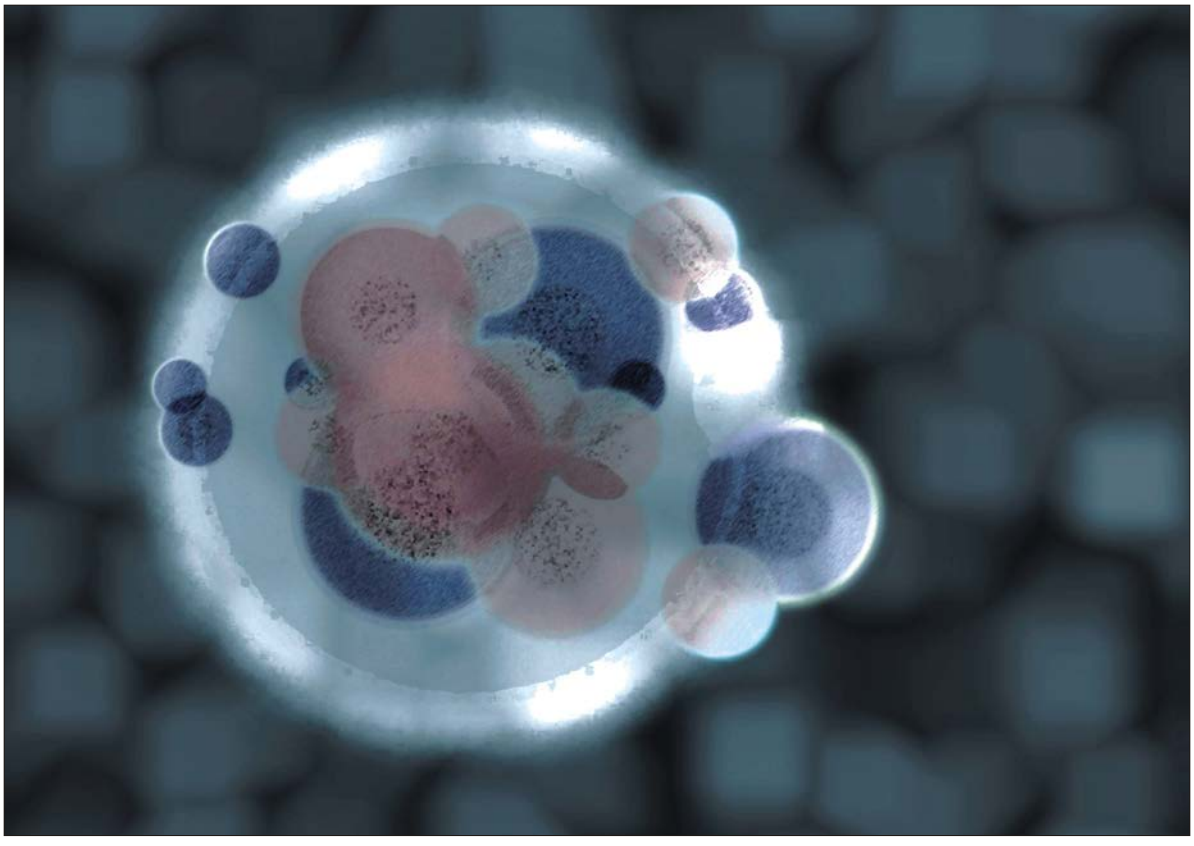

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Optimisation of Various Cultural Conditions on Growth and Antioxidant Activity Generation by *Saccharomyces cerevisiae* IFO 2373

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Abstract: The objective of this study was to optimise the cultural factors to enhance the production of antioxidant from *Saccharomyces cerevisiae* IFO 2373 (IFO 2373). Carbon and nitrogen sources of the medium were optimised along with other cultural conditions for growth and antioxidant activity generation. The antioxidant activity of the cell free extract was detected by measuring the free radical scavenging activity. Other than poly saccharide (water soluble starch), mono and di saccharide sources were observed solely responsible for antioxidant activity generation. The culture broth had higher antioxidant activity (202 u mL^{-1}) when sucrose was used as carbon source. Polypeptone containing medium was found better for antioxidant activity generation among the tested organic and inorganic nitrogen sources. The highest antioxidant activity (296 u mL^{-1}) was achieved at 30°C with 0.7 L min^{-1} of aeration rate. These results are promising because we succeeded in increasing the antioxidant activity almost 2.3 fold over the initial production obtained from half YPD (H-YPD) culture broth.

