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Permeabilization of *Aspergillus niger* by Reverse Micellar Solutions and Simultaneous Purification of Catalase

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Abstract: Reverse micellar solutions(RMS) of sodium bis- (2-ethylhexyl)-sulfosuccinate (AOT) in aliphatic organic solvents were used for permeabilization and protein removal directly from *Aspergillus niger* cells. Most of the cell wall proteins (~95–100%) were solubilized into the reverse micelles solutions. A significant fraction of intracellular catalase (26–30%) permeates out of the cells and remains on the cell surface and is recovered in a cell wash. Because of it's size the catalase in not solubilized in the RM water pools and thus is not detected in the stripping solution used to recover other proteins from the organic reverse micellar solutions. The remaining amount of catalase is recovered by breaking the cells in purer form. Multiple extractions were used for the extraction of the cell wall proteins followed by ultrasonication of the cells to recover intracellular catalase in a purified form. Therefore, catalase with 5 fold purification was recovered in 88% yield from the RMS- treated cells.

Keywords: *Aspergillus niger*, catalase, reverse micelles, permeabilization, multiple extraction

INTRODUCTION

Cell disruption becomes a necessity when a desired product is intracellular (1). This step, however, complicates the downstream processes for the recovery of target protein(s) because of the complex mixture generated by the total break-up of the cells. A promising alternative to mechanical disruption is to induce

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the protein release by chemically altering the cell envelope structure to allow diffusion of the product out of the cell. Microbial cell permeabilization has been achieved by employing osmotic shock, chaotropic agents, detergents, solvents, enzymes, antibiotics, biochemicals, and chelating agents (2–7).

Protein extraction using reverse micelles in a liquid/liquid extraction mode has been demonstrated as a potential technique for recovery of extracellular proteins from fermentation broth (4–17). Advantages like selective recovery, reduced product contamination, and denaturation have attracted attention of several researchers (8–17). The synergism of a surfactant and an organic solvent to selectively recover intracellular dehydrogenases by lysing whole bacterial cells of *Azatobacter vinelandii* was first attempted by Giovenco et al. (18). Other studies of cell permeabilization by surfactant are reported only in aqueous solutions (3–6). The reverse micellar solutions (RMS) aided permeabilization have also been reported for selective recovery of penicillin acylase and alkaline phosphatase from *Escherichia coli* and recovery of enzymes from different cellular locations of *Saccharomyces cerevisiae* cells (19–22). It is proposed that the surfactant molecules adsorb on the cellular surface when the microbial cells are suspended in the RM solutions (20). The hydrocarbon chains of these adsorbed surfactant molecules provide a passage to the hydrocarbon solvent to access the lipid membrane structure. Once some of the phospolipids from the cellular structures are solubilized into the organic solvents, diffusion of proteins from the cell wall/membrane and/or periplasmic space is facilitated. These proteins either are solubilized in the reverse micellar water pools if their sizes and charges are appropriate or remain on the cell surface and can be recovered by a water wash to the cells (21, 22). These reports reflect the potential of RMS in an easily scalable permeabilization process giving selective recovery of intracellular enzyme/proteins as a substantially purer product.

The present work investigates the ability of RMS to permeabilize *Aspergillus niger* cells for the removal of proteins from the cellular structure and purification of catalase as a model intracellular enzyme, with an objective of extending the process to other microbial species. *Aspergillus niger* is well known to produce a number of enzymes, organic acids, antibiotics, and mycotoxins. The success of *A. niger* for industrial production of biotechnological products is largely due to the metabolic versatility of this strain. Fungal cell is very rigid owing to its tough cell wall comprising of chitin and polysaccharides-glucans and mannans.

