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ORIGINAL PAPER

Construction and characterization of a thermostable whole-cell chitinolytic enzyme using yeast surface display

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Abstract To develop a novel yeast whole-cell biocatalyst by yeast surface display technology that can hydrolyze chitin, the chitinaseC gene from *Serratia marcescens* AS1.1652 strain was cloned and subcloned into the yeast surface display plasmid pYD1, and the recombinant plasmid pYD1/SmchiC was electroporated into *Saccharomyces cerevisiae* EBY100 cell. Aga2p-SmChiC fusion protein was expressed and anchored on the yeast cell surface by induction with galactose, which was verified by indirect immunofluorescence and Western blotting. The chitinolytic activity of the yeast whole-cell biocatalyst or partially purified enzyme was detected by agar plate clear zone test, SDS-PAGE zymography and dinitrosalicylic acid method. The results showed that the chitinaseC gene from *S. marcescens* AS1.1652 strain was successfully cloned and expressed on the yeast cell surface, Aga2p-SmChiC fusion protein with molecular weight (67 kDa) was determined. Tests on the effect of temperature and pH on enzyme activity and stability revealed that the yeast whole-cell biocatalyst and partially purified enzyme possessed both thermal stability and activity, and even maintained some activity under acidic and weakly alkaline conditions. The optimum reaction temperature and pH value were set at 52 °C and 5.0, respectively. Yeast surface display technology succeeded in preparing a yeast whole-cell biocatalyst with chitinolytic activity, and the utilization of chitin could benefit from this process of enzyme preparation.

Keywords Chitinase · *Serratia marcescens* · Yeast surface display · *Saccharomyces cerevisiae* · Thermostable enzyme

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Introduction

Chitin, a polysaccharide made up of many units of *N*-acetyl-D-glucosamine (GlcNAc) linked by β-(1-4) glycosidic bonds, is widely found in the exoskeletons of arthropods, such as shrimp, crabs and insects. It is the second most abundant polysaccharide in nature, after cellulose (Dutta et al. 2004). Chitin is an important renewable resource with multiple potential uses in biomedicine, agriculture, biotechnology, food additives and other fields (Khor 2001). Unfortunately, as result of strong hydrogen bonds among intermolecular, many applications of chitin have been limited due to its low solubility in water and other organic solvents. In contrast, chitin oligosaccharides, which are produced by degrading chitin, are soluble in water and easily absorbed by the body, where they demonstrate superior biological qualities, such as antioxidant, antibacterial, anti-tumor, and anti-inflammation. They have also been shown to regulate fat metabolism and blood sugar, enhance immune function, and aid the regulation of intestinal flora. Therefore, the study of chitin

oligosaccharides has been of great importance in the development of anti-tumor agents, immunomodulatory agents, anti-hypertensive drugs, anti-microbial agents, and biological preservatives in the pharmaceutical and food industries (Ahmed et al. 2012).

Chitin oligosaccharides are prepared mainly by enzymatic hydrolysis or chemical methods (Ahmed et al. 2012). Because the chemical method is laborious and leads to environmental pollution through the use of large amounts of acids, research attention has focused increasingly on enzymatic hydrolysis. Chitinase (EC3.2.1.14) is a specific hydrolase that degrades the chitin by random cutting of glycosidic bonds, and it plays an important role in chitin's biodegradability and utility as a renewable resource (Sitrit et al. 1993). Currently, researchers are studying chitin and chitinase's applications in pharmaceutical production and the food processing industry.

With the development of genetic engineering technology, cell surface display technology was established to anchor the recombinant protein or peptide on cell surface of bacteria, yeast, insect or mammalian. Yeast whole-cell biocatalysts, in which active enzymes are anchored on the cell surface by a yeast display system, have been widely applied in the fields of food, medicine and feed enzyme production. Examples include display amylase (Shigechi et al. 2004) and cellulolytic enzyme (Tsai et al. 2010) to produce ethanol, display cyclodextrin glucanotransferase to produce cyclodextrin (Wang et al. 2006), display carnosinase to synthesize functional carnosine from non-protein amino (Inaba et al. 2010), display lipase to synthesize edible spices and perform hydrolysis of esters (Kuroda and Ueda. 2011), display β-glucosidase to convert isoflavone glycosides into isoflavone aglucones (Kuroda and Ueda. 2011).

Based on the adhesion function of a-agglutinin, a commercial yeast display vector pYD1 and host strain *Saccharomyces cerevisiae* EBY100 was developed by Wittrup et al. and may be obtained from Invitrogen Life Technologies. A-agglutinin is a mating adhesion receptor that normally present on the surface of yeast cell, and is a commonly used carrier protein in *S. cerevisiae* display system. The a-agglutinin receptor consists of two subunits encoded by the Aga1 and Aga2 genes. The Aga1 protein (Aga1p, 725 amino acids) is secreted from the cell and becomes covalently attached to glucan in the extracellular matrix of the yeast cell wall. The Aga2 protein (Aga2p, 69 amino acids) binds to Aga1p through two disulfide bonds and after secretion remains attached to the cell through its contact with Aga1p (Hackel and Wittrup. 2009).

