sago starch before and after enzymatic treatment showed no significant difference. It is difficult to explain. Further experimentation in this direction is needed to solve the question.

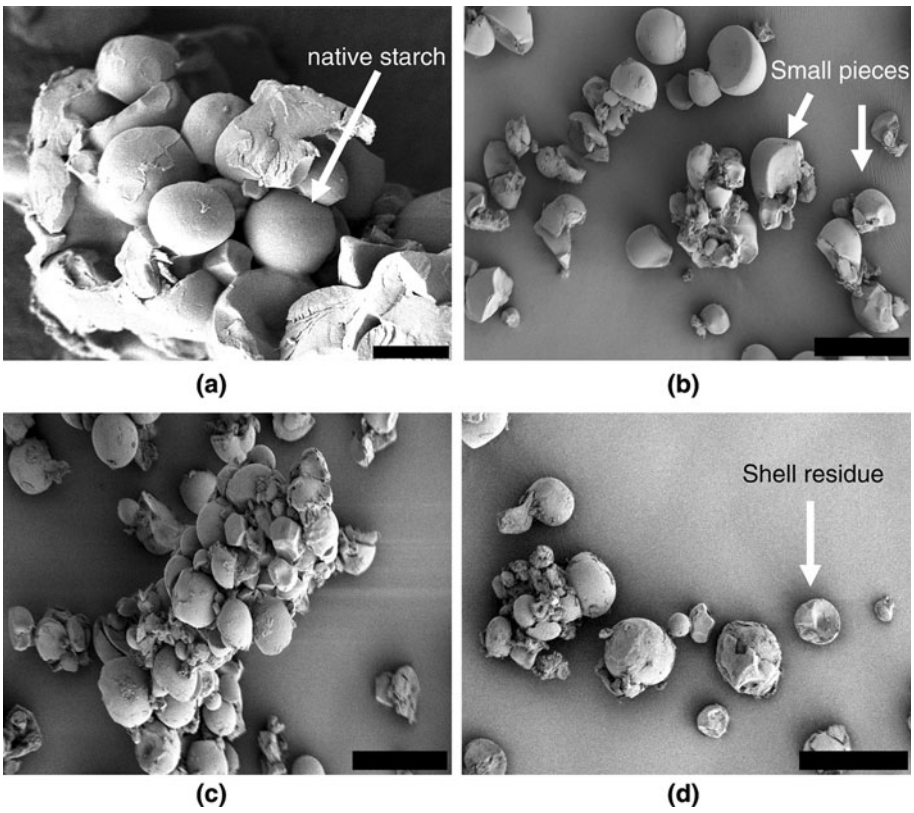

Fig. 6 Scanning electron micrographs (2,000×) of native and enzyme-treated sago starch. **a** Native sago starch granules. **b–d** Sago starch granules were hydrolyzed by α-amylase for 24, 48, and 72 h. *Bar* 10 μm

Table 3 Changes in enzymatic resistance of sago starch

Treatment	RDS (%)[a]	SDS (%)[b]	RS (%)[c]	BSRS (%)[d]
Sago starch				
Native	18.2 ± 0.6	44.3	37.5 ± 0.3	8.3 ± 0.2
E[e]-12h[f]	20.7 ± 0.4	42.7	36.6 ± 0.1	10.6 ± 0.5
E-024h	24.6 ± 0.1	42.3	33.1 ± 0.0	14.8 ± 0.1
E-048h	22.7 ± 0.1	42.4	34.9 ± 0.1	17.2 ± 0.4
E-072h	21.1 ± 0.5	42.1	36.8 ± 0.2	18.1 ± 0.6

[a] RDS stands for the rapidly digestible starch. RDS = 100% − amount of residual starch (%) after digested for 20 min

[b] SDS stands for the slowly digestible starch. SDS = 100% − RS% − RDS%

[c] RS stands for the resistant starch. RS = Amount of residual starch (%) after digested for 120 min

[d] Boiling-stable RS content (%) = (Weight of starch after boiled and digested)/(weight of native starch used) × 100%

[e] E stands for enzymatic treatment

[f] Time of enzyme treatment

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