J Ind Microbiol Biotechnol (2008) 35:799–804
DOI 10.1007/s10295-008-0350-9

ORIGINAL PAPER

Permeabilization of baker's yeast with *N*-lauroyl sarcosine

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Received: 1 June 2007 / Accepted: 28 March 2008 / Published online: 16 April 2008

Abstract *N*-Lauroyl sarcosine (LS), a cationic, non-toxic and biodegradable detergent readily permeabilized whole cells of baker's yeast (*Saccharomyces cerevisiae*). Permeabilization was carried out to increase assayable cellular catalase activity, an enzyme of great physiological and industrial importance, and to release 5′-nucleotides which find food/nutritional applications. The event of permeabilization was concentration, time and temperature dependent. Maximum permeabilization of yeast cells were observed when 1 g wet weight (0.2 g dry wt) of cells were permeabilized with 1.0 ml of 2% LS at 45 °C for 15 min. LS-permeabilized cells showed 350-fold increase in catalase activity and the supernatant obtained after permeabilization was rich in 5′-nucleotides. LS-permeabilized baker's yeast cells can be used as a source of biocatalyst and to isolate valuable by-products.

Keywords Permeabilization · *N*-Lauroyl sarcosine · Baker's yeast · *Saccharomyces cerevisiae* · Catalase

Abbreviations
LS *N*-Lauroyl sarcosine

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Introduction

Enzymes are biocatalyst which are used in industry for production of drugs and fine chemicals. Microorganisms like baker's yeast (*Saccharomyces cerevisiae*) are a rich source of many of these enzymes. The permeability barrier of the cell envelope for both substrate and products often causes very low enzyme activities, necessitating the need for extraction of these enzymes from the cells. However, the process involved in extraction and purification of these enzymes from baker's yeast cells, is time consuming and expensive. Moreover, purified enzymes can be used only once as it is expensive to re-purify them without appreciable loss of their activity. Therefore, there is continued interest in developing techniques which allow exploitation of intracellular enzymes without the need for purifying them. Permeabilization of baker's yeast cells is one such technique [1]. Permeabilization may be defined as a process that alleviates the permeability barrier of the cell wall/membrane thereby permitting free mobility of substances, including substrate(s) and product(s), across cell wall/membrane. Therefore, addition of substrates to a suspension of permeabilized cells should bring about their bioconversion. So, permeabilized cells can be used as whole-cell biocatalyst to perform biotransformation mediated by their intracellular enzymes [2]. As a biocatalyst, permeabilized baker's yeast cells can be easily collected and reused without appreciable loss in enzyme activity unlike the purified enzymes. Permeabilized baker's yeast cells also offers a good intermediate model between the intact cell and cell free system for experimental analysis of cell function [3]. The event of permeabilization is generally monitored by measuring activity of one of the intracellular enzymes. These enzymes show negligible activity in non-permeabilized cells which could be due to the permeability barrier of

cell wall/membrane to substrate and/or products. However, upon permeabilization, there is a decrease in the permeability barrier of cell wall/membrane resulting in many-fold increase of assayable enzyme activities [4–6].

Permeabilization of whole-cell yeast for use as industrial biocatalysts, in either immobilized or suspension modes, has been achieved with organic solvents such as chloroform, ether, alcohol or toluene:ethanol mixture. Detergents, antibiotics, ethylenediaminetetraacetic acid (EDTA) and osmotic shocks, also, can cause reduction in the permeability barrier [2, 5, 7–15]. However, the toxic/non-biodegradable nature of permeabilizing agents used for permeabilization, limits the use of these permeabilized yeast cells. Here we report the permeabilization of baker's yeast (*Saccharomyces cerevisiae*) cells with *N*-lauroyl sarcosine (LS), a non-toxic, biodegradable, cationic detergent. In the present study, we choose to assay intracellular catalase activity because of its relatively high activity in baker's yeast. In recent years, catalase has been gaining industrial importance in cold-pasteurization of liquid food materials such as milk and for water treatment [16, 17]. It is also used in textile industry for removing hydrogen peroxide from fabrics [18, 19]. Under the conditions reported here, permeabilization of baker's yeast cells with LS resulted in 350-fold increase in assayable intra-cellular catalase.