Saccharomyces cerevisiae EBY100 strain contains a stably-integrated Aga1 gene regulated by GAL promoter. The yeast display vector pYD1 contains Aga2 gene, and fusion of the gene of interest to Aga2 allows secretion and display of the protein of interest. When *S. cerevisiae* EBY100 transformed with pYD1 containing gene of interest induced by galactose, the Aga1p and Aga2p-fusion protein are expressed, respectively. As a result, the Aga2p-fusion protein binds to Aga1p through two disulfide bonds and anchors onto the yeast cell surface. Prototrophic genes, such as LEU2 and TRP1, are used to complement auxotrophic lesions in the host *S. cerevisiae* EBY100 as a nutritional selectable marker.

In this study, we attempted to express a thermostable chitinase from *Serratia marcescens* on the *S. cerevisiae* cell surface by a-agglutinin receptor system in order to hydrolyze chitin by enzymolysis. *S. marcescens* chitinaseC gene was inserted into the 3′ end of the Aga2 gene of the display vector pYD1 and electroporated into the *S. cerevisiae* EBY100 strain. Aga2p-SmChiC fusion protein and Aga1p were induced expression with galactose, respectively. The fusion protein was anchored on the cell wall through the disulfide bond between Aga1p and Aga2p, and thereby constructing the yeast whole-cell biocatalyst with chitin degradation activity. This process of whole-cell biocatalyst preparation shows great potential for application in utilization of chitin or bioactive chitin oligosaccharides production.

Materials and methods

Strains, plasmids and medium

The *S. marcescens* AS1.1652 strain used for cloning of the chitinaseC gene was obtained from Guangdong culture collection center, Guangzhou, China. *Escherichia coli* DH5α strain was used as a host for recombinant plasmid. Plasmid pYD1 and *S. cerevisiae* EBY100 strain (Invitrogen) were used for yeast cell surface display. PCR cloned vector, pMD®18-T, was purchased from Takara.

Escherichia coli and *S. marcescens* were grown in LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl, and 1.5 % agar for solid medium). Untransformed yeast EBY100 strains were grown in non-selective YPD medium (2 % dextrose, 1 % yeast extract, 2 % peptone, and 1.5 % agar for solid medium). Minimal Dextrose Plates (2 % glucose, 0.67 % YNB, 1.5 % agar, 0.01 % leucine) were used for selection of pYD1 transformants. *S. cerevisiae* EBY100 harboring pYD1 or its derived plasmids were grown in SD-CAA medium (pH 6.0) (2 % glucose, 0.67 % YNB, 0.5 % casamino acid, 0.54 % Na_2HPO_4, 0.856 % $NaH_2PO_4{\cdot}H_2O$, and 1.5 % agar for solid medium) (Hackel and Wittrup 2009). SG-CAA medium (pH 6.0) (2 % galactose, 0.67 % YNB, 0.5 % casamino acid, 0.54 % Na_2HPO_4, 0.856 % $NaH_2PO_4{\cdot}H_2O$, and 1.5 % agar for solid medium) was used as inducing medium for Aga1, Aga2, or Aga2-fusion protein (Hackel and Wittrup 2009).

Plasmid construction and yeast transformation

Based on nucleotide sequence of chitinaseC gene from *S. marcescens* GEI strain (GenBank number, GQ855219), the chiC gene was amplified from *S. marcescens* AS1.1652 strain chromosomal DNA by colony PCR using two primers: 5′-BSmchiC (GGATCCATGAGCACAAATAACATT AT) containing endonuclease *BamH I* restriction recognition sites and 3′-XSmchiC (CTCGAGGGCGATGAGCTG CCACAG) containing *Xho I* recognition sites (Tu et al. 2010). PCR conditions were initial denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min. The resulting PCR product was cloned into T-A cloning vector to form pMD18-T/SmchiC. The products of plasmid pMD18-T/SmchiC digested by *BamH I* and *Xho I* were inserted into the *BamH I*/*Xho I* sites of pYD1 to construct yeast surface display plasmid pYD1/SmchiC. Both plasmids were identified by double digestion and sequencing. We performed BLAST searches using software available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The isoelectric point and molecular weight of *S. marcescens* AS1.1652 ChitinaseC were calculated using the program ProtParam (http://web.expasy.org/protparam/).

The plasmid pYD1/SmchiC was transformed into *S. cerevisiae* EBY100 competent cells (1.5 kV, 7.5 kV/cm, 25 uF, 200 Ω: Gene Pulser Xcell™ Electroporation System, Bio-Rad). Leu^+ transformants were selected on MD plates containing 0.01 % leucine.

