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Cell yield and superoxide dismutase activity of the marine yeast *Debaryomyces hansenii* under different culture conditions

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Abstract. The effect of aeration, pH, stirring rate, and temperature on the biomass production and superoxide dismutase (SOD) activity of the marine yeast *Debaryomyces hansenii* strain C-11 was determined. The cell biomass yield was approximately 50% in a seawater-formulated medium using glucose as the carbon source. The SOD activity increased by application of a pulse of oxygen or 0.8 mM sulfate copper into the chemical reactor. The SOD enzyme had an activity of 400 units/mg of protein in a crude extract produced under such conditions, the best activity ever reported for this enzyme in a crude preparation.

Superoxide dismutase (SODUEC), 1.15.1.1 is a metalloenzyme that catalyzes the dismutation of superoxide radicals. This enzyme has several applications in medicine: as an antiinflammatory agent, as an immune-response modulator, in malignant-tumor regression, for protection against radiation or chemotherapy, in treatment of premenstrual syndrome and arthritis, in antiaging treatments, during the use of hyperbaric chambers, and in general against oxidative stress (Autor 1982; Burkley 1993). In addition, SOD has been used as an antioxidant in the food industry (Aurand et al. 1977; Hill 1979; Allen and Wrieden 1982; Meyer and Isaksen 1995). The main sources for this enzyme include erythrocytes, brain, and human, bovine, pig, chicken, and rat liver and heart (Fridovich 1975). It has been isolated from bacteria (Lygren et al. 1986; Lee and Lee 1988; Kroll et al. 1991; Chamnongpol et al. 1995), algae (De Jesus et al. 1989), and from yeast (Van Loon et al. 1986; Bilinski 1988; Gralla et al. 1991; Galiazzo and Labbe, 1993; Ochoa et al. 1995). An abundant and cheaper source has not yet been identified (Lee and Lee 1988; Salin and Desterhelt 1988).

Debaryomyces hansenii strain C-11 was isolated from the Pacific Ocean off the west coast of Baja California Sur, México (Hernández-Saavedra 1990). In a previous report, we proposed the use of this marine yeast as a potential source of SOD because it produces considerable amounts of the enzyme (Ochoa et al. 1995). In this report, we discuss the optimization of physical variables for biomass production and the assay of possible inducers for the enzyme.

Materials and methods

Culture medium

The yeast was grown in a semienriched medium containing 2% glucose as carbon source, 0.3% peptone as nitrogen source, and 0.2% yeast extract as source of vitamins and trace elements.

Culture conditions

Aereation was optimized by injection of different airflow rates (0, 5, and 7 liters/min). Growth at various temperatures (10, 20, 30, and 40°C) was tested also. The stirring rate was controlled automatically (New Brunswick) and the pH of the medium recorded by use of an electrode and an electronic microchip (Ingold). All experiments were done in a 2-liter chemical reactor (New Brunswick) with 900 ml of culture medium and 100 ml of cell inoculum collected at the logarithmic phase (2.4×10^7 cell/ml).

Growth kinetics

Growth kinetics were followed for 48 h, taking samples every 6 h. The analyses made included dry weight (by gravimetry), consumption of glucose (Trinder, Merck), and determination of SOD activity in the crude extract by the xanthine–xanthine oxidase method (Rainer 1975). Protein analysis was done according to Bradford (1976), and the statistical analyses of specific growth rate (μ), cell yield coefficient ($Y_{x/s}$), and productivity (X_p) were made using two-way ANOVA ($P < 0.05$).

