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Enhancement of Tannase Production on Submerged Fermentation by Red Yeast *Rhodotorula glutinis* DB2

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Present work deals with optimize the cultural condition and medium composition for enhancing the tannase production by *Rhodotorula glutinis* DB2 in submerged fermentation. A tannase-producing read yeast strain *R. glutinis* DB2 was isolated from hydrolysable tannin solution (extracted from *Rhizophora apiculate* bark) that kept for a year at room temperature (30±2°C). This yeast strain showed ability to produced extracellular tannase significantly under submerged fermentation using tannic acid as an inducer. The rapid degradation of tannic acid and production of extracellular tannase was observed in the submerged fermentation medium. *R. glutinis* DB2 produced maximum extracellular tannase activity at the optimum bioprocess condition with 0.75 U/ mL and the yeast growth of 8.7 g/L. These results indicate that an increment about 83% of tannase production was obtained compared to before optimization of cultural condition (0.41 U/ mL).

Key words: Tannase; *Rhodotorula glutinis*; submerged fermentation; yeast.

Tannin acyl hydrolase (EC 3.1.1.20) or commonly known as tannase is an extracellular, inducible enzyme that catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid to release glucose and gallic acid[1]. Hydrolysable tannins are present in most of the residues from higher plants and are polyphenolic compounds formed by the association of sugar, gallic acid, and ellagic acid through ester linkages. Tannic acid is a polyphenolic mixture of polyhydric alcohol's that can easily bind with any protein[2].

Tannase is an industrially important microbial enzyme. The enzyme is found to be useful in the manufacture of instant tea, coffee-flavoured soft drinks, clarification of beer and fruit juices[3]. Nevertheless, the current most of the commercial application of tannase is in the manufacturing of instant tea, where it is used to eliminate the water-insoluble precipitates[2]. Industrial production of gallic acid from tannic acid is performed with tannase[4]. Gallic acid, a hydrolytic product of tannin, has different uses like preparation of trimethoprim, pyrogallol, propyl gallate, dyes, *etc*[5].

Even though tannases can be extracted from plants and animals but microbial tannases are sought after because of their stability. Among the various sources of tannase production, filamentous fungi such as *Aspergillus*[6], *Trichoderma*[4], *Fusarium*[7] strains were studied extensively. Besides that, bacterial sources and lactic acid bacteria were also reported to produce tannases. However, tannase producing yeast was scarcely reported except the study done by Jacob and Pignal[8] who reported the production of tannase by six strains of yeast included *Pichia pseudopolymorpha*, *P. strasburgensis*, *P. adzetti*, *P. monospora*, *Debaeyomyces hansenii*, and *Candida nitrotivorans*. Later, Aoki *et al*.,[9] reported

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the isolation and purification as well as characterization of *Candida* sp. K1. Dechamps *et al*.,[11] then reported the tannase production by *Pichia* sp. and *Debaromyces hansenii*. There is limited yeast isolate that is capable in producing tannase ever reported.

Therefore, searching for new sources of tannases with novel biotechnological properties is very attractive, since alternative producers may be found for future or new applications. In addition, the potential of yeast biodiversity for tannases production is yet poorly known. Due to this respect, in this communication we would like to report the tannase production by a local yeast isolate *Rhodotorula glutinis* DB2 which could be a potential tannase producer. This is probably the first report of red yeast *R. glutinis* capable of producing tannase.

MATERIALS AND METHODS

Microorganisms and culture maintenance

The *R. glutinis* IBRL DB2 was isolated from a one-year old hydrolysable tannin solution extracted from the bark of mangrove plant (*Rhizophora apiculata*) that was kept at 30±2ºC. The culture was grown and maintained on a modified medium slants[9] (Aoki *et al*., 1976) containing (w/v): tannic acid, 1%; K_2HPO_4, 0.3%; $MgSO_4$, 0.05%; KH_2PO_4, 0.3%: monosodium glutamate, 1% and agar, 3% (the pH was adjusted to 6.0 by 1 M NaOH solution) for 48 hours at 37°C aerobically, before storing them at 4°C until further use. The subculturing was performed every 3 weeks.