Induced expression and purification of Aga2p-SmChiC fusion protein

Untransformed *S. cerevisiae* EBY100, EBY100/pYD1, and EBY100/pYD1-SmchiC strains were cultivated to mid-log growth phase in SD-CAA medium for large scale production of yeast biomass. The cells were collected and induced in SG-CAA medium for 48 h at 22 °C, and then Aga1 and Aga2-SmChiC fusion protein were expressed by inducing with galactose. The induced yeast cells were used to immunofluorescence staining, determine the chitin degradation activity and prepare partially purified enzyme.

According to the instruction manual of Invitrogen (pYD1 Yeast Display Vector Kit, Catalog no. V835-01), the partially purified enzymes were produced by releasing the Aga2p-SmChiC fusion protein from the cell wall using dithiothreitol (DTT), which can break the disulfide bond between Aga2p and Aga1p. The cells were collected from 10 mL of the induced culture by centrifugation. The precipitate was suspended in 400 µL PBS buffer containing 100 µmol/L of DTT and incubated for 2 h with constant shaking at 37 °C to release Aga2p-SmChiC fusion protein from the cell walls. After centrifugation, the supernatant containing Aga2p-SmChiC fusion protein was used to perform Western-blotting and SDS-PAGE zymography analysis.

Yeast immunofluorescence

Yeast immunofluorescence was according to the instruction manual of Invitrogen, except the cells coated on glass slide and fixed with glutaraldehyde. The induced cells were coated on a glass slide after they were washed using PBS buffer and fixed with glutaraldehyde. After the glass slide was air-dried at room temperature, aliquots of PBS (pH 7.4, containing 0.5 % BSA, 0.5 % ovalbumin) were added to it, keeping it in a humid box for blocking overnight. Next the blocking solution was discarded, and the slide was washed three times with PBS. The cells were incubated in 1:2500 dilution of His-Tag (2A8) Mouse IgG (Abmart) for 2 h, followed by incubation in 1:1,000 Alexa Fluor 555-labeled Goat Anti-Mouse IgG (H + L) for 2 h more after washing the cells three times again. After rinsing completely, one drop of anti-fluorescence quenching reagent was added to the slide before placing a coverslip. The slide was observed and photographed under a fluorescence microscope (555 nm).

Western-blotting analysis of expression products

Partially purified enzyme was run on 15 % SDS-PAGE and electrotransferred to PVDF membrane. The protein marker lane was cut and stained with amino black. The rest of the membrane was washed and blocked with nonfat dry milk followed by incubation in 1:2,500 dilution of His-Tag (2A8) Mouse IgG (Abmart) for 2 h. Next, the PVDF membrane was washed three times and incubated in dilution 1:1,000 HRP-IgG for 2 h. After washing three times, the membrane was stained with diaminobenzidine (DAB) and compared to protein molecular weight standards.

Qualitative detection of chitinolytic activity

The chitinolytic activity was detected using water-soluble chitin as substrate by agar plate clear zone test and SDS-PAGE zymography. Water-soluble chitin was prepared as follows, according to the method reported in literature (Guo et al. 2002). We suspended 2 g of chitin powder in 40 g of 48 % NaOH solution and kept it at −20 °C for dipping overnight, and then added 86 g of crushed ice and stirred till a clear solution formed. The solution was kept at 4 °C for 10 days, and then 4 mol/L HCl was added to make pH within 7.5–8.0. The solution was boiled to form swollen gels. The centrifuged precipitate was washed with

deionized water until the eluate was neutral, and then it was suspended in 10 times volume of H_2O again. The pH value of the suspension was adjusted to 6.0–6.5 with 2 mol/L HCl until a clear solution formed. The filtrate from the gauze was reprecipitated with acetone at room temperature. The white fibrous precipitate obtained was washed with 90 % acetone solution three times and dried at 60 °C. At last the resultant chitin could be soluble in water to a 0.5 % (w/w) solution for enzyme assays.

The *S. cerevisiae* EBY100/pYD1-SmchiC and EBY100 (control) were inoculated on SG-CAA agar plates containing 0.025 % water-soluble chitin, and then cultured at 25 °C for 48 h. The agar plate was stained in an aqueous solution of Congo red (1 mg/mL) for 10–15 min, and then soaked with 1 mol/L NaCl solution to visualize the chitin degradation zone (Teather and Wood 1982).

Partially purified product was run on 15 % SDS-PAGE (containing 0.025 % water-soluble chitin). Subsequently the gel was kept in 0.1 mol/L sodium acetate buffer (pH 5.0) containing 1 %Triton X-100 at 37 °C overnight. The gel was stained with Coomassie Brilliant Blue G250, and then destained to visualize the chitin degradation zone (Liau and Lin 2008).

