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## Optimisation of Cultural Conditions and Some Properties of Radical Scavenging Substance from *Sporobolomyces salmonicolor*

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**Abstract:** *Sporobolomyces salmonicolor* produced high activity ( $101 \text{ u mL}^{-1}$ ) in the stationary culture while cultivated in the half YPD (H-YPD) medium. This activity production was maximised while optimising cultural condition. The effects of nutrients including several carbon and nitrogen sources were assayed along with medium pH and cultivation temperature. The optimised medium consisted of 0.5% yeast extract, 1% polypepton and 1% fructose. A maximum radical scavenging activity ( $176 \text{ u mL}^{-1}$ ) was obtained when cells were cultivated with this medium in the stationary condition for 9 days at  $25^{\circ}\text{C}$  with an initial pH of 6.0. This was 1.7-fold increase of initial production from H-YPD. The active substances were partially purified by means of ion exchange chromatography and gel filtration. Two active substances were isolated from gel filtration, namely, DG I and DG II. The molecular mass of DG I is expected to be high. The activity was stable between pH 6.0-7.0 and until  $60^{\circ}\text{C}$  for 30 min incubation. On the contrary, the molecular mass of the DG II was estimated to be 281 m/z. This substance was stable in the acidic condition (pH 4.0-6.0) and almost 90% of the initial activity was lost above pH 8.0. Its activity remained stable at  $100^{\circ}\text{C}$  for 60 min. The spectrometric analysis shows that this substance might have aromatic structure.

**Key words:** Radical scavenging activity, optimisation, *Sporobolomyces salmonicolor*

### INTRODUCTION

Antioxidants are utilized to prevent the atmospheric oxidation of food and its components. They protect food either by inhibiting the generation of reactive oxygen species (ROS) or by scavenging the preformed free radicals<sup>[1]</sup>. The most widely used synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) etc., now a days, are suspected to have toxic effect<sup>[2]</sup>. Therefore, search for an effective antioxidant from natural origin is desired. Both plant and microbial origin have been found to be the plenty source of natural antioxidants. A great number of aromatic, spicy, medicinal and other plants contained phenolic compounds<sup>[3-5]</sup> exhibiting primary (chain-breaking) antioxidant activity. Microbe also produces phenolic compounds as free radical scavenger<sup>[6,7]</sup> and antioxidant<sup>[8,9]</sup>.

During our screening for radical scavenging activity in the culture supernatant of various yeast strains, we observed that in the half YPD (H-YPD) medium, *Saccharomyces cerevisiae* IFO 2373 ( $127 \text{ u mL}^{-1}$ ) and *Sporobolomyces salmonicolor* ( $101 \text{ u mL}^{-1}$ ) produced

marked activity in the shaking and stationary culture, respectively<sup>[10]</sup>. It is well known that microbial growth and metabolite production depend concurrently on nutritional status and environmental conditions<sup>[11]</sup>. Therefore, we successfully increased the initial activity production to 2.3 fold by optimising the cultural conditions of *S. cerevisiae* IFO 2373. The highest activity of this strain was achieved at  $30^{\circ}\text{C}$  with  $0.7 \text{ L min}^{-1}$  of aeration rate while cultivated in the medium composed of 0.5% yeast extract, 1% polypeptone and 1% sucrose<sup>[12]</sup>. We have also purified the radical scavenging substance produced by this strain and observed that it produced two kinds of substance, namely, heat stable and unstable substance as radical scavenger (Gazi *et al.* unpublished). These findings insisted to investigate the initial production improvement of *S. salmonicolor* by optimising its suitable cultural conditions. On the above, this strain produced activity in the stationary condition so we suspect that the radical scavenger might be different from that of *S. cerevisiae* IFO 2373.

In our knowledge, this is the first report on the optimisation of the cultural conditions for radical

scavenging activity production by *S. salmonicolor*. This study, mainly focused on the optimisation of the cultural conditions to stimulate the production of the free radical scavenging activity and some properties of the scavenger.

## MATERIALS AND METHODS

**Microorganism and culture conditions:** *S. salmonicolor* was used from stock cultures maintained on potato dextrose agar slant and stored at 4°C for subsequent use. A loopful culture was taken from the slant and directly inoculated into the test tube (2.2x20 cm) containing 10 mL H-YPD medium. Cultivation was done in an incubator at 30°C for ten days. For the partial purification, Cells were cultivated in 300 mL Erlenmeyer flasks containing 300 mL of culture medium consisting of 0.5% (w/v) yeast extract, 1% (w/v) polypepton and 1% (w/v) fructose (YPF), pH 6.0, at 30°C for 10 days. After the incubation period the culture broth was centrifuged at 3000 rpm at 4°C for 15 min and the supernatant regarded as cell free extract was used for activity assay.