Key words: Antioxidant activity, optimisation, *Saccharomyces cerevisiae* IFO 2373

INTRODUCTION

Antioxidants are generally used for the protection of foods from oxidative damage and rancidity. They protect food either by inhibiting the generation of reactive oxygen species (ROS) or by scavenging the preformed free radicals[1]. 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[2]. Therefore, search for an effective antioxidant from natural origin is desired. Antioxidants from natural substances such as edible plants, spices and herbs have been widely investigated, because those natural substances have been eaten safely for long time[3,4]. Microbes are also expected as an origin of antioxidants because of their numerous species and ease of handling[5]. We therefore, search antioxidants from the microbial organisms utilized for manufacturing food. As a result we found that some strains of *Saccharomyces cerevisiae,* which are used for brewing and baking industry, produced antioxidant activity. Among them *Saccharomyces cerevisiae* IFO 2373 (IFO 2373) showed marked antioxidant activity in the half YPD (H-YPD) medium[6].

On our screening, we attempted to detect extra cellular antioxidant activity from culture broth by measuring the radical scavenging activity, using DPPH as free radical. It is well known that normally microbial growth and metabolite production depends concurrently on nutritional status and environmental conditions[7]. IFO 2373 produced water-soluble antioxidant in the culture broth and thus, there is a potential for the production improvement by optimising suitable cultural and nutritional requirement of this organism.

A limited number of reports are available on the optimisation of the cultural condition for antioxidant production[8]. In this study, we therefore, mainly focused on the optimisation of the cultural condition to stimulate the generation of antioxidant by IFO 2373.

MATERIALS AND METHODS

Microorganism: IFO 2373 was used from stock cultures maintained in our Laboratory. This strain was maintained on potato dextrose agar slant and stored at 4°C for subsequent use.

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

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nitrogen sources were polypeptone, tryptone, meat extract, urea, ammonium nitrate and ammonium sulfate.

Culture condition and measurement of cell growth: A loopful culture of IFO 2373 was taken from the slant culture and directly inoculated into the test tube (2.2x20 cm) containing 10 mL H-YPD medium. Cultivation was done in a reciprocal shaker RTR No-1-P (Iwashiya) at 30°C and 210 rpm for eight days. To assess the cell growth, cells were inoculated into the L-tube and the cell density at various time interval was measured at 660 nm using a Spectronic 20^{+} (spectronic instrument, USA).

Preparation of cell free extract: Cell free extract was prepared by centrifugation at 3000 rpm for 15 min at 4°C. Supernatant regarded as cell free extract was used for activity assay.

Medium pH: The initial pH of the culture broth was maintained at 4-11 by adding either 1N HCl or 1N NaOH. Fifty milliliter of the culture broth was taken in 300 ml Erlenmayer flask and initially the cell concentration was 10^6 cells mL^{-1}. The flasks were placed in an orbital shaker (Innova incubator shaker 4430, Japan) maintaining 200 rpm and 30°C for incubation. The cell growth and antioxidant activity was measured at various time intervals.

Aeration effect assay: One liter of culture broth was taken in 2 L culture flask. Initially the cell concentration was 10^6 cells mL^{-1}. The constant air supply in the culture device was maintained by using aeration pump, compressor MAU-1 (Eyela). Vent filter (Millex-FG_{50}) was used to protect air contamination. The culture device was set in an incubator and the temperature was maintained at 30°C. The agitation was done with magnetic stirrer. The cell growth and antioxidant activity was measured at every 6 h interval.