Quantitative determination of chitinolytic activity

The chitinolytic activity of whole cell chitinase and DTT-released chitinase (partially purified enzyme) was detected by DNS method according to literature (Fukuda et al. 2007). The induced cells collected by centrifugation were washed with deionized water, suspended in PBS, and adjusted OD_{600} to 20. Next, 1 mL of cell suspension was centrifuged, and 1 mL of substrate solution (PBS containing 0.5 % water-soluble chitin) was added into the cell precipitate or 10 μL partially purified enzymes. After incubation at 52 °C for 3 h with frequent shaking and centrifugation, the supernatant (0.5 mL) was added to 0.5 mL of DNS reagent (containing 1 % dinitrosalicylic acid, 0.2 % phenol, 0.05 % sodium sulfite, 1 % sodium hydroxide). The mixture was boiled in a water bath for 5 min and cooled on ice. By measuring the absorbance of the solution at 540 nm, the amount of reducing sugar released from the substrate was calculated by the standard curve obtained in the same way as above, using *N*-acetylglucosamine (GlcNAc) as standard analyte. One unit of chitinolytic activity was defined as the amount of enzyme required to release 1 μmol GlcNAc per hour at 52 °C. Deionized water and EBY100/pYD1(not display SmChiC) was used as control, respectively. The protein concentrations of the partially purified enzymes were determined by Bicinchoninic acid (BCA) Assay Kit (BioTeke, China).

Effect of temperature and pH on enzyme activity and stability

Induced cells were collected by centrifugation, and the chitinolytic activities of the whole cell chitinase and DTT-released chitinase were determined at different temperatures ranging from 25 to 80 °C (interval of 5 °C) and 44 to 66 °C (interval of 2 °C) by the DNS method described above.

To test the effect of temperature on the stability, the whole cell chitinase and DTT-released chitinase were incubated at 50, 60, 70, 80, 90 and 100 °C, in the absence of substrate. Next the samples were removed and placed on ice at 1, 2, 3, 4 h. Residual activities were measured according to the DNS method described above.

To test the effect of pH values on activity of whole cell chitinase and DTT-released chitinase, the chitinolytic activities were also assayed in sodium hydrogen phosphate-citrate buffer containing 0.5 % water-soluble chitin with pH values 2.2, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 at optimal temperature by the DNS method described above.

To qualitative test the effect of temperature on enzyme stability of partially purified enzyme, we incubated the DTT-released chitinase at 37, 50, 60, 70, 80, 90 and 100 °C for 1 h in the absence of substrate. Chitinolytic activity of partially purified enzyme was detected using SDS-PAGE zymography, as described above.

Results

Construction of expression plasmids

The chitinaseC gene of *S. marcescens* AS1.1652 strain (chiC) was amplified, sequenced and cloned into T-A cloning vector to form pMD18-T/SmchiC, and then the yeast surface display plasmid pYD1/SmchiC was constructed (Fig. 1). Both plasmids were identified by double digestion and sequenced (Fig. 2). The gene was submitted to GenBank (GenBank access number: KJ155833).

The sequencing results showed that chiC has one open reading frame (ORF) composed of 1,440 base pairs, which encodes a protein of 480 amino acids. The isoelectric point and molecular weight of the protein were predicted to be around 5.36 and 52 kDa, respectively, and the protein does not contain signal peptide. The degrees of identity to the four previously described chiC were 96, 96 96, 98 % at the DNA level and 99, 98, 98, 99 % at the protein level for chiC from strain 2170, strain 141, strain xd1 (Sezen et al. 2008) and strain BJL200 (Synstad et al. 2008), respectively. This clearly demonstrated that the gene cloned here was the counterpart of the four chiC genes previously described.

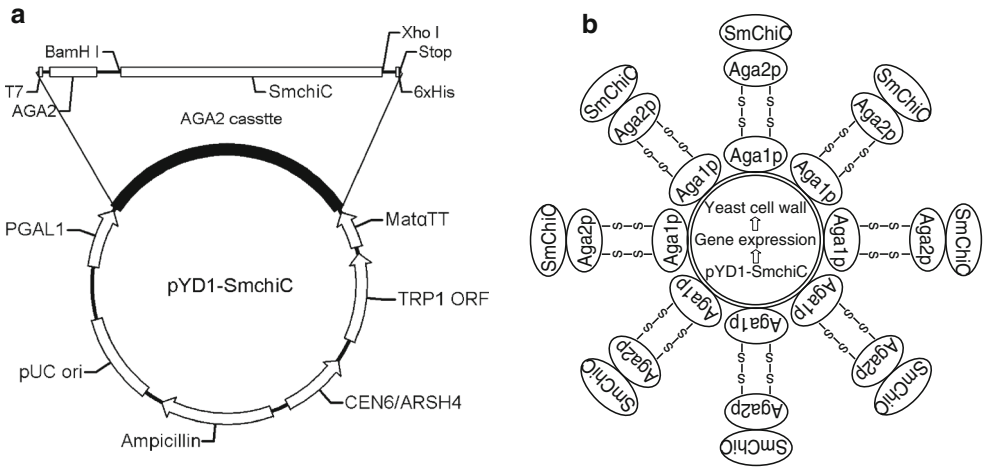

Fig. 1 Design of a yeast surface display system for chiC gene of *S. marcescens*. **a** Construction of plasmid pYD1-SmchiC. **b** Schematic representation of the recombinant protein anchoring on the yeast cell surface