Inoculums preparation

The inoculums was prepared by adding 5.0 mL of sterile distilled water to the culture agar slants and shaken vigorously. The cell suspension was adjusted spectrophotometrically to the absorbance equivalent to 1.0 at 640 nm (equivalent to 1 x 10^8 cells/ mL or 0.3 g dry weight cells/L) and used as the inoculums.

Medium composition and cultivation conditions

The cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of cultivation medium which consisted of (g/L): 8.0 Urea; 3.0 KH_2PO4; 0.5 $MgSO_4 \cdot 7H_2O$; 10 monosodium glutamate; 15 tannic acid; 3.0 lactose and in µg/L: 500 H_3BO_3; 40 $CuSO_4 \cdot 5H_2O$; 100 KI; 200 $FeCl_3 \cdot 6H_2O$; 400 $MnSO_4 \cdot 4H_2O$; 200 $Na_2MoO_4 \cdot 2H_2O$ and 400 $ZnSO_4 \cdot 7H_2O$. The initial pH of the medium was adjusted to pH 6.0 before autoclaving. The inoculated flasks were incubated at 30±2°C and shaken at 150 rpm for 6 consecutive days. The tannase production, growth of *R. glutinis*, pH, reducing sugars and residual tannic acid concentration was determined after 4 days of cultivation. The growth of yeast was monitored by measuring the dry weight cells (mg/mL).

Optimization of fermentation process for enzyme production

Various process parameters influencing enzyme production during submerged fermentation were optimized. The strategy followed was to optimize each parameter (tannase activity and biomass), independent of the others and subsequently optimal conditions were employed in all experiments.

Effect of incubation period and incubation temperature

Fermentation was carried out at various temperatures such as 22, 25, 30, and 35ºC. Samples were withdrawn after 4 days of cultivation and analyzed for tannase activity and biomass production.

Effect of agitation

Agitation speed in the range of 0, 100, 150, 200 and 250 rpm were studied in order to maximize the yield of tannase.

Concentration of tannic acid in medium

Various concentrations of tannic acid (0.25, 0.50, 1.0, 0.8, 1.5, 2.0 and 2.5 %, w/v) were used to enhance the production of tannase.

Effect of sugar additives

To study the effect of carbon source on enzyme production, simple and complex carbon source like glucose, xylose, lactose, sucrose, maltose, cellulose, starch, mannitol and sorbitol (0.3%, w/v) were incorporated in the cultivation medium.

Effect of nitrogen source

Various nitrogen sources such as sodium nitrate, ammonium nitrate, sodium nitrate, urea, aspartic acid, monosodium glutamate, ammonium sulphate, ammonium chloride and potassium nitrate (%, w/v) were incorporated in the cultivation medium.

Crude enzyme extraction

The culture broth was filtered through a

Buchner funnel containing a pre-weighted filter paper (Whatman No. 1) to separate the cells. The cell-free culture filtrate containing the crude enzyme was then assayed for extracellular tannase activity.

Fungal growth determination

The growth was estimated on the basis of dry weight (g/L) of cell biomass. The retained biomass was then washed with distilled water to remove any remaining substrate and then both the cell biomass and the filter paper were dried to a constant weight at 65°C. Subsequently, they were re-weighted and the weight of the dry biomass was calculated by subtracting the weight of the filter paper. The growth was then expressed as gram of dry weight per liter culture.

Tannase activity assay

Extracellular tannase activity was assayed according to the procedure described by Iibuchi *et al*.[10] (1967). Enzyme solution (0.5 ml) was added to 2.0 mL of 0.35% (w/v) tannic acid in 0.05 M citrate buffer (pH 6.0) in a test tube. The substrate solution was pre-incubated at 40ºC for 5 minutes before the enzyme solution was added. Reaction mixture (0.05 mL) was withdrawn and added into 5 mL of 95% ethanol to stop the enzyme reaction after 10 minutes of incubation. The absorbance at 310 nm was read immediately (t_1). After 40 minutes of incubation (t_2), 0.05 ml of the reaction mixture was again withdrawn and the enzyme reaction was stopped by adding 5 mL of 95% ethanol. The absorbance was again read at 310 nm. The difference between the absorbencies at t_1 and t_2 were determined. One unit of tannase activity was defined as the amount of enzyme which was able to hydrolyse one micromole of ester bond per minute per mL of fermentation broth under standard assay conditions. Each sample was tested at least in triplicate and the results were represented as an average value with standard deviation.