To assess the cell growth (turbidity), cells were inoculated into the L-tube and the cell density at various time interval was measured at 660 nm using a Spectronic 20<sup>+</sup> (spectronic instrument, USA).

**Medium and chemicals:** Initial cultivation of the cells was carried out in H-YPD broth, which contained 0.5% yeast extract, 1% polypepton and 1% glucose, pH 6.0. Other carbon and nitrogen sources were used in the medium to assess their impacts on the antioxidant production instead of glucose and polypepton of the medium. Glucose, fructose, galactose, maltose, sucrose and starch were used as mono, di and poly saccharide sources. The tested nitrogen sources were polypepton, tryptone, meat extract, urea, ammonium nitrate and ammonium sulfate.

**Medium pH:** The initial pH of the culture broth was maintained at 4–11 by adding either 1N HCl or 1N NaOH. Ten milliliter of the culture broth was taken into the test tube and initially the cell concentration was 10<sup>6</sup> cells mL<sup>-1</sup>. The cell growth and radical scavenging activity was measured at various time intervals.

**Radical scavenging activity determination:** To measure the radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as free radical<sup>[13]</sup>. Eight mg of DPPH was completely dissolved in 100 mL of ethanol (95%) and then 100 mL of de-ionized water was added. The cell free extract (0.2 mL) was mixed vigorously with 1.8 mL of DPPH solution and was kept in dark for

30 min. The medium itself was used as control and it was treated similar to that of cell free extract. Absorbance was measured at 517 nm by using Ultrospec-2000 (Pharmacia biotech) and the reduction of absorbance was expressed as unit activity per milliliter according to the formula; (units/mL) = (Abs<sub>c</sub> – Abs<sub>s</sub>)/S × 100. Here Abs<sub>c</sub> and Abs<sub>s</sub> are the absorbances of control and sample and S is the quantity (mL) of the sample.

**Partial purification in 2 steps:** Supernatant obtained by centrifugation of the culture broth was used as crude sample.

**Step 1; DEAE cellulofine A 500 column chromatography:** The sample was loaded on the ion exchange chromatography carried out with DEAE cellulofine A-500 column (2.4×21.4 cm) equilibrated with 20 mM Tris-HCl buffer pH 7.4. After washing, the substance was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer. Fractions of 10 mL each were collected at a flow rate of 0.5 mL min<sup>-1</sup>. Active fractions were pooled and concentrated by rotary evaporator at 30°C.

**Step 2; Sephadex G-15 column chromatography:** The concentrated sample was applied on a Sephadex G-15 column (1.4×79 cm) and eluted with distilled water. Fractions of 5 mL each were collected at a flow rate of 0.2 mL min<sup>-1</sup>. The active fractions were pooled and freeze-dried as partial purified free radical scavenger from *S. salmonicolor*.

**Some properties of the purified substance:** The effect of pH on the radical scavenging activity was determined by incubating the reaction mixture at pH values ranging from 3.0–11.0 using different buffer systems. The pH effect on the stability of radical scavenging activity was determined by measuring the remaining activity of the substance after an overnight incubation in the buffers at 4°C.

The temperature effect for the radical scavenging activity was determined by conducting the assay at various temperatures from 40 to 100°C. The sample (0.1 mg mL<sup>-1</sup> in distilled water) was taken in a test tube and incubated at 40, 60, 80 and 100°C for 60 min. The tested sample (0.2 mL) was taken at every 20 min interval to detect the remaining scavenging activity.

**Spectrometric identification of the substance:** The UV-vis absorption spectra of the active components were recorded on a Shimadzu 8705 S electro micro analyzer. The IR spectra of the sample in KBr pellets were obtained using a Shimadzu 8600 PC fourier transform infra red spectrophotometer. The mass spectra of the components

were obtained using the electro spray ionization mode on a Nippon Denshi JMS-700 mass spectrometer.