Materials and methods

N-Lauroyl sarcosine was obtained from Sigma Chemical Co., USA, hydrogen peroxide (30%) was obtained from BDH Chemicals, Glaxo Laboratories Bombay Division, India, compressed baker's yeast was obtained from Vasavi Soft Drinks Pvt. Ltd., India. All other chemicals used were of reagent grade.

Permeabilization of yeast cells

One gram (wet wt) of baker's yeast cells were suspended in 1.0 ml of water containing 20 mg of freshly prepared *N*-lauroyl-sarcosine and incubated at 45 °C for 15 min with occasional shaking. After detergent treatment, cells were separated by centrifugation. The supernatant obtained was used for measuring 260 nm absorbing compounds and estimation of leaked nucleotides. The LS-treated cells were washed twice with water to remove excess detergent and was used to measure catalase activity.

Catalase assay

Catalase activity was assayed by the method of Aebi [20] in which decomposition of H_2O_2 was monitored by recording the decrease in absorbance at 240 nm ($\varepsilon_{240} = 0.00394 + 0.0002$ l/mmol per mm). The initial absorbance was approximately 0.5 OD. The decrease in absorbance was monitored continuously for at least 60 s in a spectrophotometer fitted with a recorder. One unit of enzyme activity is defined as that quantity which degrades 1 μmol of H_2O_2 per min under standard conditions.

HPLC of 5′-nucleotides

5′-Nucleotide content in the supernatant was estimated on a Shimadhzu high performance liquid chromatography (HPLC) system using a 5μ Zorbax ODS (15 cm × 4.6 mm *i.d.*) C_{18} column at 254 nm. Five microliters of supernatant was injected directly into an HPLC column. Nucleotides were eluted with 100 mM ammonium phosphate buffer (pH 4.5) at a flow rate of 1.0 ml/min at room temperature. Freshly prepared known concentrations of standard 5′-GMP, 5′-IMP, 5′-CMP, 5′-UMP and 5′-AMP were run on HPLC. Areas under the chromatogram were calculated to plot a standard graph for absolute quantification.

The results presented in this manuscript are an average of at least three experiments.

Results

Effect of detergent concentration on permeabilization

Non-permeabilized baker's yeast cells did not show any measurable catalase activity. There was also no significant amounts of 260 nm absorbing compounds in the medium in which these cells were suspended. This is mainly due to the permeability barrier of the cell wall/membrane to substrate and/or products. However, treating baker's yeast cells with LS caused perturbation of membranes, resulting in measurable amounts of catalase activity in cells. When the medium containing permeabilized yeast cells was separated by centrifugation and analyzed, it gave significant absorbance at 260 nm indicating leakage of molecules from the cell.

In order to determine whether concentration of LS in the permeabilizing medium and/or ratio between detergent and cell are important in permeabilizing baker's yeast cells, the following experiments were carried out.

1. Baker's yeast cells (1.0 g wet wt) were treated with varying amounts of LS in 1.0 ml water. Catalase activity and amount of 260 nm absorbing materials leaching out increased rapidly above 0.5% of LS and reached maximum with 2% of LS. Higher LS concentrations did not significantly increase catalase activity or the amount of low molecular weight compounds leaking out from cell (Fig. 1a). LS-permeabilized cells