Estimation of tannic acid degradation

The remaining tannic acid content in the fermented broth was estimated by the methods described by Hagerman and Butler[11]. The residual tannic acid was derived from a standard curve. Cell free cultivation broth (1.0 mL) was added into 2.0 mL of bovine serum albumin solution (1 mg/mL in 0.05 M acetate buffer, pH 5.0, and containing 0.17 M sodium chloride) in a 15 mL centrifuge tube. The mixture was mixed and allowed to stand at 4ºC for 15 minutes and was then centrifuged for 15 minutes at 5000 rpm. The supernatant was discarded and the surface of the pellet and the walls of the tubes were washed with acetate buffer without disturbing the pellet. The precipitate was dissolved in 4 mL of SDS-TEA solution (1% SDS, w/v and 5% TEA, v/v). One millilitre of the ferric chloride reagent (0.01 M $FeCl_3$ in 0.01 M HCI) was added, and the solution was mixed immediately. Thirty minutes after the addition of the ferric chloride, the absorbance at 510 nm was read. Each sample was tested at least in triplicate and the results were represented as an average value with standard deviation.

Estimation of reducing sugar content

Reducing sugar content was assayed by the method described by Miller[12] using dinitrosalicylic acid (DNS) reagent containing (w/v): DNS, 1 %; NaOH, 1 %; sodium potassium tartrate, 30 %. The reagent was stored in an amber bottle at 4ºC. The total reducing sugar content was derived from a standard curve using glucose as standard and expressed as percentage of initial concentration. Cell free cultivation broth (3 mL) was added into 3 mL of dinitrosalicylic acid reagent. The mixture was vortexed and heated for 5 minutes in a boiling water bath. The reaction mixture was then cooled down under running tap water to ambient temperature. The colour intensity was measured at 575 nm. Each sample was tested in triplicate and the results were represented as an average value with standard deviation.

RESULTS AND DISCUSSION

Effects of cultivation temperature

The effect of temperature on the growth and tannase production by *R. glutinis* DB2 is shown in Figure 1. Tannase production was maximal at 25°C of about 0.41 U/mL of tannase activity. The growth was also achieved its maximal value at 25°C of 6.10 g/L. Tannase activity decreased when the temperature increased. There were a decreased of about 51% when cultivated at 30°C and about 76% when cultivated at 35°C. However, the temperature below 25°C also produced lower tannase activity compared to 25°C. The growth temperature conditions described for *R. glutinis* for extracellular polysaccharide production was 22°C [13] and at 28°C [14] for arabinofuranosidase production.

Normally yeasts need lower optimal

temperature for growth compared to filamentous fungi such as *A. niger* which had optimal temperature of 30ºC [15]. Yamada *et al.*[16] reported an optimum of 30 ºC for tannase production by *A. flavus* and Rajakumar and Nandy[17] found 28 ºC as the optimum temperature for tannase production by *Penicillum chrysogenum* NCIM 722. The higher temperature range is preferred for the industrially useful enzymes. But in the case of tannase enzyme the activity did not increase continuously along with the temperature nature. When the temperature increases, the kinetic energy of the substrate and enzyme molecules also increase which affects the reaction rate. The number of collisions per unit time of tannase activity and its substrate, tannic acid increases, resulting in a higher activity with the continuous increases in the temperature level. When the optimum level of temperature obtained, the energy of the molecules increased thorough out the process, but the chemical potential energy increases enough, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation of the tannase protein causing its inactivation. Thus, an increase in temperature beyond the optimum value caused reduces in the catalytic rate of tannase as either the enzyme or substrate became denatured and inactive. Here the temperatures above the optimum (25ºC) level also affect the protein active state, which resulted in a reduction in enzyme activity. Therefore, 25°C was selected for subsequent experiments.