## RESULTS AND DISCUSSION

**Influence of carbon source:** Glucose of the H-YPD medium was replaced with other carbon sources at a concentration of 1%. The effects of different carbon sources on the cell growth and radical scavenging activity are given in Table 1. *S. salmonicolor* produced a maximum radical scavenging activity ( $125 \text{ u mL}^{-1}$ ) and cell growth (OD 2.20) in the medium containing fructose. Lower radical scavenging activity was detected in the medium containing disaccharides such as, maltose ( $38 \text{ u mL}^{-1}$ ) or sucrose ( $55 \text{ u mL}^{-1}$ ) and polysaccharide such as starch ( $25 \text{ u mL}^{-1}$ ). On the other hand, no activity was found in the medium containing galactose. It is noticeable that the cell growth (OD 0.14) in this medium was low. This strain is known to show negative response for cell growth in galactose<sup>[14]</sup>. *S. salmonicolor* could not produce radical scavenging activity without carbon source although the cell growth (OD 0.69) was at fair level. Carbon source was known inevitable for radical scavenging activity production in *S. cerevisiae* IFO 2373 and also for *S. salmonicolor*. One point five percent and 0.5% fructose containing medium showed activity of 88 and  $69 \text{ u mL}^{-1}$ , respectively, although the cell growth was sufficient. From the above findings it is also suspected that monosaccharide is possibly potent carbon source for radical scavenging activity production by this strain. Nevertheless, this observation revealed that carbon source in the medium is essential for radical scavenging activity.

**Influence of nitrogen source:** Polypepton of the basal H-YPD medium was replaced with a number of organic and inorganic nitrogen sources such as tryptone, meat extract, urea, ammonium nitrate and ammonium sulfate as shown in Table 2. The radical scavenging activity detected from tryptone ( $83 \text{ u mL}^{-1}$ ) and meat extract ( $81 \text{ u mL}^{-1}$ ) containing medium are higher than that of ammonium sulfate ( $61 \text{ u mL}^{-1}$ ) and ammonium nitrate ( $58 \text{ u mL}^{-1}$ ) containing medium. On the other hand, no activity was detected when cultivated in the urea containing media. One point five percent and 0.5% polypepton containing medium was found to produce 102 and  $62 \text{ u mL}^{-1}$  of radical scavenging activity with sufficient cell growth. Although this strain was capable of growing in both inorganic and organic nitrogen sources but no significant activity was detected compared to 1% polypepton ( $125 \text{ u mL}^{-1}$ ). Gazi *et al.*<sup>[12]</sup> also found that using polypepton in the medium is significant for

Table 1: The influence of carbon sources on growth and radical scavenging activity production by *S. salmonicolor*

Source (concentration %)	Growth OD 660	Activity ( $\text{u mL}^{-1}$ )[ratio] <sup>b</sup>
Glucose (1)	1.75	101[1.00]
Fructose (1)	2.20	125[1.24]
Galactose (1)	0.14	00[0.00]
Maltose (1)	1.40	38[0.38]
Sucrose (1)	1.52	55[0.54]
Starch (1)	1.03	25[0.25]
None	0.69	00[0.00]

The basal medium was prepared in deionized water containing 0.5% yeast extract and 1% polypepton. Data reported were the average of experiments repeated three times. <sup>b</sup>Ration was calculated with respect to 1% glucose in the basal medium

Table 2: The influence of nitrogen source and yeast extract on growth and radical scavenging activity production by *S. salmonicolor*

Source (concentration %)	Growth OD 660	Activity ( $\text{u mL}^{-1}$ )[ratio] <sup>b</sup>
Polypepton (1)	2.20	125[1.00]
Ammonium nitrate (1)	1.42	58[0.46]
Ammonium sulfate (1)	1.47	61[0.49]
Meat extract (1)	1.71	81[0.65]
Trypton (1)	1.56	83[0.66]
Urea (1)	0.31	00[0.00]

The basal medium was prepared in deionized water containing 0.5% yeast extract and 1% fructose. Data reported were the average of experiments repeated three times. <sup>b</sup>Ratio was calculated with respect to 1% glucose in the basal medium.

antioxidant activity production for *S. cerevisiae* IFO 2373.

**Influence of incubation temperature:** *S. salmonicolor* was cultivated at various incubation temperatures up to ten days (Fig. 1). Although the radical scavenging activity was progressively increased up to nine days of cultivation both at 25 and 30°C, a maximal activity was found at 25°C. On the contrary, both the cell growth and the activity ( $42 \text{ u mL}^{-1}$ ) production was very low at 37°C. It is well known that *S. salmonicolor* cannot grow well at 37°C<sup>[14]</sup>. It was also observed that this yeast produced activity for a short period of time and no further progress in activity was observed when culture temperature was maintained at 37°C. The results reflects that the culture temperature should be kept a round 25°C for highest radical scavenging activity generation by *S. salmonicolor*.