Antioxidant activity determination: The antioxidant activity of the cell free extract was detected by measuring the free radical scavenging activity. For this purpose, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as free radical[9]. Eight mg of DPPH was completely dissolved in 100 mL of ethanol 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^{-1}) = (Abs_c-Abs_s)/S 100. Here Abs_c and Abs_s are the absorbances of control and sample and S is the quantity (mL) of the sample.

RESULTS AND DISCUSSION

Effect of carbon source: Strikingly, no antioxidant activity was detected without carbon source although the growth of IFO 2373 was at fair level (Table 1). When glucose was replaced with other carbon sources at a concentration of 1% a maximum antioxidant activity (202 u mL^{-1}) was found in sucrose containing medium. Lower but almost same antioxidant activity was detected in the medium containing either fructose (160 u mL^{-1}) or maltose (161 u mL^{-1}). On the other hand, no activity was found in the medium containing starch. Carbon source is inevitable for cell growth. It is noticeable that the maximum cell growth occurred in the medium containing fructose (OD 2.00). Yen and Chang[8] found sucrose as suitable carbon source for antioxidant activity production from *Aspergillus candidus*. Makkar and Cameotra[10] obtained maximum yield of biosurfactant from medium containing sucrose as a carbon source. Comparatively low growth was observed in the starch contain medium and the organism could not generated antioxidant activity. Therefore, we suspect that IFO 2373 might not assimilate starch as carbon source. Nevertheless, this observation revealed that carbon source in the medium is probably responsible for antioxidant activity. No reference was found regarding this phenomenon.

Effect of nitrogen source: The influence of various nitrogen sources on cell growth and antioxidant activity is shown in Table 2. Polypeptone of the 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 antioxidant activity detected from tryptone (128 u mL^{-1}) and meat extract (118 u mL^{-1}) containing medium are

Table 1: The effect of carbon sources on growth and antioxidant activity production

Source (concentration %)	Growth[a]	Activity (ratio)[b]
Control	1.40	127 (1.00)
Fructose (1%)	2.00	160 (1.20)
Galactose (1%)	1.38	144 (1.10)
Maltose (1%)	1.32	161 (1.20)
Sucrose (1%)	1.30	202 (1.59)
Starch (1%)	0.96	0 (0.00)
None	0.74	0 (0.00)

The control is H-YPD medium. The basal medium was prepared in deionized water containing 0.5% yeast extract and 1% polypeptone. [a] The cells growth was measured by the turbidity of cells at 660 nm. [b] Activity was calculated as unit ml^{-1}. Data reported were the average of experiments repeated three times

Table 2: The effects of nitrogen source and yeast extract on growth and antioxidant activity production

Sources (concentration %)	Growth[a]	Activity (ratio)
Control	1.40	127 (1.00)
Ammonium nitrate (1%)	1.34	108 (0.85)
Ammonium sulfate (1%)	1.38	83 (0.65)
Meat extract (1%)	1.51	118 (0.93)
Polypeptone (1%)	1.40	202 (1.59)
Tryptone (1%)	1.50	128 (1.00)
Urea (1%)	0.12[b]	22 (0.17)

The control is H-YPD medium. The basal medium was prepared with 0.5% yeast extract and 1% sucrose. [a]The cell growth was the turbidity at 660 um. [b] The initial turbidity was 0.21. Data reported were the average of experiments repeated three times

Table 3: The effects of aeration on growth and antioxidant activity production

Aeration rate (L min^{-1})	Growth[a]	Activity (u mL^{-1})
0	0.9	67
0.3	8.6	171
0.5	9.0	191
0.7	5.3	296
1.0	1.4	119

Cells were cultivated in the YPS (0.5% yeast extract, 1.0% polypeptone and 1.0% sucrose, pH 7.0) medium. [a]The cell growth was measured by the turbidity of the cells at 660 um. The initial cell concentration was 10^6 cells ml^{-1}. The cell growth and antioxidant activity shown in the table was measured after 24 h of cultivation. Data reported were the average of experiments repeated three times

higher than that of ammonium nitrate (108 u mL^{-1}) and ammonium sulfate (83 u mL^{-1}) containing medium. The antioxidant activity detected from urea containing medium was the lowest. No enhancement of activity was achieved in compared to polypeptone. This result indicated that polypeptone is better nitrogen source.