Agitation

Like all aerobic microorganisms, oxygen is required as the final electron receptor in the respiratory chain. In the liquid medium, the availability of dissolve oxygen can be varied by changing the agitation speed in the cultivation

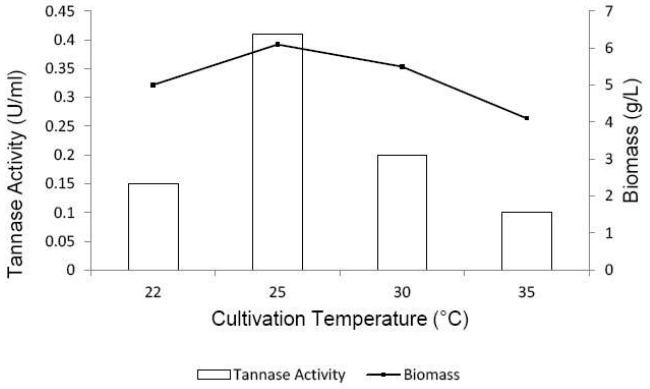

Fig. 1. Effect of cultivation temperature on tannase and biomass production by *R. glutinis* DB2

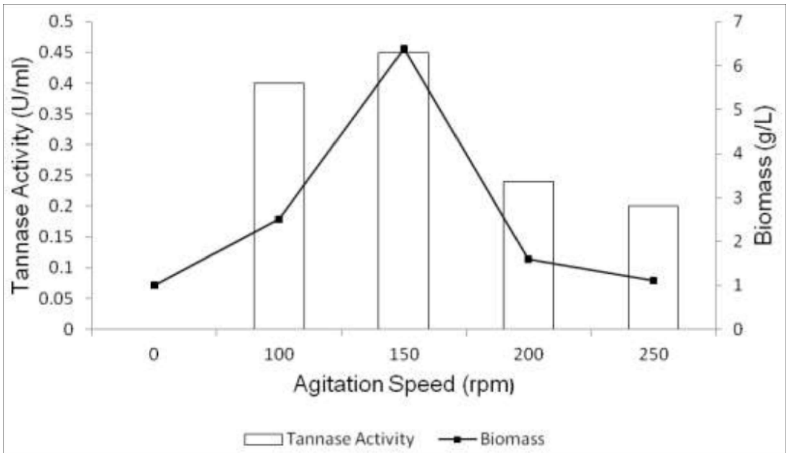

Fig. 2. Effect of agitation speed on tannase and biomass production by *R. glutinis* DB2

system. Figure 2 shows the tannase production and growth by *R. glutinis* DB2 and the results showed that a maximum tannase production was obtained at the agitation speed of 150 rpm with about 0.44 U/mL. The results also showed that tannase production increased gradually as the agitation speed increased until achieved its maximum production at the agitation speed of 150 rpm, and dropped after that. The tannase production decreased as the agitation speed was more than 150 rpm. Agitation speed of 200 and 250 rpm, produced about 0.23 and 0.20 U/mL,

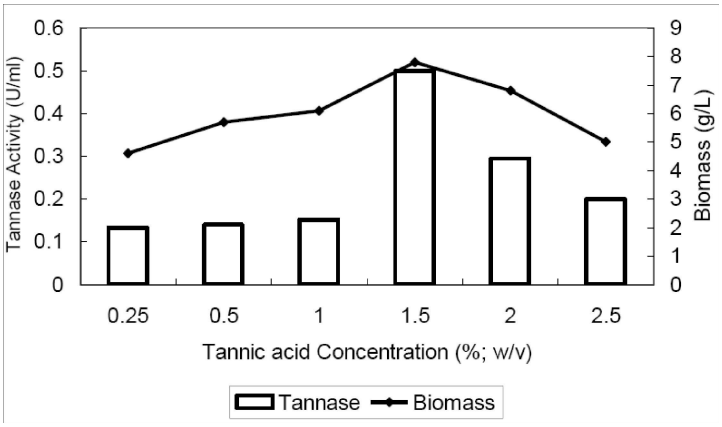

Fig. 3. Effect of tannic acid concentration on tannase and biomass production by *R. glutinis* DB2