**Influence of initial pH:** Figure 2 indicated the influence of medium pH on the cell growth and radical scavenging activity production. *S. salmonicolor* was capable of growing and producing radical scavenging activity in the acidic condition. The optimum pH for activity production was 6.0. Decreasing the pH from this value also caused to decrease the cell growth and as well as the activity production. Increasing the value also showed same phenomenon. It is noteworthy that the increase of culture fluid pH onward to 7.0 caused serious effect on cell

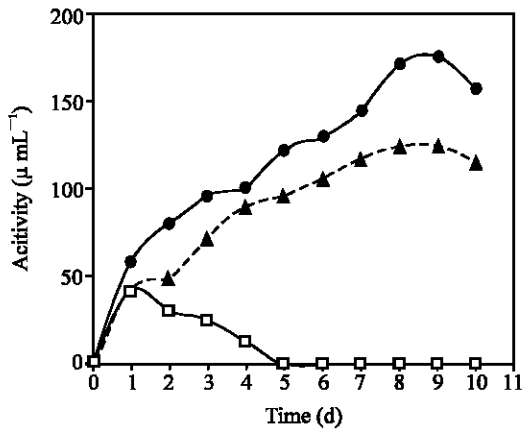


Fig. 1: Showing the radical scavenging activity production pattern by *Sporobolomyces salmonicolor* at different temperature. The cells were cultivated in the YPF (yeast extract 0.5%, polypeptone 1% and fructose 1%) medium. (●), Activity at 25°C; (▲), Activity at 30°C; (□) Activity at 37°C

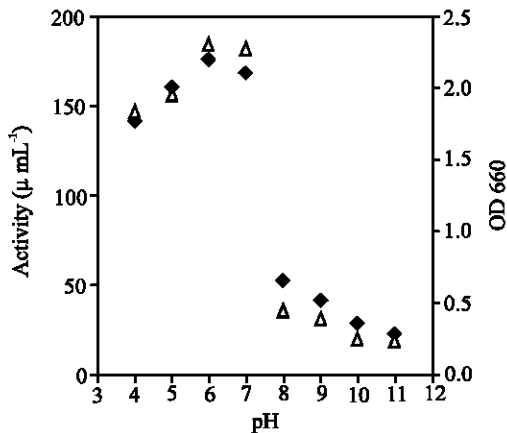


Fig. 2: Influence pHs on growth and radical scavenging activity production of *Sporobolomyces salmonicolor*. The yeast was grown at various initial pHs of the YPF medium containing 0.5% yeast extract, 1% polypeptone and 1% fructose. The turbidity of cell was measured at 660 nm. (▲), Activity (u mL<sup>-1</sup>); (◆), Absorbance at 660 nm

growth as well as activity production. pH has a profound effect on the growth and viability of microbial cells. Present observations support this phenomenon for cell growth as well as radical scavenging substance production. Moreover, it is conceived that acidic condition is favorable for *S. salmonicolor* for radical scavenging activity production.

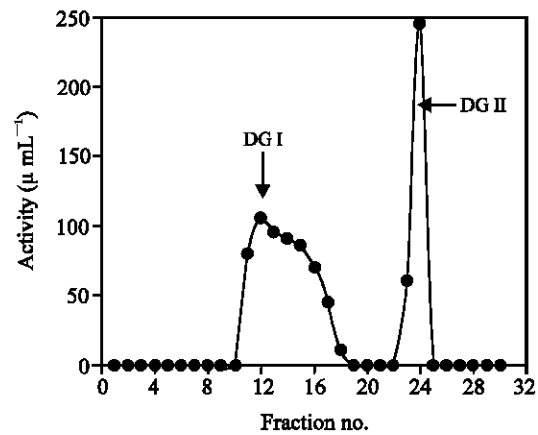


Fig. 3: Showing the partial purification of free radical scavenger from *S. salmonicolor* using gel filtration (Sephadex G-15) column chromatography