SOD extraction

The enzyme was extracted by mixing 35 mg of biomass, 35 mg of glass beads (0.5 mm diameter), and 1 ml of 50 mM phosphate buffer, pH 7.8, in

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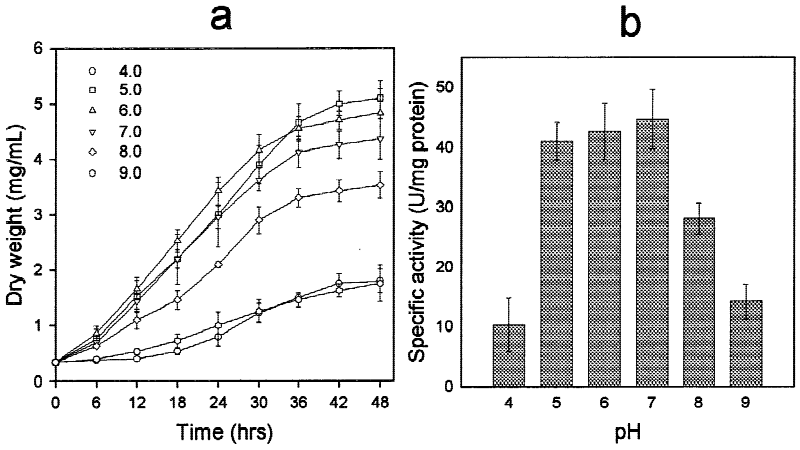

Fig. 1. The effect of culture media pH on the growth **(a)** and on the specific activity of the enzyme superoxide dismutase **(b)** of the marine yeast *Deb. hansenii.*

an Eppendorf tube. The mixture was stirred for 15 min in a Vortex mixer at 5°C, centrifuged for 10 sec in a microfuge E (Beckman), and the supernatant separated and dialyzed against the same buffer for 18 h.

Induction of SOD activity

The enzyme activity throughout the growth cycle was established. For induction, the yeast culture was first incubated for 24 h as above and then treated with 100% oxygen or 0.8 mM copper sulfate.

Results and discussion

Effect of culture medium pH on cell biomass and SOD activity

Yeast generally grows well at pH 3.5–6.5, although it can tolerate pH values from 0.9–11 (Davenport 1980). Some yeasts are restricted to small ranges of pH values. For example, *Schizosaccharomyces* spp. requires a pH of 5.45 and *Torulopsis pintolepessi* a pH of 0.9–1.1 (Van Uden and Vidal 1970). The environmental pH at which the microorganisms are isolated, as a general rule, cannot be considered optimal for growth; therefore such values need to be determined.

In our case, and in spite of the average pH reported for the Pacific Ocean being 8–8.7, *Debaryomyces hansenii* strain C-11 was found to tolerate pH between 4 and 9 (Fig. 1a). At both extreme pH values, it grows slowly. Our results are in contrast to those reported by Hobot and Jennings (1981), who concluded reproduction of the *Deb. hansenii* strain was higher as the pH of the medium increased, with a maximum at pH 8.3. Norkrans (1966) found *Deb. hansenii* grew better at a slightly acidic pH, which is more in agreement with our findings. For the effect of medium pH on SOD activity of *Deb. hansenii* strain C-11, we found the highest activity within a pH range of 5–7 (Fig. 1b).

Effect of stirring rate on cell biomass and SOD activity

The stirring rate is a variable of major importance in yeast growth. A well-stirred process promotes the maximum growth rate, not only by increasing oxygen diffusion but also by the homogeneous dispersal of the cells and nutrients throughout the reactor. Figure 2a shows that when the stirring rate is low cell reproduction is greatly reduced. If the process is stirred in excess, bubbles can form that may create dead spaces in the culture medium, to the detriment of cell growth (Fig. 2a). In Fig. 2b, we can observe the effect of the stirring rate on SOD activity, which reaches a maximum at 500 rpm and remains fairly constant thereafter, even if the stirring rate is increased to 700 rpm.

Effect of temperature on cell biomass and SOD activity

Yeast are considered ubiquitous. When studying the effect of temperature on the growth of *Deb. hansenii* (Fig. 3a), we found the best temperature was 30°C, so our yeast strain can be considered as mesophilic (Watson 1987). SOD activity is also affected by the medium temperature (Fig. 3b) and appears to increase up to 40°C. This effect has been observed for *Porphyromona gingivalis* (Amano et al. 1994), which shows an increase in the SOD activity at 40°C. It would seem SOD is produced as a heat-shock protein to protect the microorganism against temperature stress.