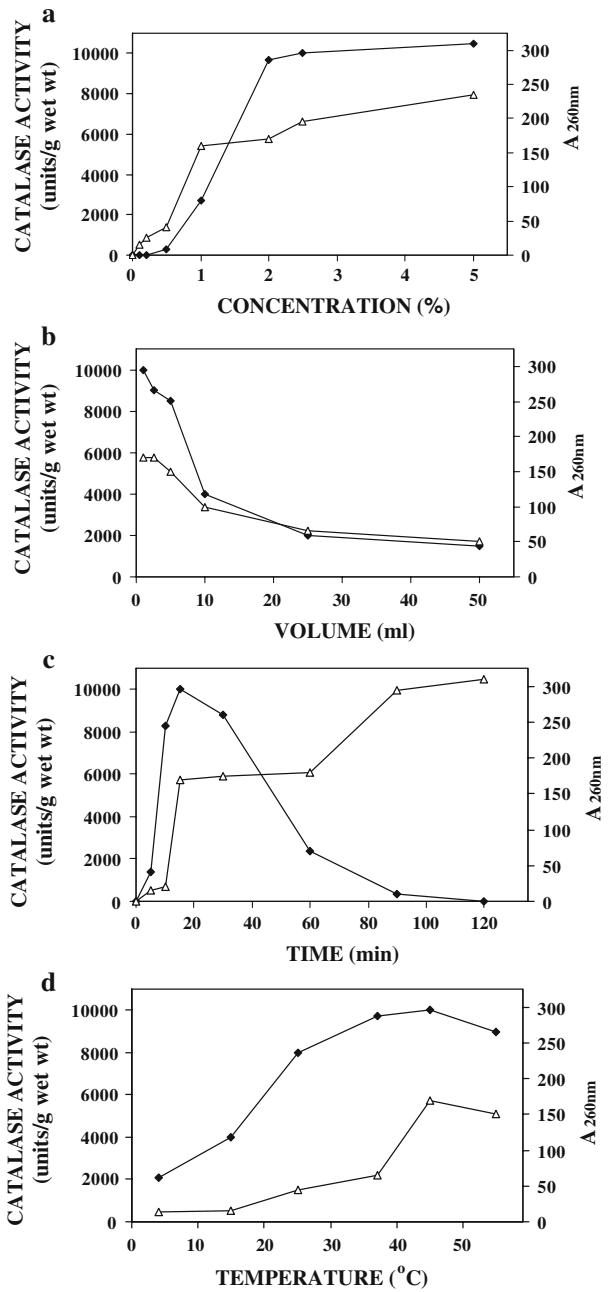

Fig. 1 Optimum conditions for permeabilization of baker's yeast (*Saccharomyces cerevisiae*) cells using LS. Baker's yeast cells were treated with detergent, *N*-lauroyl sarcosine (LS), under varying conditions. After treatment, cells were separated by centrifugation and catalase activity was measured in cells and the absorbance of the supernatant was measured at 260 nm. **a** Effect of varying concentration. Baker's yeast (1.0 g wet wt) cells was treated with 1.0 ml of different concentration of LS at 45 °C. **b** Effect of varying volume of permeabilizing medium. Baker's yeast (1.0 g wet wt) cells were treated with 20 mg of LS in increasing volumes of permeabilizing media at 45 °C for 15 min. **c** Effect of time. Baker's yeast (1.0 g wet wt) cells were treated with 1.0 ml of 2% LS at 45 °C for various time intervals. **d** Effect of temperature. Baker's yeast (1.0 g wet wt) cells were treated with 1.0 ml of 2% LS at different temperatures for 15 min. Cellular catalase (*filled square*) and $A_{260\ nm}$ absorbing compounds released (*open triangle*) from cells were determined as described in "Materials and methods"

showed approximately 350-fold increase in catalase activity compared to control cells that were not treated with LS.

2. Baker's yeast cells (1.0 g wet wt) were treated with 20 mg LS in increasing volumes (1–50 ml) of permeabilizing medium, for 15 min at 45 °C. Results showed that catalase activity and 260 nm absorbance of supernatant was maximum when yeast cells were treated in 1.0 ml of detergent medium (Fig. 1b).