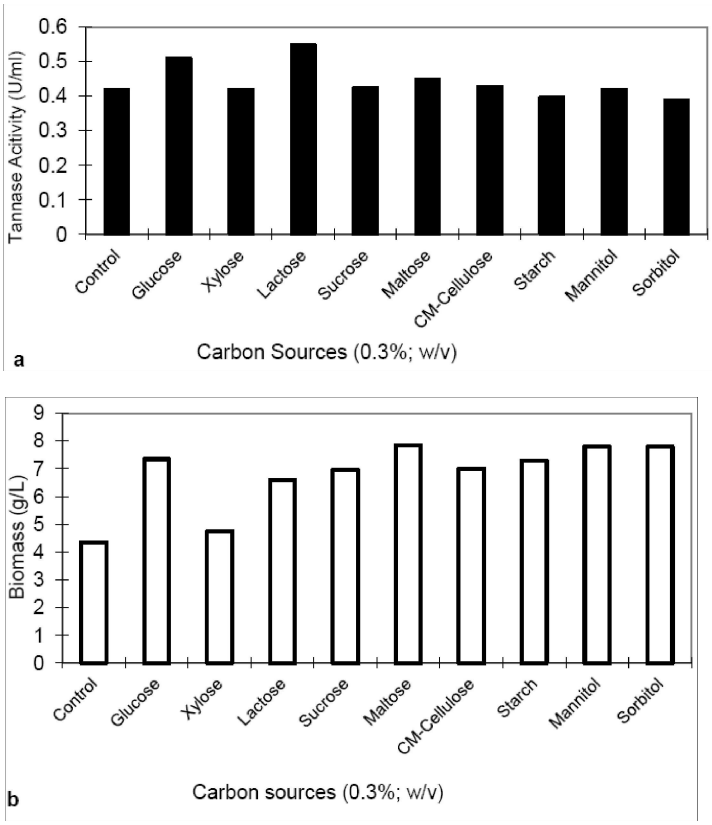

Fig. 4. Effect of various sugar additives (0.3%, w/v) on tannase and biomass production by *R. glutinis* DB2. (A) tannase production and (B) biomass

respectively. The growth of the *R. glutinis* DB2 also achieved its maximum value at the agitation speed of 150 rpm. Lower or higher agitation speed than 150 rpm produced lower growth of *R. glutinis* DB2. Therefore, agitation speed of 150 rpm was chosen for the subsequent experiments.

It is well documented that the agitation speed influence the formation of pellet for filamentous fungi and homogenous turbidity growth for unicellular cells. Moreover, the formations of the products are depending on morphological structure of the macroscopic growth of the microorganisms[18]. *R. glutinis* is yeast, hence the optimal agitation speed will make them grow in a homogenous form with high turbidity. Furthermore, the optimal agitation speed also involved in good mixing, mass and heat transfer that require in the submerged fermentation system.

Effect of substrate (tannic acid) concentration

Tannase is an inducible enzyme, and tannic acid as an inducer for tannase production play an important role in this matter. Therefore, the optimal tannic acid concentration has to be studied before proceed with the subsequent experiments. Furthermore, tannic acid also can act as a sole carbon source for the cultivation Various concentration of tannic acid as substrate were studied (0.25%, 0.50%, 1.00%, 1.50%, 2.00% and 2.50%) keeping other parameters constant at temperature 25°C, pH 6.0 and agitation speed of