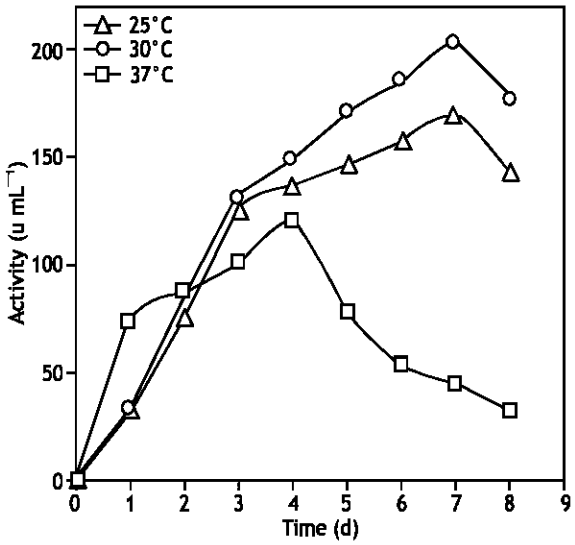

Fig. 1: Showing the antioxidant activity production pattern by *S. cerevisiae* IFO 2373 at different temperature. The cells were cultivated in the YPS (yeast extract 0.5%, polypeptone 1% and sucrose 1%) medium

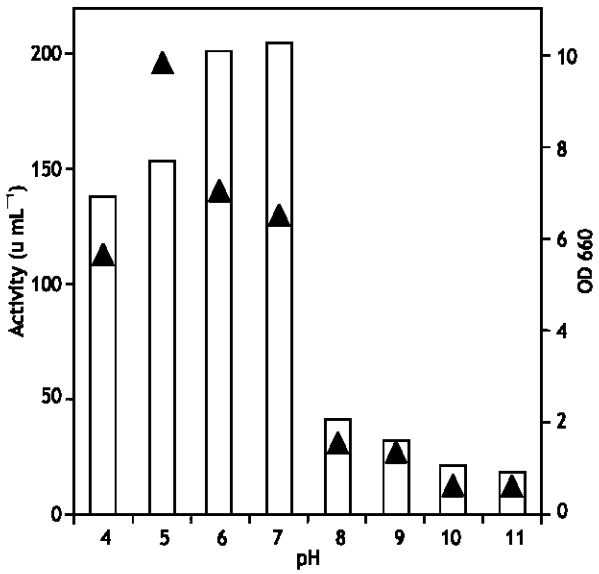

Fig. 2: Exhibit the medium pHs effects on growth and antioxidant activity generation of *S. cerevisiae* IFO 2373. The yeast was grown at various initial pHs of the YPS medium containing 0.5% yeast extract, 1% polypeptone and 1% sucrose. The turbidity of cell was measured at 660 nm
Symbols: (□), Activity (u mL^{-1}) ; (▲), Absorbance at 660 nm)

