Protoplasts Obtained from *Candida tropicalis* Grown on Alkanes

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A method for the preparation of protoplasts from *Candida tropicalis* cultivated on *n*-tetradecane is described. This essentially consists of replacing the mannitol-sorbitol solution of the classical helicase technique by 1 M magnesium sulfate and lowering the pH to 4.1 during incubation in the presence of helicase. The protoplasts thus prepared behave like intact cells and are capable of consuming oxygen in the presence of *n*-tetradecane, *n*-decane, 1-decanol, and glucose.

The first step in the detailed study of the location of enzymes from yeasts, as from bacteria, consists of the preparation of protoplasts. Since the publication of the work of Eddy and Williamson (3) on the use of an extract of *Helix pomatia* for the solubilization of the cell walls of microorganisms, many other workers (6) have tried to replace this source of lytic enzymes with more appropriate mixtures obtained from bacteria.

The action of helicase on yeasts other than *Saccharomyces* has given disappointing results. Aguirre and Villanueva (1) and Gascon et al. (4) have therefore tried to use lytic enzymes produced by strains of *Micromonospora* and *Streptomyces*, and in particular have obtained protoplasts of *Candida utilis* in good yield. It is evident from all this work that the preparation of protoplasts depends on a number of factors and, in particular, on the growth substrate used and the physiological condition of the yeast.

So far as we know, no information has been obtained on the preparation of protoplasts from cells grown on hydrocarbons. Volfova et al. (7), in studying the penetration of hydrocarbon into cells, prepared protoplasts of *C. lipolytica* grown on glucose and reported that protoplasts do not oxidize *n*-hexadecane, although intact cells do.

This paper describes a method of preparation of protoplasts from cells of *C. tropicalis* cultivated on *n*-tetradecane and gives some properties of these protoplasts.

**MATERIALS AND METHODS**

**Organisms.** Bakers' yeast *Saccharomyces cerevisiae* (strain U) was given to us by the Société de Biologie et Synthèse de Marseille. *C. tropicalis* strain 101 from our own collection oxidizes hydrocarbons constitutively.

**Culture media.** The following minimal medium was used: NH₄Cl, 2.5 g; KH₂PO₄, 7 g; NaHPO₄, 7.2 g; MgSO₄·7 H₂O, 0.2 g; NaCl, 0.1 g; water, 1 liter. For cultures of *S. cerevisiae*, 1 g of yeast extract per liter was added; for *C. tropicalis* 100 mg per liter was added. The carbon source used was either glucose (2 g/liter) or *n*-tetradecane (1.5 g/liter). The cultures were incubated at 32°C with vigorous agitation.

Growth was measured by the change in optical density at 450 nm and was expressed as milligrams (dry weight) of cells produced by means of a reference curve. The cells were harvested during the exponential growth phase, washed with a 0.9% solution of NaCl, and used for protoplast studies.

**Preparation of protoplasts.** The washed yeast cells were suspended in a buffer solution of tris(hydroxymethyl)aminomethane-hydrochloride (pH 9.3) in a concentration range of 50 to 100 mg (dry weight) per ml. These suspensions were used for the preparation of protoplasts by two different techniques.

(i) The suspensions were treated under the same experimental conditions as those described by Kovac et al. (5). This consisted of incubating 25 ml of the preparation for 30 min at 32°C in the presence of 0.5 m sodium thioglycolate and then centrifuging. The recovered cells were washed with a phosphate-citrate buffer (0.1 M, pH 5.8) containing sorbitol (0.7 M), mannitol (0.3 M), and ethylenediaminetetraacetic acid (0.001 M). Cells were then suspended in 10 ml of the same solution containing 1 ml of helicase (commercial solution of *H. pomatia* supplied by the Industrie Biologique Française) and then were incubated at 30°C with agitation.

(ii) Protoplasts were prepared by treating the cells with sodium thioglycolate as before, centrifuging at 6,000 X g for 15 min, suspending the cells in 10 ml of phosphate-citrate buffer (0.05 M, pH 4.1) containing a molar concentration of MgSO₄, and adding 1 ml of helicase. This mixture was then incubated at 30°C with agitation.
Measurement of activity. The formation of protoplasts was followed by the measurement of optical density at 450 nm after dilution of samples of the reaction mixture with 500 parts of water. Yields in protoplasts are calculated approximately at any intermediate period from the percentage of the final change in optical density.

Measurement of respiratory activities. The respiratory activities of suspensions of cells or of protoplasts was measured by the Warburg manometric method by determining the consumption of oxygen under the following conditions: gaseous phase, air; temperature, 32°C; carbon substrate, 200 μmoles; and phosphate buffer (0.05 M, pH 5.6) for cell suspensions or phosphate-citrate buffer (0.05 M, pH 5.6) containing 1 M MgSO₄ for the protoplast suspensions.

RESULTS

Treatment of cells of C. tropicalis cultivated on glucose with helicase under the experimental conditions defined by Kovac et al. (5) gives low yields of protoplasts (10 to 15%).

This result is in strong contrast with that obtained with glucose-grown cells of S. cerevisiae which are transformed quantitatively into protoplasts under the same conditions. Applied to C. tropicalis cultivated on n-tetradecane, this treatment gives negative results (Fig. 1, curve 2). Therefore, some modifications were made.