The unique mechanical, morphological and biological properties of fungal cell walls are based on their chemical composition and spatial arrangement of the individual cell wall polymers (3). Polysaccharides constitute almost 80–90% of the dry matter of the fungal cell wall. Chitin and glucans impart mechanical strength to the cell wall while the amorphous homo- and hetero-polysaccharides are the cementing substances. The general picture is that of microfibrillar skeletal wall components, such as β-glucan, chitin, and/or cellulose, embedded in an amorphous

polysaccharide, and protein-polysaccharide matrix. The outer surface of the wall is usually smooth or slightly rough, whereas the polysaccharide microfibrils are more prominent on the inner surface of the wall. The lipid content in the fungal cell walls is much less as compared to 8% lipids in the yeast cell wall which makes fungal cells very hydrophilic (3, 23).

The effect of RMS on fungal cell is studied in the present work for total protein removal and to improve the purity of catalase. Aqueous solutions of various surface active compounds have also been investigated for their efficacy in fungal cell permeabilization for comparison.

MATERIALS AND METHODS

Materials

Organic solvents, sodium bis-(2-ethylhexyl)-sulfosuccinate (AOT), KH_2PO_4 and NaH_2PO_4, were obtained from S.D. Fine Chemicals, Mumbai. Cetyl trimethyl ammonium bromide (CTAB) was obtained from Spectrochem, Mumbai and sodium dodecyl sulfate (SDS) from SRL, Mumbai. Potato dextrose agar, sucrose, $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, citric acid, and $CaCO_3$ were procured from HiMedia Lab. Ltd., Mumbai. Hydrogen peroxide and sodium nitrate were obtained from Merck (I) Ltd. Corn steep liquor (CSL) was procured from Anil Starch Ltd., Ahmedabad.

Aspergillus niger (NCIM 545) was obtained from the National Chemical Laboratory, Pune. Its culture was maintained on potato dextrose agar in test tubes at 25–30°C. The fungus was grown in an optimized medium containing sucrose (7%), KH_2PO_4 (0.025%), $MgSO_4 \cdot 7H_2O$ (0.025%), $FeCl_3 \cdot 6H_2O$ (0.001%), CSL (2%), citric acid (0.025%), $NaNO_3$ (0.4%), and $CaCO_3$ (2%) at pH = 7.0 and temperature 28–30°C. Fermentation was carried out in 50 cm^3 medium in 250 cm^3 conical flasks for 48 hours on a rotary shaker (200 rpm). The cells were harvested from the broth by centrifugation at 15,000 × g for 25 minutes and the harvested cells were washed thrice with distilled water to remove broth proteins adhering to the cells, if any, and again centrifuged.

Cell Disruption by Ultrasonication

The cells (~1 g of wet mass), recovered by centrifugation from 50 cm^3 of broth, were suspended in 20 cm^3 distilled water and subjected to ultrasonication using an ultrasonic horn of 20 mm diameter (Dakshin make, Mumbai) at a frequency of 22.5 kHz in an ice-bath for 60 minutes. During the ultrasonication, the temperature of the suspension was allowed to increase to a maximum of 12°C by frequently pausing the process. The cell debris was separated by centrifugation at 15,000 × g for 20 minutes and the supernatant was subjected to the enzyme and protein analysis. The enzyme recovery reached

to a maximum after 40 minutes of ultrasonication followed by a drop in the enzyme activity. However, the protein content increased continuously, as monitored up to 60 minutes. The highest specific activity of the catalase (168 Units/mg), defined as the ratio of the enzyme activity (Units $\cdot$ cm^{-3}) to the protein content (mg $\cdot$ cm^{-3}), was obtained after 40 minutes of ultrasonication of the cells. These values of the enzyme/protein content in the crude extract, as obtained by directly ultrasonicating the cells, are used as a basis for comparison of the recovery of proteins and the enzyme in further studies using reverse micellar solutions.