Effect of aeration on cell biomass and SOD activity

The quantity of air supplied to the reactor is an important factor if we assume that *Deb. hansenii* needs oxygen for growth (Fig. 4a). If the airflow is excessive, the observed effects are the same as observed with a fast stirring rate: bubble and foam formation. In general, yeast are facultative anaerobic microorganisms (Tilbury 1980). Javor (1989) believes *Deb. hansenii* has an exceptional ability to grow under anaerobic conditions. Our results, however, are not in agreement with this. In Fig. 4a, we observe when air is not supplied (0 liters/min), the yeast grows only slightly, which may be just a consequence of the stirring rate allowing the cells to remove whatever oxygen is present in the reactor. This was confirmed by an experiment done under an anaerobic environment generated by bubbling nitrogen gas into the medium and following the biosynthesis of sterols by adding Tween 80 and oleic acid into the culture medium. Under these conditions, we failed to observe any biosynthesis, indicating the absence of cell growth (data not shown).

SOD activity is known to be modified by the aeration rate in the culture of microorganisms (Chamnongpol et al. 1995). In our case, *Deb. hansenii* C-11 is no exception, because we observed a clear correlation between enzyme

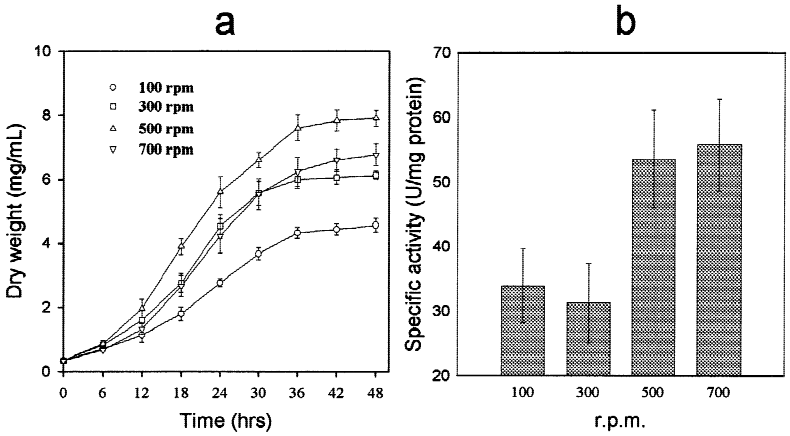

Fig. 2. The effect of the stirring rate on the growth **(a)** and on the specific activity of the enzyme superoxide dismutase **(b)** of the marine yeast *Deb. hansenii.*

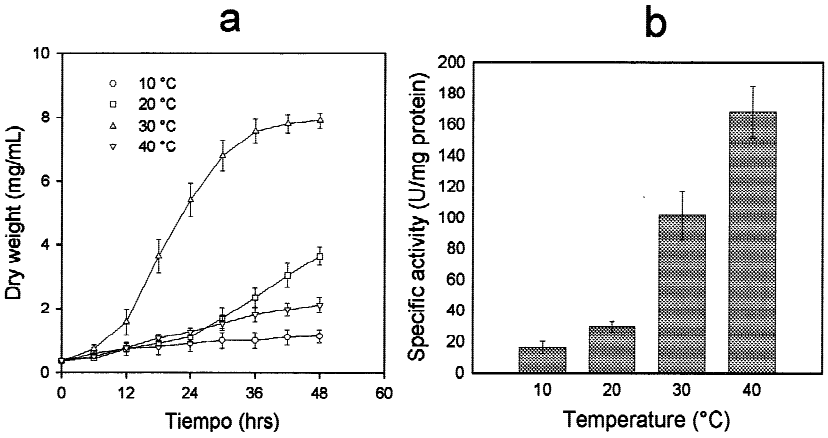

Fig. 3. The temperature effect of the culture media on the growth **(a)** and on the specific activity of the enzyme superoxide dismutase **(b)** of the marine yeast *Deb. hansenii.*