Effect of contact time for incubation of cells with detergent on permeabilization

When baker's yeast cells (1.0 g wet wt) were incubated with 1.0 ml of 2% LS at 45 °C, for different time intervals, maximum assayable catalase activity was achieved within 15 min. Thereafter, there was a decrease in assayable catalase activity and almost no cellular catalase activity was detected after 90 min of incubation. Permeabilization monitored by measuring 260 nm absorbing material indicated that maximum permeabilization took place within 15 min. Thereafter, released UV-absorbing material was almost constant up to 60 min, after which there was a significant increase in the 260 nm absorbing materials (Fig. 1c).

Effect of temperature on permeabilization

To study the effect of temperature on LS-permeabilization, yeast cells were treated with 1.0 ml of 2% LS for 15 min at different temperatures. Maximum catalase activity was detected at 45 °C. Above 45 °C, a slight decrease in catalase activity was observed. Release of 260 nm absorbing material was also maximum at 45 °C (Fig. 1d). There was significant catalase activity even at lower temperatures (97% at 37 °C and 80% at 25 °C), compared to very low release of 260 nm absorbing materials (40% at 37 °C and 28% at 25 °C).

Absolute quantification of 5′-nucleotides leaked during permeabilization

Supernatant obtained by centrifuging the LS-treated baker's yeast cells, under optimum conditions, was analyzed for 5′-nucleotide (5′-GMP, 5′-IMP, 5′-CMP, 5′-UMP and 5′-AMP) content by HPLC. The different 5′-nucleotides were identified based on retention time of standard compounds and by spiking the supernatant with standard 5′-nucleotides. One gram wet weight of baker's yeast cells released about 37 mg of 260 nm absorbing materials, of which 6.5 mg was 5′-GMP, 5.5 mg was 5′-IMP, 6.0 mg was 5′-CMP, 6.4 mg was 5′-UMP and 7.0 mg was 5′-AMP (Fig. 2).

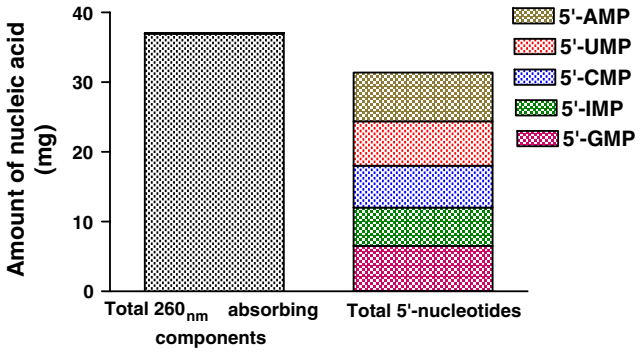

Fig. 2 Amount of 5′-nucleotides released during the process of permeabilization. Total nucleic acid content (quantitated from 260 nm absorbance) and amount of 5′-nucleotides (quantitated by HPLC, as described in "Materials and methods")

Discussion

Permeabilized baker's yeast (*Saccharomyces cerevisiae*) cells can be a rich source of number of industrially important enzymes [21, 22]. However, when whole-permeabilized cells are to be used for bioconversion of substrates for human consumption, it is very important that cells are permeabilized using agents that are not toxic and have GRAS status. In this study, we have used LS, a non-toxic, biodegradable detergent with GRAS status, to permeabilize baker's yeast cells. LS is readily metabolized by humans to sarcosine and corresponding fatty acids. Sarcosine, which is ubiquitously present in the body, is rapidly degraded to glycine [23].

Different permeabilization treatments result in different permeability alterations, thereby making it difficult to follow the same protocol for permeabilizing cells with different agents. Therefore, it is very important to optimize conditions for permeabilizing whole cells with various agents which will not induce the leakage of intracellular enzymes. Results to the contrary would indicate disintegration of yeast cell membranes rather than their permeabilization which will not serve our purpose of producing whole-cell catalyst. Also whole-cell catalyst can be separated by centrifugation and reused, something that cannot be achieved with cell-free extracts. To determine the optimum conditions of permeabilization of the baker's yeast cells (*Saccharomyces cerevisiae*) with LS, effect of detergent concentration, incubation time and temperature were investigated in this study.