Cell Permeabilization using Aqueous Surfactant/Hydrotrope Solutions

The cells ($\sim$1 g of wet mass), recovered by centrifugation from 50 cm^3 of broth, were resuspended in 20 cm^3 aqueous solution of a surface active agent and mixed on a rotary shaker at 200 rpm for 60 minutes. After harvesting the cells from the suspension, the supernantent solution was analyzed for the protein content and enzyme activity.

Cell Permeabilization using Reverse Micellar Solutions

A 200 mM reverse micellar (RM) solution of AOT in a selected organic solvent was prepared. The water content (w_o, moles of water/mole of surfactant) was adjusted by directly adding 10 mM phosphate buffer as forward extraction(FE) buffer solution of pH = 7.0 to the AOT solutions.

For the forward extraction, thoroughly washed cells ($\sim$1 g) were suspended in 10 cm^3 of a RM solution in a 100 cm^3 stoppered conical flask and subjected to agitation for 1 hr on a rotary shaker at 180–200 rpm at 28–30 °C. After sufficient time of contact, the cells were separated from the solution by centrifugation at 15000 $\times$ g for 20 minutes.

The supernatant organic RM solution was contacted with an aqueous stripping solution of equal volume (pH = 7.0 and 0.5 mM NaCl) on a rotary shaker for 15 minutes for recovery of proteins from the RM solutions into the back extract aqueous phase. The aqueous and organic phases were separated from each other using a separating funnel. The aqueous phase was analyzed for the enzyme and protein contents. No catalase was detected in any of these back extract solutions.

The cells, harvested from the RM solution suspension, were washed with distilled water to recover proteins/enzyme adhering to the cell surface, if any. The enzyme/protein, that may have been liberated from the fungal cells but because of bigger size are unable to get solubilized in the RM phase, get recovered in such cell wash. The cell wash was also subjected to the protein and enzyme analysis.

The washed cells were then resuspended in 15 cm^3 of distilled water and subjected to ultrasonication for 10–15 minutes. This was done to ensure the complete release of intracellular proteins, in addition to the above two

partial protein-recovery steps. Cell debris was removed by centrifugation at $15{,}000 \times g$ for 15 minutes and the supernatant was subjected to the enzyme/protein analysis.

The effect of agitation and that of cell suspension density on cell dispersion quality and permeabilization in RM solutions, was studied in a 100 cm^3 round-bottom fully baffled cylindrical vessel with an inner diameter of 6 cm and a height of 15 cm, fitted with a 2 cm pitch 6-bladed turbine impeller. While studying the effect of agitation the impeller speed was varied from 250–1000 rpm and 5% wet cell loading was maintained in 200 mM AOT/iso-octane RM solution. The water content ($w_o = 50$) of the RM solution was adjusted with 10 mM phosphate buffer of pH = 7.0 as a forward extraction(FE) buffer. After 1 hr of the forward extraction, the cells were separated from the RM solution. The cell-free clear RM solution was contacted with an aqueous stripping solution for the back extraction using an orbital shaker at 200 rpm for 20 minutes at 30°C. The aqueous phase, after separation from the organic phase, was analyzed for total proteins and the catalase activity.

For the multiple extraction experiments, the entire procedure was repeated thrice with the same cell mass except the cell washing step, each time using a fresh RM solution of the same composition. Our objective here was to solubilize as much proteins/enzyme as possible by multiple contacts with RM solutions. After the third extraction, the cells were thoroughly washed and resuspended in 20 cm^3 distilled water before sonication for 15 minutes. The sonicated samples were then centrifuged at $15{,}000 \times g$ to remove the cell debris. The supernatant after sonication and the cell wash obtained before the sonication of the cells were analyzed for the total proteins and the catalase recovery.

Analytical Techniques

The total protein content was determined by a modified Folin-Lowry method (24). In the case of surfactant- and hydrotrope-treated cell samples, because of their interference with the Folin-Lowry method, the protein content was determined by direct absorbance measurement at 280 nm by taking appropriate reference solutions.