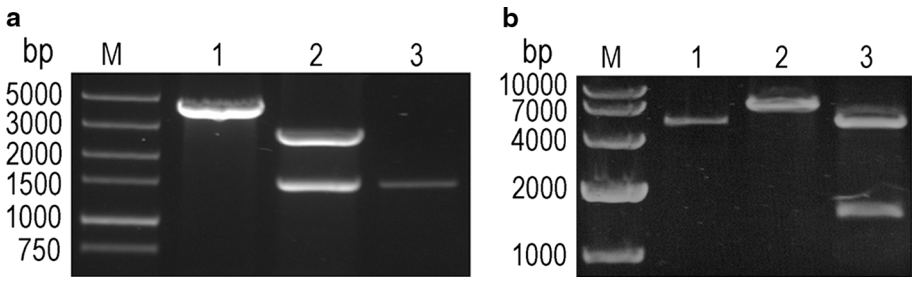

Fig. 2 Agarose gel electrophoresis of recombinant plasmids. **a** pMD18-T/SmchiC. *Lane M* DNA marker; *lane 1* pMD18-T/SmchiC digested by *Xho*; *lane 2* pMD18-T/SmchiC digested by *BamH I* and *Xho I*; *lane 3* PCR product of SmchiC gene. **b** pYD1/SmchiC. *Lane M* DNA marker; *lane 1* pYD1 digested by *Xho*; *lane 2* pYD1/SmchiC digested by *Xho*; *lane 3* pYD1/SmchiC digested by *BamH I* and *Xho I*

Confirmation of active chitinase displayed on yeast surface

After the yeast cells were induced with galactose, the presence of Aga2p-SmChiC on the cell surface of *S. cerevisiae* was verified by indirect immunofluorescence (Fig. 3).

The red fluorescent signal can be clearly observed in induced *S. cerevisiae* EBY100/pYD1-SmchiC cells with Aga2p-SmChiC fusion protein anchoring on cell surface, and fluorescence on the cell surface was significantly diminished after fusion protein released by DTT-treatment, while fluorescent labeling was negligible for the *S. cerevisiae* EBY100 cells (only Aga1p was expressed). Western blot analysis of partially purified product showed the molecular weight of Aga2p-SmChiC fusion protein meets our expectation of approximately 67 kDa (Fig. 4).

The chitinolytic activity of the expression product was detected by agar plate clear zone test and SDS-PAGE zymography. After the two clones had grown on water-soluble chitin agar plates for 48 h, the *S. cerevisiae* EBY100/pYD1-SmchiC strains could form a larger clear zone (Fig. 5a). Furthermore, the results from SDS-PAGE zymography confirmed that DTT-released chitinase of EBY100/pYD1-SmchiC strains formed a chitin degradation zone on SDS-PAGE gel (Fig. 5b). These results indicated that *S. marcescens* ChitinaseC was successfully displayed on *S. cerevisiae* cell surface.

Effect of temperature and pH on enzyme activity and stability

Our results demonstrated that both whole cell chitinase and DTT-released chitinase hold chitinolytic activity in a wide temperature range, from 25 °C to 80 °C (Fig. 6a, b). The optimum reaction temperature was determined to be 52 °C by a further test in a narrower temperature range, 44–66 °C, as shown in Fig. 6c, d. The effect of pH on enzyme activity of