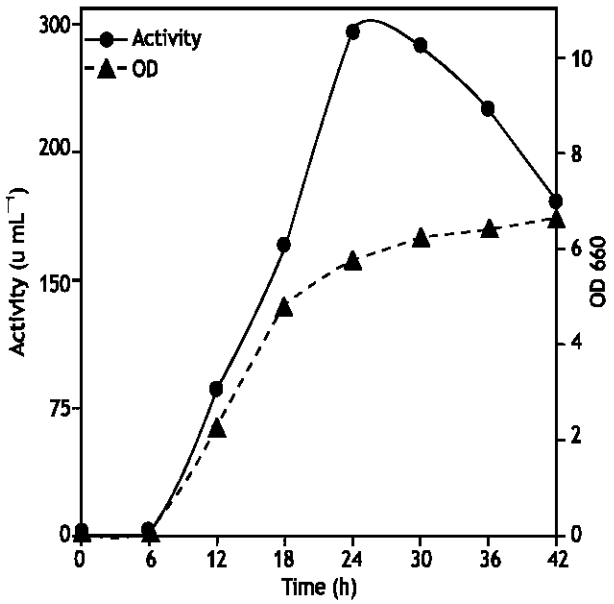

Fig. 3: Influence of aeration on growth and antioxidant activity of *S. cerevisiae* IFO 2373 in the culture flask incubated at 30°C. The aeration rate was maintained at 0.7 L min^{-1}. Basal medium composition was 0.5% yeast extract, 1% polypeptone and 1% sucrose. The turbidity of cell was measured at 660 nm

IFO 2373 was observed to grow well in medium containing both inorganic nitrogen sources like ammonium sulfate, ammonium nitrate and in organic sources like tryptone and polypeptone but no significant activity was detected compared to polypeptone. Urea containing medium was observed less effective for cell growth as well as antioxidant activity. Adding the vitamin biotin to the culture medium with urea assist to assimilate[7]. Therefore, lack of biotin along with urea might be the reason for low cell growth and antioxidant activity.

Effect of incubation temperature: IFO 2373 was cultivated at various incubation temperatures up to eight days (Fig. 1). A differential pattern of activity was observed with different incubation temperature. Though the activity progressively increased up to seven days both at 25 and 30°C, a maximal activity was found at 30°C. However, at 37°C, the antioxidant activity was lower compared to 30°C and the cell growth were also low. Although optimum temperature for maximum growth is strain dependent, it is usually in the range of 25-40°C[11]. In this experiment optimum cell growth and high antioxidant activity was detected at 25 and 30°C on the other hand, it was retarded at 37°C. Nevertheless, culture temperature should be kept around 30°C for highest antioxidant activity generation by IFO 2373.

Effect of initial pH: Figure 2 indicates that cell growth and antioxidant activity was not affected in the range of pHs 4-7. On the other hand, a short fall of growth along with activity was observed at pH 8 to on ward. Although the activity progressively increased up to pH 7, the activity reached its maximal in the culture fluid with an initial pH 7. Almost strains of the genus *Saccharomyces* are able to grow within 3 to 7 pH[12]. In bulk medium, growth and fermentation rate were not affected due to change in pH values within 3.5 to 6 and this is suspected due to the tight control over intracellular pH[7]. Our observation also supports this phenomenon for cell growth. Moreover, it should be assumed that acidic condition is favorable for IFO 2373 for antioxidant activity generation.

Effect of aeration: Both aerobic and anaerobic cultivation was done to assess the impact of aeration on antioxidant activity. The results in Table 3 exhibits cell growth and antioxidant activity of IFO 2373 in both aerobic and anaerobic conditions. Comparatively a higher cell growth and antioxidant activity was detected in the aerobic condition. The cell growth was increased until the aeration rate of 0.5 L min^{-1} but the highest activity was observed at the aeration rate of 0.7 L min^{-1}. Increasing the aeration above this level caused the cell growth and antioxidant activity to decrease sharply. Therefore, these results suggest that aeration effect on antioxidant activity generation and excess aeration might influence the substance to auto oxidised. Although *Saccharomyces* is known to grow in both aerobic and anaerobic conditions[13, 14] we observed a sharp antioxidant activity generation in aerobic condition by IFO 2373.

The time course of the antioxidant activity (Fig. 3) shows that the activity was increased until 24 h of cultivation and then markedly decreased. Moreover, the cells were also observed in the stationary phase after 24 h of cultivation. The above results are indicating that antioxidant activity might be generated during exponential phase of growth.

On the basis of the results of this study, it is clearly indicated that the antioxidant activity of IFO 2373 could be increased with optimised cultural conditions. High antioxidant activity is helpful for mass production and purification. Currently we are in progressing experiment regarding purification and characterization of antioxidative substance produced by IFO 2373 with this optimised condition.

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