The catalase activity was determined by measuring the decrease in the absorbance with time, due to the decomposition of the substrate H_2O_2 with catalase at 240 nm. One unit of catalase activity is defined as the amount of enzyme that catalyzed the decomposition of one μmole of H_2O_2 per minute at 25°C and pH = 7.0 (25).

The purification factor of the enzyme is defined as the ratio of specific activity of the catalase recovered after the RM treatment to that obtained in the crude extract after direct ultrasonication of the untreated cells for 40 minutes.

RESULTS

A. niger cells subjected to direct ultrasonication for 40 minutes without the RM-treatment released 28 mg of total proteins and 4700 units of catalase activity per gm of the fungal cell mass [specific activity = 168 IU/mg].

Effect of Aqueous Surfactant and Hydrotrope Solutions

Figure 1 shows the effect of surface active compounds in aqueous solutions on the protein recovery from the cells. The anionic surfactants, AOT (10 mM) and SDS (10 mM), released 26% and 80% of the total proteins from the cells, respectively, compared to ~30% of the proteins released by aqueous solutions of a cationic surfactant, CTAB, of the same concentration. The non-ionic surfactant, Triton X-100 (0.5%), caused ~40% release of total proteins. Since SDS is known to bind and denature the proteins, it was not tested further even though it could release a substantial amount of the proteins from the cells.

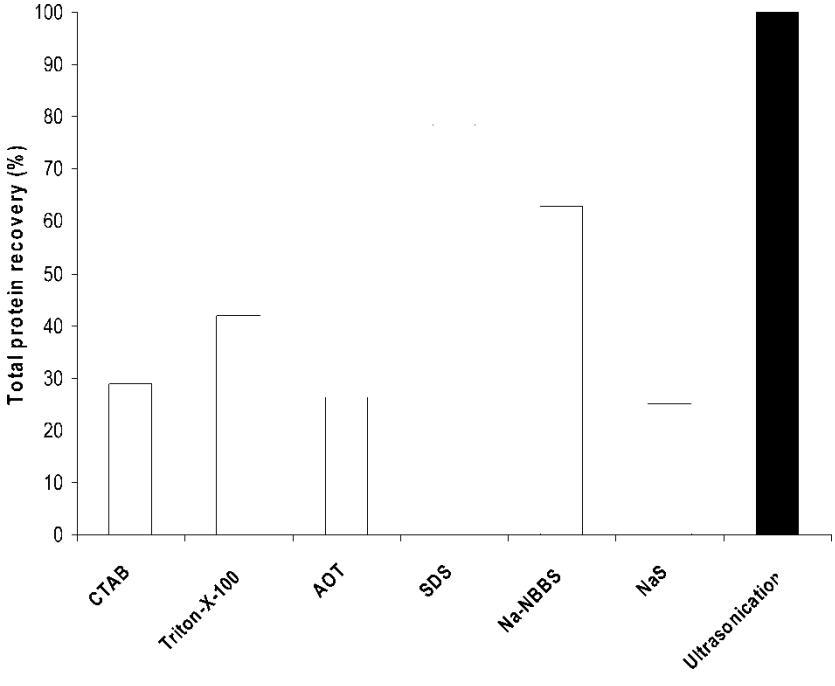

Figure 1. Effect of aqueous solutions of various surfactants and hydrotropes on *A. niger* cell permeabilization. [CTAB] = 10 mM; [SDS] = 10 mM, [AOT] = 10 mM; [Tritin-X100] = 0.5% [NaNBBS] = 50 mM' [Na-S] = 400 mM; Time of treatment = 60 min; Cell loading = 5%.