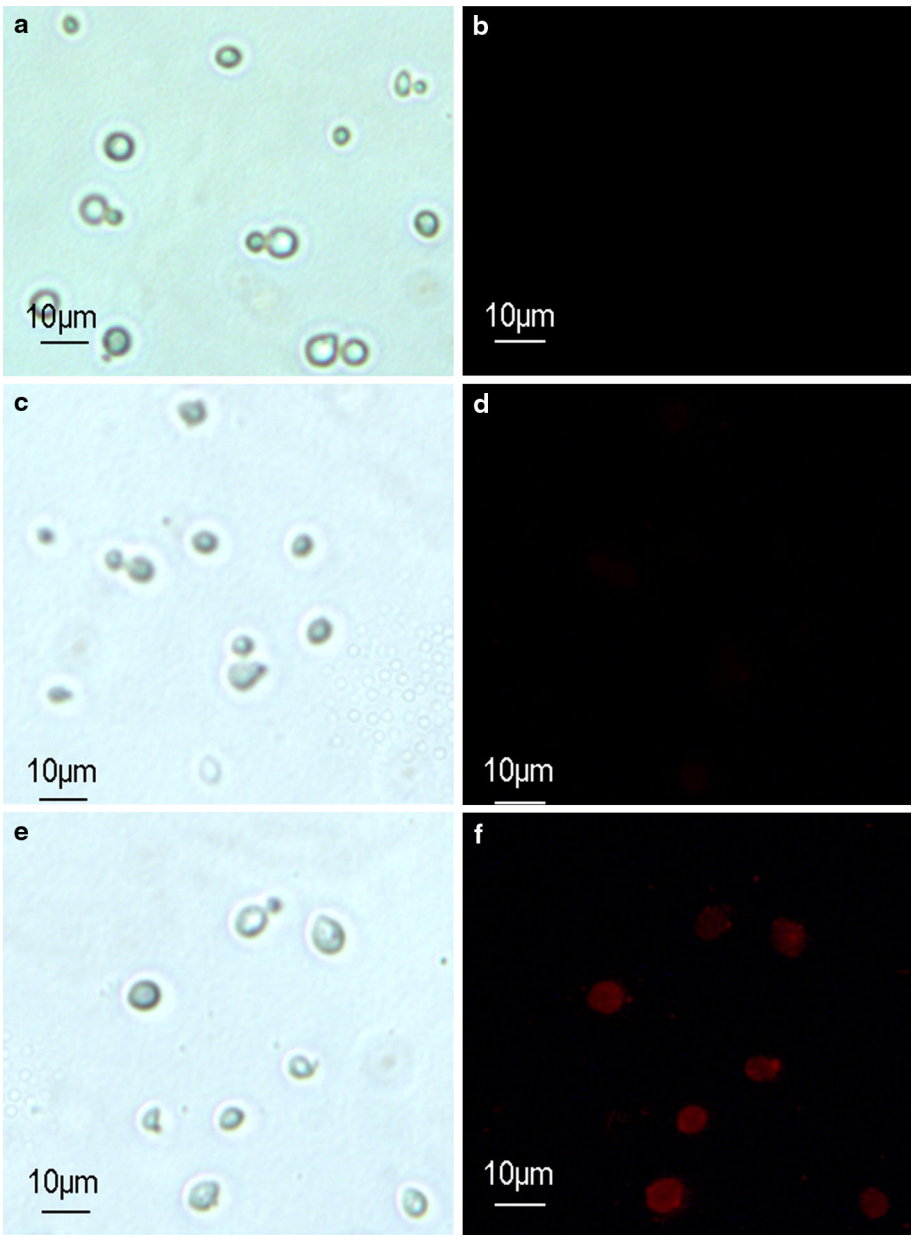

Fig. 3 Immunofluorescence labeling of recombinant enzyme displyed on the yeast cell surface. **a**, **c**, **e** Bright-field micrographs. **b**, **d**, **f** Immunofluorescence micrographs. **a**, **b** Control cells, *S. cerevisiae* EBY100. **c**, **d** Induced *S. cerevisiae* EBY100/pYD1-SmchiC cells treated by DTT. **e**, **f** Induced *S. cerevisiae* EBY100/pYD1-SmchiC cells

whole cell chitinase and DTT-released chitinase was examined at pH values ranging from 2.2 to 8 at 52 °C. The whole cell chitinase and DTT-released chitinase had some chitinolytic activity between pH 2.2–8.0 while the optimum pH value was 5.0, as shown in Fig. 6e, f. Tests on thermal stability showed that whole cell chitinase and DTT-released chitinase retained some chitinolytic activity after heat treatment, for example after being boiled for 1 h. However, the thermal stability of whole cell chitinase was slightly higher than DTT-released chitinase (Fig. 6g).

The results from zymography also confirmed that DTT-released chitinase of EBY100/pYD1-SmchiC strains formed a chitin degradation zone on a SDS-PAGE gel after being treated at different temperatures. Figure 7 shows that the partially purified recombinant SmChiC is a thermostable enzyme.

Discussion

Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin and may be used to convert chitin into bioactive chitin oligosaccharides, which have a variety of potential applications. Many researchers are dedicated to isolating and screening potential chitinolytic microorganisms. In another approach, a number of

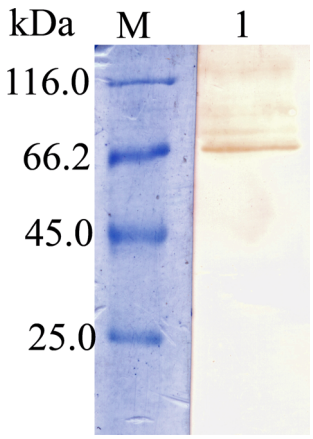

Fig. 4 Western-blot analysis of the Aga2p-SmChiC fusion protein displayed on the cell surface. *lane M* Protein Ladder; *lane 1* Aga2p-SmChiC fusion protein

heterologous chitinase genes have been recombinant expressed in bacterial or yeast systems with the aim of increasing production and expanding biotechnological applications (Suzuki et al. 2002; Dahiya et al. 2006; Horn et al. 2006; Synstad et al. 2008; Gutierrez-Roman et al. 2014). Some conventional recombinant technologies have been used to prepare enzyme, but they are limited to use in industry due to the requirement for cumbersome downstream processes, such as extraction and purification. However, the preparation and use of yeast whole-cell biocatalyst can avoid the tedious procedures such as extraction, concentration, coating and adsorption (Kuroda and Ueda. 2011; Shigemori et al. 2013). In this study, yeast cells with recombinant chitinase anchored on their cell surfaces possess chitinolytic activity, whereas control cells hardly degrade chitin. It has been demonstrated that the yeast whole-cell chitinolytic enzyme was produced by anchoring the recombinant ChitinaseC on the cell surface. The yeast whole-cell chitinolytic enzyme has a great deal of potential use in the medical and food industries, such as production of chitin oligosaccharides or bioactive compounds from shrimp and crab processing waste.