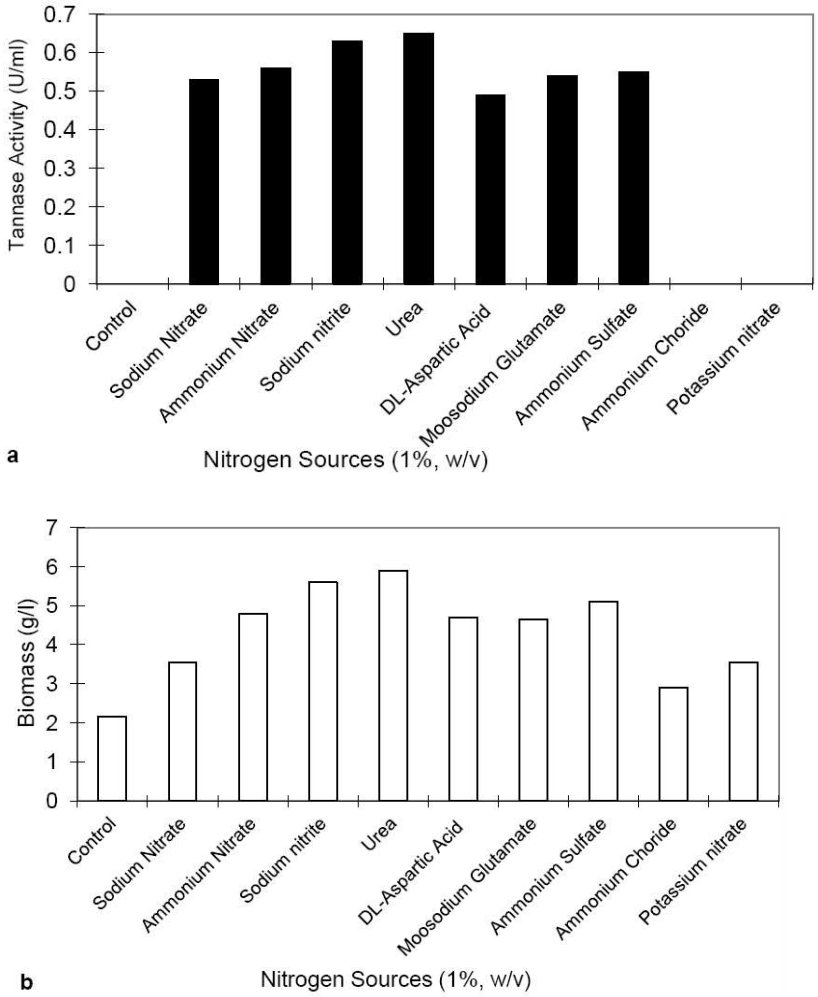

Fig. 5. Effect of various nitrogen sources (1.0%, w/v) on tannase and biomass production by *R. glutinis* DB2. (A) tannase production and (B) biomass

150 rpm. Figure 3 shows tannase production and growth by *R. glutinis* DB2 increased as the concentration of tannic acid increased until achieved its maximal production of about 0.50 U/ml at the concentration of 1.5% (w/v). However, the enzyme production decreased when the tannic acid concentration was higher than 1.5% (w/v). The growth of *R. glutinis* DB2 also increased and achieved its maximal growth production at the tannic acid concentration of 1.5% (w/v). Higher and lower than 1.5% (w/v) of tannic acid resulted in low tannase production.

Mohapatra *et al.*[3] have reported maximum tannase production by *Bacillus licheniformis* KBR6 in liquid submerged fermentation containing 1% tannic acid. Tannase from fungal strains were found to be maximum in the medium containing higher concentration of tannic acid[19, 20]. However, at higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation as tannic acid at higher concentration produces complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited[20]. Ayed and Hamdi[21] reported that increase in substrate concentration induced an increase in tannase activity followed by a decrease because the enzyme synthesis. Similarly, Selwal *et al.*[22] observed an inhibitory effect due to higher concentration of tannic acid in the tannase synthesis by a bacterium *Pseudomonas aeruginosa* IIIB 8914.

Effect of sugar additives

Various carbon sources which can be divided into four groups: monosaccharides (glucose and xylose), disaccharides (lactose, maltose, and sucrose), polysaccharides (carboxymethyl cellulose and soluble starch) and sugar alcohol (mannitol and sorbitol) were added at a concentration of 0.3% (w/v) to the medium containing 1.5% (w/v) tannic acid [Fig. 4] as sugar additives. It was observed that the supplementation of additional sugar additives (glucose, galactose, mannose, sucrose, lactose and fructose) slightly increased the enzyme production [Fig. 4a] compared to control (only tannic acid without the addition of carbon sources) which was 0.42 U/mL. However, the addition of lactose showed the highest tannase activity of 0.55 U/mL.

The addition of carbohydrate as sugar additives also increased the cell biomass [Fig. 4b] compared to control, and the highest growth was obtained in the cultivation medium with 0.3% (w/v) maltose. The results of this experiment showed that tannase production by *R. glutinis* DB2 was not growth dependent. Therefore, lactose was chosen to be used in the subsequent experiments, because its addition to the medium enhanced the tannase activity.