Hydrotropes are hydrophilic and weak surface-active agents, which have the ability to permeate plant cells and extract active constituents (26, 27). Our recent attempts of permeabilization of yeast cells using hydrotropes have shown up to 70% protein recovery with sodium n-butyl benzene sulfonate(Na-NBBS) (22). Sodium salts of *n*-butyl benzene sulfonate (Na-NBBS)(50 mM) and salicylate (NaS)(400 mM) were, therefore, tried to permeabilize *A. niger* cells. Na-NBBS caused 63% protein recovery while NaS released only ~25% of the total proteins from the cells in single stage operations. However, the catalase release from the cells could not be checked due to interference by the hydrotropes in the analysis. The enzyme denaturation by hydrotropes also can not be ruled out.

Solvent Effect

A. niger cells were initially contacted with pure organic solvents — hexane, heptane, and isooctane. In the absence of the surfactant, there was no dispersion of the cells at all in any of these solvents. The cells remained clumped at the bottom of the vessel suggesting that the solvent alone is unable to wet the cells owing to the aqueous coating on the cell surface. In the absence of insufficient dispersion, the organic solvent would not have any permeabilizing effect on the cells. However, in the presence of AOT, the cells spontaneously dispersed in hexane, heptane, and isooctane, indicating a definite relation between the surfactant adsorption on the cell surface and the cell dispersion. All the further experiments were conducted using AOT which is a well characterized surfactant for the formation of reverse micelles.

The AOT/organic solvent solutions with w_o of 50 formed a transparent micro-emulsion. Only in the case of hexane, the AOT/hexane/water system formed a biphasic *macro-* and *micro-* emulsion. The effect of the RM treatment of *A. niger* cells with different hydrocarbon/AOT/water systems on the total protein release in the RM solution (recovered in the aqueous stripping solution after back extraction), and the catalase recovery in the cell wash before ultrasonication of the cells as well as the catalase recovery by sonication of the RM treated cells are given in Table 1. The cell wash of the RM-treated cells showed 26–30% catalase activity recovery but no enzyme activity was detected in the aqueous back extract solution, i.e. the reverse micellar solution showed no enzyme activity (Table 1). RM solutions are, therefore, indeed able to permeabilize the cells to an extent that the catalase is translocated from the cytoplasm to the cell surface but is not solubilized in the reverse micellar water pools but probably remains on the cell surface. This catalase is recovered in the cell wash before breaking the cells. Upon sonicating the RM-treated cells, the remaining catalase (~60%) was recovered giving a total of 90% enzyme recovery. All the further enzyme recovery values are given as total recovery of the enzyme in

Table 1. Effect of reverse micellar solutions in aliphatic hydrocarbons on fungal cell permeabilization and catalase recovery/purification

System	% Proteins recovered	% Catalase activity recovered in cell wash of RM treated cells	% Catalase activity recovered after ultrasonication of RM treated cells
AOT/Hexane	61	26	60
AOT/Heptane	68	33	61
AOT/Iso-octane	65	29	62
AOT/Decane	25	8	—

200 mM AOT; Cell loading: 10%; $w_o = 50$.
FE buffer: 10 mM phosphate buffer, pH = 7.0; Contact time: 1 hour.
BE buffer: 0.5 mM NaCl in phosphate buffer, pH = 7.0.
Sonication time: 40 minutes.

the cell wash and after the sonication of the RMS treated cells. There was no significant difference in the total proteins and the catalase recovery with hexane, heptane, and isooctane. This suggests that the aliphatic hydrocarbon chain length has no apparent effect on the extent of cell permeabilization unlike that reported in previous studies on the permeabilization of *E. coli* cells (19–21). However, the purification factor was relatively lower in the case of AOT/heptane system (~1) as compared to that with AOT/hexane (~2.5) and AOT/isooctane (~2) systems.

Decane, on the other hand, formed a highly viscous reverse micellar system. The total protein and enzyme recoveries were the least with decane as the solvent. Since this solution was viscous, the suspension of the cells was not proper which leads to poor recovery in AOT/decane/water system.

Effect of Water Content

The AOT/iso-octane/water system was selected for further studies because of the formation of larger reverse micelles and less volatility of isooctane compared to that of hexane (20). A transparent monophasic system was formed up to $w_o = 70$, beyond which the system became biphasic.