We measured the amount of 260 nm absorbing compounds leaking from cells to monitor the event of permeabilization. They were analyzed spectrophotometrically in the supernatant that was obtained by centrifuging cells after detergent treatment. Permeabilization is known to release endogenous low molecular weight compounds, mainly believed to be nucleotides, from the cell's interior. So monitoring them can give a measure of the progress of permeabilization. However, since only small molecular weight compounds leach out, the amount of leachate alone may be an inaccurate measurement of optimum permeabilization conditions. Also, certain permeabilizing agents like CTAB, ethyl alcohol, isopropanol, etc., are known to precipitate nucleotides [24]. Therefore, when such detergents and solvents are used for permeabilization, measurement of 260 nm absorbance of leachate cannot give an accurate measurement of the degree of permeabilization. Alternatively, assay of intracellular enzyme activity, such as catalase, lactose dehydrogenase, alcohol dehydrogenase, etc., [25] in the permeabilized cells, is employed for monitoring the process of permeabilization. In the present study, we monitored catalase activity in cells to measure permeabilization. However, if there is loss of enzyme activity due to inactivation, monitoring enzyme activity alone may give a false indication of the progress of permeabilization. Therefore, in our current study we monitored permeabilization event both by measuring intracellular catalase activity and by analyzing the 260 nm absorbing compounds leaking out during permeabilization process.

Presence of salts in the solution in which the detergent is dissolved can effect permeabilization. But in our study, we observed that buffer salts had little to no effect on the event of permeabilization. Catalase activity and amount of 260 nm absorbing materials released during permeabilization were equivalent when either buffer (50 mM sodium or potassium phosphate buffer pH 7.0) or distilled water was used as the permeabilizing medium (results not shown). Since it is easier and less expensive to use water in an industrial setting, we carried out all our experiments in water.

The concentration of detergent plays an important role in determining if the cell is being permeabilized or lysed during the treatment with detergent. Also differential affinity of the detergent to the cell wall/membrane could affect the permeabilization process. In order to determine whether concentration of LS in permeabilizing medium and/or ratio between detergent and cell was important in permeabilizing baker's yeast, the cells were incubated with varying concentrations of the detergent (Fig. 1a, b). With decreasing concentration of detergent, there was a decrease in assayable catalase activity and in the amount of low-molecular weight compounds leaking out of cells (Fig. 1b). This suggests that the affinity of LS to yeast cell wall/membrane is low. This is in contrast to earlier reports of CTAB permeabilization of *Saccharomyces cerevisiae* and *Kleuveromyces fragilis* [4, 26] where only the cell to detergent ratio was found to be crucial for permeabilization and not the final concentration of detergent in the medium. Similar observations on the importance of effec-

tive detergent concentration were also reported on the permeabilization of *Rhodotorula gracilis* with CTAB [27] and in *Yarrowia lipolytica* cells by Triton X-100 [28], where effective detergent concentration was found to be a key factor rather than the cell to detergent ratio, for optimum permeabilization.

The amount of time a cell is exposed to detergent also plays a role in determining whether the cell is permeabilized or getting lysed. We found that the time required for LS to reach optimal permeabilization was about 15 minutes. Though, catalase activity started to decrease after 15 min of incubation with detergent, no corresponding increase in catalase activity was detected in the supernatant. This suggests self-inactivation of catalase or degradation of catalase protein by the endogenous proteases. Amount of 260 nm absorbing materials leaking from cells reached a maximum within 15 min of incubation with LS and was constant up to 60 min. Thereafter, there was a significant increase in 260 nm absorbing materials (Fig. 1c). This later increase may be due to release of digested intracellular components/organelles by endogenous hydrolytic enzymes like proteases.