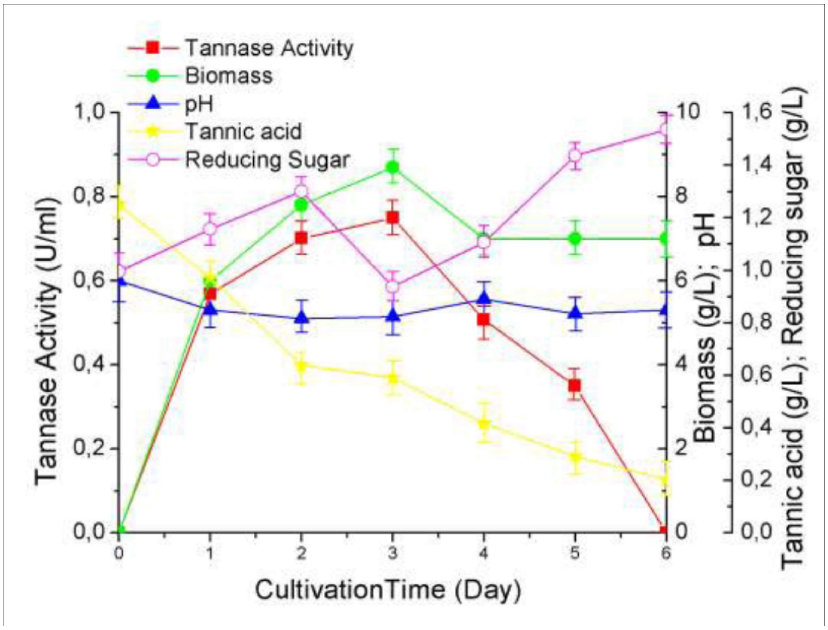

Fig. 6. Time course profiles after optimization of cultural conditions and medium compositions

Available reports on the role of carbon sources or in this study we called it sugar additive, on the extracellular secretion of tannase are contradictory. Repression of tannase activity by glucose, sucrose, lactose etc. was also reported by Sabu *et al*.,[23] and Kumar *et al*.,[24]. Lokeswari and Raju[25] found that glucose at higher concentration repressed tannase synthesis while the lower concentration was not repressive. This may be due to the fact that high concentrations of additional carbon sources such as glucose changes the carbon/nitrogen ratio as well as creates an osmotic stress, which depresses the enzyme synthesis by microorganisms[26]. However, Battestin and Macedo[27] and Bradoo *et al*.,[28] have reported that external carbon sources did not affect the tannase production. Van de Lagemaat and Pyle[29] reported that the glucose if present in the media will be exhausted rapidly and this may lead to the partial induction of tannase.

Effect of nitrogen source

Fig. 5 shows the effect of seven different nitrogen sources, consisting of sodium nitrate, ammonium nitrate, sodium nitrite, monosodium glutamate, ammonium sulphate, ammonium chloride, potassium nitrate, and urea. The results showed that the addition of nitrogen sources enhanced the production of tannase by *R. glutinis* DB2. There was no tannase activity detected in the cultivation medium without the addition of any nitrogen source (a control medium). It was found that the addition of urea (an organic nitrogen source) into the cultivation medium produced maximum tannase activity of about 0.65 U/mL [Fig. 5a].

Even though, *R. glutinis* DB2 still able to grow in the control medium (without the addition of any nitrogen source), the addition of various nitrogen sources did increase the growth of *R. glutinis* DB2, and the highest biomass production obtained when urea was added into the medium [Fig. 5b] of about 5.90 g/L. Ammonium chloride, urea, creatinin and ammonium nitrate were comparatively less significant for tannase production, presumably because of the release of ammonium ions. Tannic acid by itself could produce only a moderate increase in tannase production whereas by interacting with $NaNO_3$, the tannase activity could be increased significantly[30]. Bradoo *et al*.[28] also reported $NaNO_3$ as the preferred nitrogen source for both growth and tannase production by *Aspergillus japonicus*. Paranthaman *et al*.,[20] observed maximum tannase production by *Aspergillus flavus* in the medium containig $NaNO_3$. Different concentrations (0.5–5.0 g/L) of sodium nitrate were used in the production medium. Optimum concentration of sodium nitrate for the production of tannase and gallic acid was 2.0 g/L. Higher concentrations of sodium nitrate in the fermentation medium did not significantly increase enzyme and gallic acid yield.