Thermal stability is a critical factor for enzymes to be utilized successfully in industry. In commercial applications, thermostable enzymes have some advantages over mesophilic enzymes (Littlechild et al. 2007) such as higher levels of activity, greater stability, and faster reaction rates at high temperature. Many thermostable enzymes can be produced by genetic engineering to meet demand from industries. In this study, yeast whole-cell biocatalyst displayed good chitinolytic activities within 25–80 °C, and the optimum reaction temperature was found to be 52 °C. Most interesting is the enzyme's ability to retain some activity even after boiling for 1 h, moreover, the thermal stability of yeast whole-cell biocatalyst was higher than partially purified enzymes. The results from both thermal activity and stability tests showed that the yeast whole-cell biocatalyst can act as a good thermostable enzyme. The bioconversion efficiency of chitin to chitin oligosaccharide could be promoted by thermophilic fermentation, where thermal activity and stability at high temperature are a great benefit in industrial applications (Sutrisno et al. 2004; Songsiriritthigul et al. 2010). Furthermore, thermostable enzymes are resistant to organic solvents, detergents, low and high pH, and other denaturing agents in terms of thermal activity and stability (Songsiriritthigul et al. 2010). In this work, the yeast whole-cell biocatalyst could maintain a certain level of chitinolytic activity under acidic or weakly alkaline conditions (pH 2.2–8) too. The unique enzymatic properties of the yeast whole-cell biocatalyst,

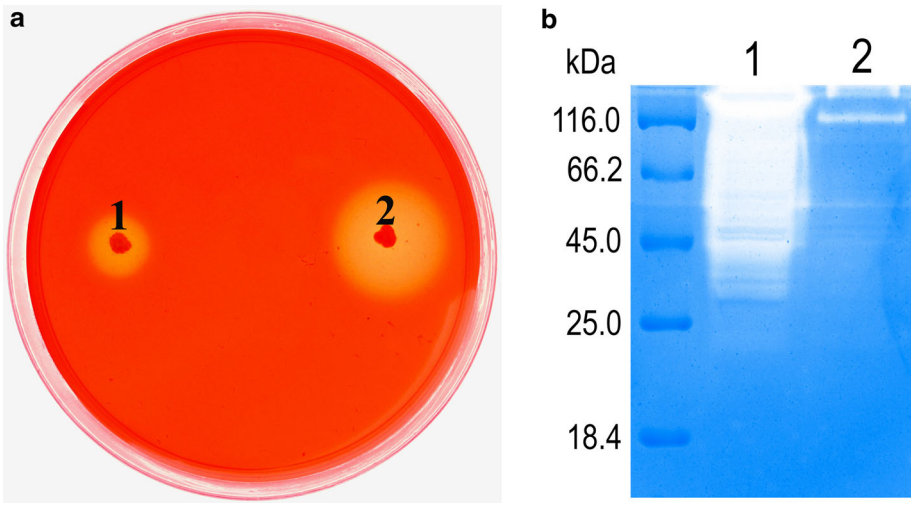

Fig. 5 Qualitative detection of chitinolytic activity. **a** Chitinolytic activity was detected by agar plate assay. *1*, EBY100/pYD1 strain; *2*, EBY100/pYD1-SmchiC strain. **b** Chitinolytic activity assay using SDS-PAGE zymography. *lane M* Protein molecular weight marker; *lane 1* DTT-released chitinase of EBY100/pYD1-SmchiC cells; *lane 2* DTT-treated mixture of EBY100/pYD1 cells