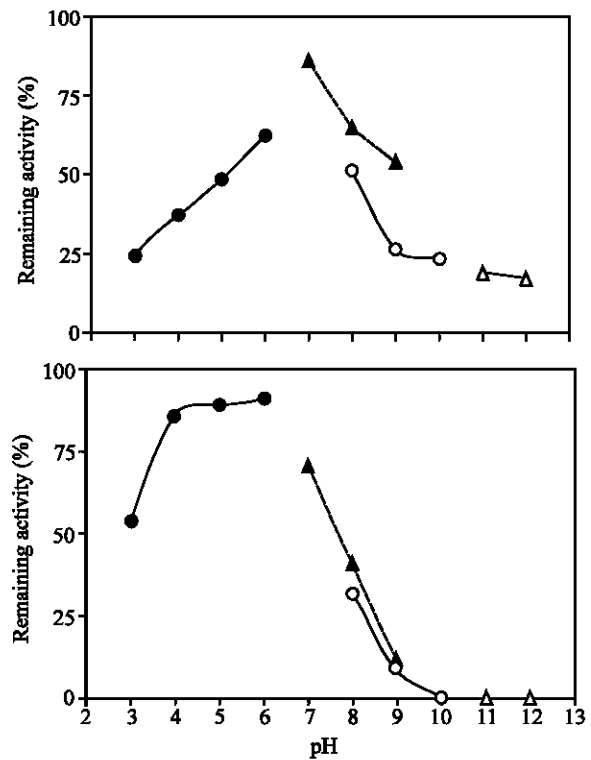


Fig. 4: The effect of pH on the radical scavenging activity of the partially purified substance. Sample was incubated with the following 50 mM buffers: Citrate buffer (●) pH 3-6; Tris-HCl (▲) pH 7-9; Boric acid buffer (○) pH 8-10; Phosphate buffer (Δ) pH 11-12 (A) Sample used from fraction DG I. (B) Sample used from fraction DG II

**Partial purification of the radical scavenger:** The radical scavenging substance from *S. salmonicolor* was partially purified by means of ion exchange chromatography and gel filtration. The cultured supernatant (70 mL) was applied on DEAE cellulofine A-500 at pH 7.4 using a NaCl gradient (figure not shown). The active fractions were accumulated and concentrated by rotary evaporator. It was applied on Sephadex G-15 column (Fig. 3). Two active peak, DG I (fraction no. 11-17) and DG II (fraction no. 23-24), showed radical scavenging activity. DG I was released earlier and thereby it reflects that the molecular mass of the substance is higher than DG II which was released to the end. DG I showed broaden peak and possessed lower activity than DG II. Both active peaks were separately accumulated and after freeze drying the substance was tested for its temperature and pH stability. Finally the substance from DG II was tested for some spectrometric analysis.

**Some properties of the partially purified substance:** The influence of pH on the stability of radical scavenging activity was studied (Fig. 4A and B). The remaining activity of DG I showed its stability in the range between 6.0-7.0. The substance lost its activity in the acidic and basic conditions. The radical scavenging activity was stable until 60°C for 30 min (figure not shown). On the other hand, the remaining activity of DG II was decreased with an increase of pH above 6.0. The radical scavenging activity was comparatively higher in the acidic pH values (pH 4.0-6.0) but showed very low activity above pH 9.0. The substance showed stability until 100°C for 60 min incubation (figure not shown).

**Spectrometric identification of the substance:** The UV absorption spectra of the radical scavenging substance from DG II showed peaks at 200, 235 and 290 nm. A large peak at 290 nm indicated that the substance might have aromatic proton. The IR spectrum exhibits absorptions at 3400, 2935, 1639, 1402, 1076, 1029 and 918 cm<sup>-1</sup> (figure not shown). The absorption at 3400 cm<sup>-1</sup> reflects that the substance might have hydroxyl bond. The estimated molecular mass was obtained ESI (Pos.) 281 m/z (figure not shown). Therefore, we suspect that this could be a phenolic substance.

On the basis of the results of present study, it is clearly indicated that the radical scavenging activity could be increased with optimised cultural conditions. It is note worthy that the optimised condition for *S. salmonicolor* is different from that of *S. cerevisiae* IFO 2373. Therefore, It can be concluded that each strain could be enhanced its radical scavenging activity production by optimising its cultural condition. During screening for radical

scavenging activity in yeast we suspected that yeast strains might produce different substance as radical scavenger. Some of them might be temperature stable and unstable. Therefore, the spectrometric results of this study also confirm that *S. salmonicolor* produced different substance as scavenger that was produced by *S. cerevisiae* IFO 2373. Therefore, from this study it also could be assumed that each strain might produce different substance as radical scavenger. So, microbial origin is a big opportunity to search natural antioxidant.

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