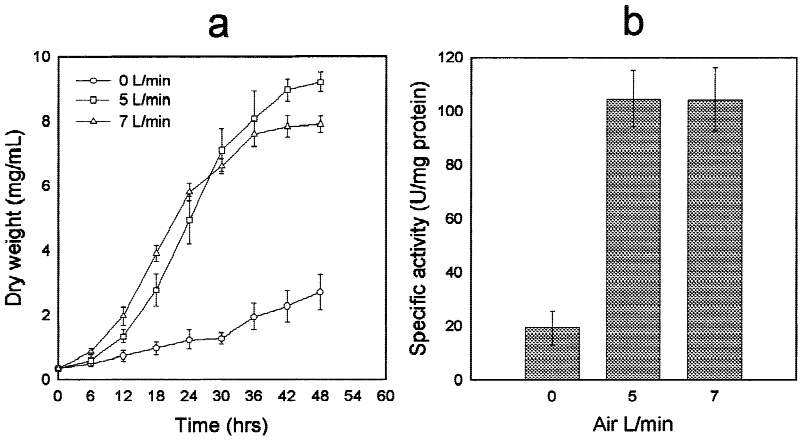

Fig. 4. The airflow rate effect on the growth **(a)** and on the specific activity of the enzyme superoxide dismutase **(b)** of the marine yeast. *Deb. hansenii.*

activity and airflow rate (Fig. 4b). This may be a consequence of the varying quantities of oxygen present under such conditions.

SOD activity throughout cell cycle of Deb. hansenii

In Fig. 5a, we illustrate the variation of SOD activity throughout the growth cycle of *Deb. hansenii* at optimum conditions (pH 5.0, 30°C, 500 rpm, 5 liters/min ambient air). At the beginning, SOD activity is low, which may be explained by the relatively slow metabolism of the yeast cell at such an early growth phase (recognized as an adaptation phase). It is beleived, at this stage, that the cell devotes most of its energy to regulating the biochemical machinery for the synthesis of enzymes that catabolize the substrate present in the culture medium. After regulation is achieved, growth and cellular reproduction start. At this point, the logarithmic phase begins and the quantity of cells increases.