These consisted essentially of replacing the sorbitol-mannitol mixture by a molar solution of MgSO₄ and using a more acid medium (pH 4.1). Under these conditions, treatment with helicase gives protoplasts of C. tropicalis cultivated on n-tetradecane (Fig. 1, curve 3). The yield, measured by means of the reduction in optical density, is 60% in 10 min, after which the rate of protoplast formation diminishes; by the end of 30 min, the optical density does not change any further and the reaction is considered to be complete. A microscopic examination of the resulting preparation shows that all the cells have been transformed into protoplasts which are different from intact cells (Fig. 2). To establish, by a method other than microscopic examination or optical density, whether any intact cells remained, respiratory activity was determined in the presence of hydrocarbons. It has been shown that only intact cells and protoplasts consumed oxygen in the presence of hydrocarbon and that cell-free extracts obtained by treatment with a French pressure cell or from burst protoplasts did not. Therefore, to detect the presence of intact cells it is sufficient to measure the respiration rate of this suspension on n-tetradecane in a hypotonic medium of 0.05 M phosphate buffer (pH 5.6). Under these conditions, we never observed oxygen consumption; taking into account the limited sensitivity of the method, we conclude that the preparation contained no residual intact cells after treatment with helicase.

Protoplasts obtained from C. tropicalis cultivated on n-tetradecane. In a recent publication, Volkova et al. (7) showed that protoplasts of C. lipolytica cultivated on glucose will not oxidize hexadecane, whereas the intact cells will. We have carried out the same experiment with protoplasts obtained from our strain of C. tropicalis which oxidizes hydrocarbons constitutively, and we have found that the protoplasts behave in exactly the

![Fig. 1. Kinetics of protoplast formation by helicase from C. tropicalis grown on n-tetradecane. (1) Control without helicase; (2) with helicase in sorbitol-mannitol medium at pH 5.8; (3) with helicase in MgSO₄ medium (pH 4.1).]

![Fig. 2. Photomicrographs obtained with phase contrast. (a) C. tropicalis cells grown on n-tetradecane. (b) Protoplasts prepared with helicase.](http://jb.asm.org/)
same manner as whole cells (Table 1). That is, protoplasts prepared from cells cultivated on either glucose or n-tetradecane oxidize both n-tetradecane and glucose.

Figure 3 shows the oxidation of n-tetradecane, n-decane, and glucose by cells of C. tropicalis grown on n-tetradecane. Figure 4 shows that the same substrates are oxidized by protoplasts obtained from the same cells; it should be noted that 1-decanol, a metabolic intermediate of n-decane oxidation, is also oxidized. To eliminate the possibility that fragments of cell wall are still remaining on the protoplasts and to check their influence on consumption of oxygen by protoplasts, we treated the protoplasts a second time with helicase; there was no diminution in their ability to oxidize n-tetradecane, n-decane, and glucose.

**DISCUSSION**

The method described for the preparation of protoplasts from C. tropicalis contains some modifications of the helicase procedure. These consist of the use of 1 M MgSO₄ as an osmotic support and a more acid pH (4.1). By using the method described by Volfova et al. (7) or by replacing the MgSO₄ by KCl or by the sorbitol-mannitol mixture in our method, we obtained low yields (15 to 20%) and we were unable to separate the protoplasts from the remaining cells. The role of MgSO₄ is not limited to that of an osmotic support but is probably related to the influence of Mg²⁺ on the membrane of microorganisms. Our experiments give no information on its action.

Our experiments prove that protoplasts are

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**Table 1. Oxygen uptake by cells of Candida tropicalis and by protoplasts**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Oxygen uptake onb</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-Tetradecane</td>
<td>n-Decane</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>84</td>
<td>88</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>54</td>
<td>74</td>
</tr>
<tr>
<td>n-Tetradecane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>60</td>
<td>103</td>
</tr>
</tbody>
</table>

a Amt: 1 mg (dry weight).
b Expressed as microliters per hour.

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**Fig. 3. Oxidation of different substrates (200 μmoles) by C. tropicalis (1.2 mg, dry weight) after growth on n-tetradecane. (1) n-Tetradecane, (2) n-decane, (3) glucose, (4) endogenous respiration.**

**Fig. 4. Oxidation of different substrates (200 μmoles) by protoplasts prepared from 1.6 mg (dry weight) of cells of C. tropicalis grown on n-tetradecane. (1) Glucose, (2) n-tetradecane, (3) n-decane, (4) 1-decanol, (5) endogenous respiration.**
able to oxidize hydrocarbons and that they behave in exactly the same manner as intact cells; however, it should be noted that our strain oxidizes hydrocarbons constitutively. On the other hand, Volfova et al. (9), using a strain of C. lipolytica which oxidizes hydrocarbon adaptively, showed that protoplasts, unlike the whole cells, are not capable of oxidizing hydrocarbon; in fact, they showed that their cells cultivated on glucose were able to adapt themselves to hydrocarbon oxidation and that protoplasts were not. This is in agreement with the fact that induction in protoplasts appears to be a rare phenomenon as proved by Duercksen (2) in studying the induction of penicillinase with Bacillus cereus. These protoplasts serve as raw material for the study and the localization of different enzymes implicated in hydrocarbon metabolism.

LITERATURE CITED