Based on catalase activity and amount of 260 nm absorbing materials that leaked from the baker's yeast cells, optimal temperature for permeabilization with LS seem to be 45 °C. However, significant catalase activity was seen even at lower temperatures, about 97% at 37 °C and 80% at 25 °C, compared to very low release of 260 nm absorbing materials, about 40% at 37 °C and 28% at 25 °C (Fig. 1d). This indicates that at lower temperatures, LS cause enough membrane distortion or alteration through which relatively neutral H_2O_2 can cross cell wall/membranes, whereas negatively charged, larger nucleotides are held back within the cells. This result further proves that monitoring one event of permeabilization is not sufficient to correctly gauge permeabilization process.

Under the conditions standardized in this study, catalase activity was seen only in LS-treated cells. No catalase activity was found in the supernatant, which was obtained when baker's yeast cells treated with LS, was centrifuged. This further indicates that baker's yeast cells were permeabilized and not lysed. Lysis of yeast cells would have resulted in measurable catalase activity even in supernatant. Catalase is known to undergo self-inactivation during catalysis which could also account for non-measurable catalase activity in the supernatant. However, significant catalase activity was retained within the cells even after 11 cycles of reuse of permeabilized cells for catalase activity [5]. This further implies that the yeast cells are not lysed under the conditions employed for permeabilization.

The exact mechanism by which LS permeabilizes baker's yeast cells is not known. Based on the structure of LS, at least three different mechanisms by which LS permeabilize baker's yeast, can be proposed. First, detergents at or above their critical micellar concentration (CMC) interact with cell membranes and form micellar structures of cooperatively bound detergents and membrane lipids [29–31] which causes breakdown of cell wall/membrane. Second, dodecanoyl tail (C_{12} group) of LS is hydrophobic in nature, and can penetrate into the lipid bilayer through interactions with hydrophobic tails of the phospholipid component of yeast cell wall/membrane. These hydrophobic inclusions perturb the transversal organization of membrane which may result in the formation of well defined pores [32, 33] through which smaller molecules can get in and out of the cells. Third, electrostatic forces can also permeabilize yeast cells. Phosphate groups of cell wall/membrane protein renders a negative charge to yeast cell surface. Cationic ends of LS can bind to negatively charged membranes through electrostatic interactions [33]. This creates a disturbance in the cell wall/membrane which facilitate the permeabilization process. Actual permeabilization could be due to a combination of the above mechanisms.

Yeast is a rich source of 5′-nucleotides which find applications as flavor enhancers, wound healers and immunoprotective agents in food and pharmaceutical industry. Since these 5′-nucleotides are relatively small compounds there is a possibility that these nucleotides are released during the event of permeabilization. So we analyzed the supernatant that was obtained by centrifuging baker's yeast cells treated with LS under the optimum conditions, for 5′-nucleotides. About 80% of the measured low molecular weight, 260 nm absorbing components released during permeabilization were in fact 5′-nucleotides (Fig. 2). Since the protocol standardized above utilizes non-toxic detergent, we could exploit this supernatant without further purification for its 5′-nucleotides content (Indian Patent No. IN192068).

In permeabilized whole cells, enzymes are in close proximity with other cellular organelles which stimulate in vivo environment of the enzymes. Therefore, enzyme activity within permeabilized whole yeast cells tend to be more stable than in purified form. In fact appreciable enzyme activity is observed even after repeated use of permeabilized cells for bioconversion [5, 34]. This allows use of permeabilized cells as an economically viable alternative to purified enzymes. It also alleviates problems and cost associated with enzyme isolation and purification. The method, described here, is simple enough for industrial level scale-up (Indian Patent No. IN178895). Though we have standardized the permeabilization technique by assaying catalase activity, these permeabilized cells can be exploited for any endogenous enzymes present in baker's yeast. In conclusion, the LS-permeabilized baker's yeast cells could be used as an alternative biocatalyst for analytical and preparative purposes.

Acknowledgments The authors would like to thank Dr. Lalitha R Gowda, Scientist, Protein Chemistry and Technology, CFTRI, for useful suggestions and discussions. Jessy Abraham would like to thank DBT for the award of Junior Research Fellowship and CSIR for the award of Senior Research Fellowship.

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