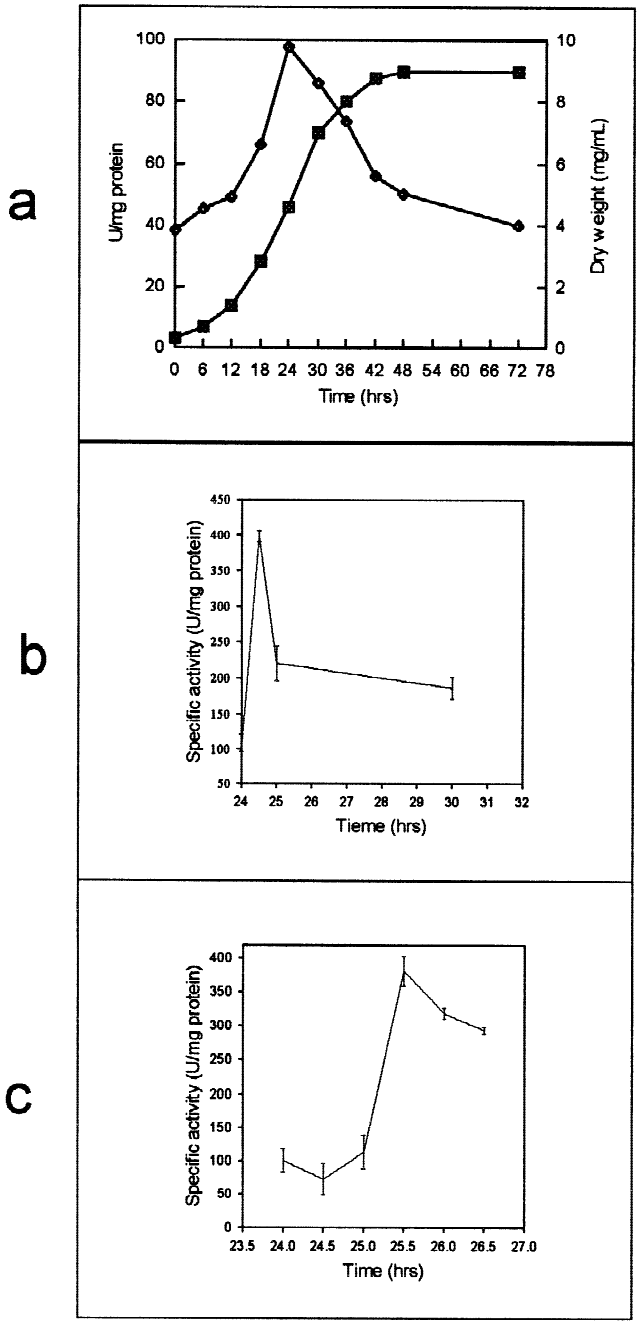

Fig. 5. The SOD activity of the marine yeast *Deb. hansenii* under different culture conditions: **a:** Cell growth (■) and SOD activity (◆) at optimum conditions (pH 5.0, 500 rpm, 30°C, 5 liters/min of filtered air); **b:** effect of adding 0.8 mM $CuSO_4$ to the culture media; and **c:** effect of the substitution of the oxygen source by 100% oxygen.

Not only is yeast reproduction highest at this point, but the demand for oxygen and, of course, the production of free radicals are also at their maximum. For this reason, Fig. 5a shows that the maximum SOD activity in *Deb. hansenii* is found at the top of the logarithmic phase.

Copper ions have been reported to induce the expression of the SOD gene in prokaryotes and eukaryotes (Fridovich 1975; Galiazzo et al. 1991; Paphasri et al. 1992; Gordon et al. 1993). Such an increase is about 2.4–3 times higher than the original SOD activity present in the cells in the absence of this inducer. In our case, when 0.8 mM copper sulfate was added to the culture medium at the logarithmic stage (i.e., hour 24 of the culture), for 30 min after the addition of copper, the activity increases rapidly to approximately 400% above the control where copper was not added (Fig. 5b). This respponse of the cells to copper ions may be explained in two ways: first, the copper ion is an essential element for certain biochemical processes in the cell where it serves as a cofactor for many enzymes, i.e., Cu-Zn SOD and some oxidases, such as polyphenol oxidase, ascorbate oxidase, diamine oxidase and, tyrosinase, that favor the oxidizing conditions under which free radicals are generated (Paphasri et al. 1992); second, the induction of the SOD activity occurs at the transcription–translation level, where the control over the gene codifying the synthesis of Cu-Zn SOD is regulated by the copper ion and the cofactor ACE1, a protein regulator that specifically binds to the gene in the presence of copper ions (Gralla et al. 1991).

The maximum efficiency of the biochemical machinery is modulated by the quantity of a particular enzyme needed to transform its substrate. When a particular substrate is not present, the cell avoids the expenditure of energy to synthesize the enzyme that would catabolize it. Consequently, induction of the production of an enzyme can be done by the addition of its particular substrate (Fridovich 1982). In Fig. 5c it can be observed that when a cell culture of *Deb. hansenii* C-11 in the logarithmic phase (i.e., at 24 h) is exposed to 100% oxygen, during the first 30 min SOD activity variation is almost negligible; however, after this period cells are able to respond to such a condition with a 400% increase in the SOD activity.

Conclusions

The pH of the culture medium is a key factor for optimal biomass production in the growth of *Deb. hansenii* C-11. This yeast strain can grow at pHs from 5 to 8 but grow best at pH 5.0. The stirring rate of the culture medium must also be controlled and preferably maintained at 500 rpm to obtain a high cell yield and SOD activity. This yeast also grows from 10 to 40°C; however, the best temperature for cell biomass production is 30°C, but the superoxide dismutase activity is highest when the yeast is grown at 40°C. The airflow rate is an important factor as an oxygen source for cell growth. Our results show *Deb. hansenii* C-11 can be a good alternative source for SOD with a high specific activity. The cell biomass yield of this yeast is comparable to that reported for similar organisms but, as shown previously, it possesses an interesting ability to grow in either a seawater- or freshwater-formulated medium (Ochoa et al. 1995), which adds some advantages in reducing contamination risks.

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