Time course profiles of tannase production by R. glutinis DB2 after optimization of parameters

Using all of the optimised physical parameters (cultural conditions) and medium compositions, the time course profiles of tannase production and growth of *R. glutinis* DB2 were determined. The utilisation of tannic acid and the concentration of reducing sugar, as well as pH of the cultivation medium also determined. The optimised medium consisted of (g/L): 8.0 Urea; 3.0 KH_2PO4; 0.5 $MgSO_4.7H_2O$; 10 monosodium glutamate; 15 tannic acid; 3.0 lactose and in mg/l: 500 H_3BO_3; 40 $CuSO_4 \cdot 5H_2O$; 100 KI; 200 $FeCl_3 \cdot 6H_2O$; 400 $MnSO_4 \cdot 4H_2O$; 200 $Na_2MoO_4 \cdot 2H_2O$ and 400 $ZnSO_4 \cdot 7H_2O$. The initial pH of the medium was adjusted to pH 6.0 before autoclaving. About 100 ml of the cultivation medium was used in a 250 ml Erlenmeyer flask with inoculum size of 4% (w/v) of 1×10^8 cells/mL. The inoculated flasks were incubated at room temperature (25±2°C) and shaken at 150 rpm for 6 consecutive days. The tannase production, growth of *R. glutinis*, pH, reducing sugars and residual tannic acid concentration were determined at every 24 hour intervals.

As shown in Figure 6, the tannase production was only observed after 24 hour of incubation (day 1). The production increased gradually and a maximal tannase production was obtained on day 3 of cultivation with about 0.75 U/ml. The tannase production was dropped drastically after achieving its maximal production. The growth of *R. glutinis* DB2 also increased rapidly until achieved it maximal value of about 8.70 g/l on day 3 of cultivation. The growth was dropped slightly after that and consistently produced from day 4 onward. The results showed that the tannase production occurred during the stationary phase of growth.

The residual tannic acid in the cultivation medium was also determined. The results showed that the amount of tannic acid decreased as the time of cultivation prolonged, and only 0.28 g/L tannic acid was detected on day 5 of cultivation. The result showed a correlation between tannic acid concentration in the medium and tannase production. As the tannase production increased, the concentration of tannic acid in the medium decreased. In fact on the day 3 of cultivation, where tannase production was maximum, the concentration of tannic acid in the cultivation medium was about 0.6 g/L only. The tannase activity dropped when the concentration of tannic acid in the cultivation medium became low. The results confirmed that tannase is an inducible enzyme and its production induces by the presence of tannic acid in the cultivation medium.

The concentration of the reducing sugar seemed to increase in the cultivation medium. At the early phase of growth, the concentration of reducing sugar seemed to be reciprocate with tannic acid, where the concentration of tannic acid decreased and the concentration of reducing sugar increased until day 2 of cultivation (1.3 g/L reducing sugar). Then its level dropped slowly. Surprisingly, the level of reducing sugar increased again after day 3 of cultivation (0.97 g/L), even though the level of tannic acid kept on decreasing. The pH of the cultivation medium decreased slightly until day 2 of cultivation, and started to increase slightly until day 4 of cultivation with about pH 5.7. The results showed some correlations between tannase production, tannic acid concentration, reducing sugar concentration and pH of the medium. The production of tannase increased and depended on the concentration of tannic acid in the cultivation medium. The utilisation of tannic acid usually produced some reducing sugars as a by-product. Therefore, the concentration of reducing sugar increased slightly as the concentration of tannic acid decreased. The pH of the cultivation medium was depended on the reducing sugar level and remained between 5.1 to 5.7 throughout the cultivation.

The results after optimisation were compared with cultivation system before optimisation in a shake flask system. The increment in tannase production of about 83% obtained after optimisation of both physical parameters and medium compositions (0.75 U/ml) compared to before optimisation (0.41 U/ml).

CONCLUSION

This is the first report of yeast tannase production by *R. glutinis* which produced significant tannase activity. From the results obtained the best favourable conditions for the production of tannase by *R. glutinis* DB2 (0.75 U/mL on day three of cultivation) were temperature of 25ºC, initial pH of medium 6.0, agitation speed of 150 rpm, inoculums size of 4% ($1x10^8$ cells/mL), 1.5% of tannic acid which acted as carbon an inducer sources, 0.3% of lactose as a sugar additive and 5.0% urea as a nitrogen source.

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