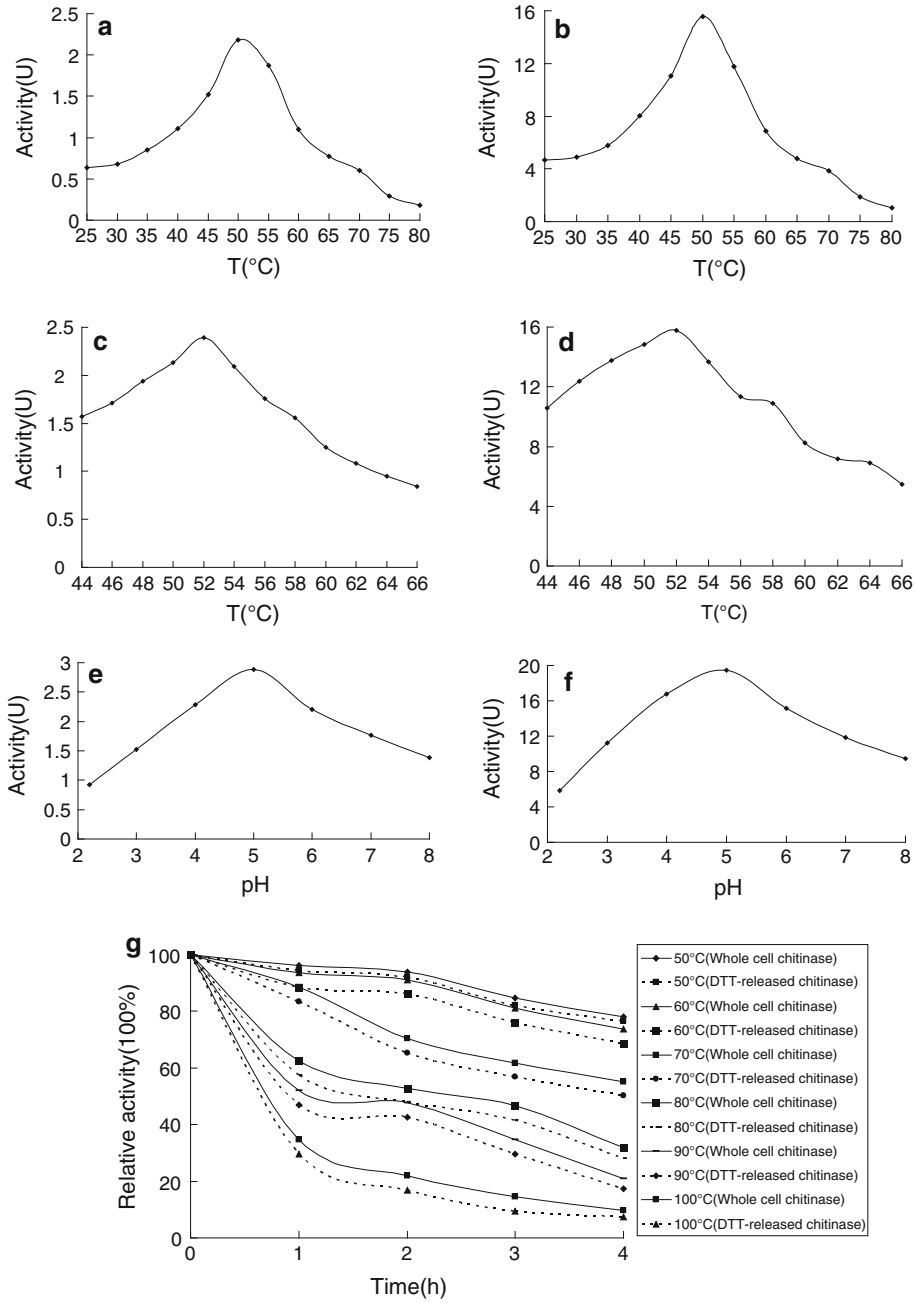

Fig. 6 Effect of temperature and pH on activity and stability of whole cell chitinase and DTT-released chitinase. **a** Effect of reaction temperature on the activity of whole cell chitinase (25–80 °C). **b** Effect of reaction temperature on the activity of DTT-released chitinase (25–80 °C). **c** Effect of reaction temperature on the activity of whole cell chitinase within a narrower temperature range (44–66 °C). **d** Effect of reaction temperature on the activity of DTT-released chitinase within a narrower temperature range (44–66 °C). **e** Effect of reaction pH on the activity of whole cell chitinase. **f** Effect of reaction pH on the activity of DTT-released chitinase. **g** Thermal stability of whole cell chitinase and DTT-released chitinase at different temperatures

such as high-temperature resistance and acid-resistance, offer great advantages in the bioconversion of chitin and other applications.

In conclusion, we succeeded in displaying thermostable chitinase from *S. marcescens* on the cell surface of *S. cerevisiae*. These chitinase-displaying yeast strains offer the possibility of producing large amounts of whole-cell chitinolytic enzyme rapidly and easily, which should be suitable for utilization of chitin and production of bioactive chitin oligosaccharides for medical and pharmaceutical usage.

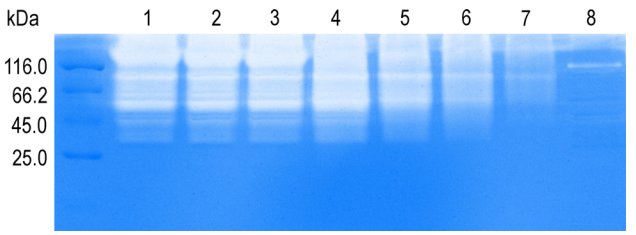

Fig. 7 Thermal stability of DTT-released chitinase at different temperatures (SDS-PAGE zymography). *Lane M* Protein molecular weight marker; *lanes 1–7* DTT-released chitinase of EBY100/pYD1-SmchiC cells treated at 37, 50, 60, 70, 80, 90, and 100 °C for 60 min, *lane 8* DTT-treated mixture of EBY100/pYD1 cells

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