Figure 2 shows that the total protein recovery decreased from ~56% (at $w_o = 10$) to ~39 % (at $w_o = 120$). At $w_o = 120$, the system was highly viscous and formed a stable *macro*-emulsion.

Effect of Cell Loading

Table 2 shows the effect of the cell loading on the total protein recovery. With 10% cell loading, 30% of the total proteins could be recovered from the

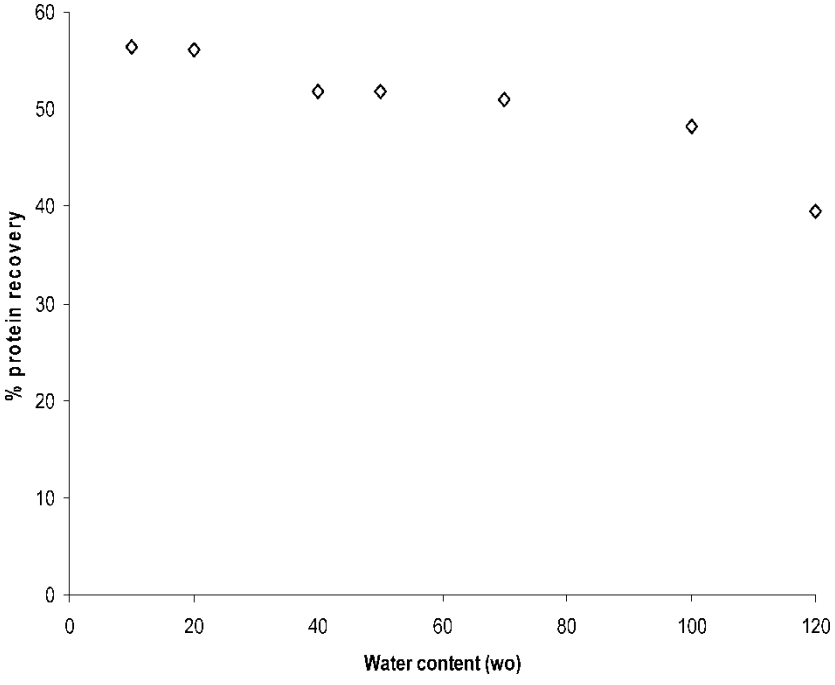

Figure 2. Effects of water content of AOT reverse micelles on release of total proteins from *A. niger* cells; Forward extraction: 200 mM AOT; Cell loading: 10%; w_o adjusted with 10 mM phosphate buffer, pH = 7.0; Contact time: 1 hour; Backward extraction from RM solution: stripping solution = 0.5 mM NaCl in phosphate buffer, pH = 7.0.

reverse micellar solution while 70% protein recovery was obtained with 0.5% cell loading. There was no catalase activity observed in the back extracted solutions indicating that the catalase was not released from the cells into the reverse micellar water pools during this step. There was a mild effect of the cell loading on the catalase activity recovered after ultrasonication of the RM-treated cells, which increased from 78% (at 0.5% cell loading) to 95% (at 10% cell loading). The low cell loading led to a better cell dispersion in the RM solutions but the biomass recovery decreased by 20–25%. It was also noticed that the enzyme recovery was the best within 10 minutes of the sonication to break cells. A longer time of ultrasonication led to the loss of the enzyme activity. Thus sonication time of the RM treated cells was restricted to 10 minutes.

Effect of Speed of Agitation

A 5% cell loading was chosen for multiple extractions and for studying the effect of agitation. A minimum of 250 rpm was required to render proper

Table 2. Effect of cell loading of *A. niger* in reverse micellar solutions on release of total proteins and catalase recovery

	After back extraction from RM solutions	After sonication	
% cell loading	% protein	% Protein recovery	% Total catalase recovery
0.5	73	26	88
1.0	65	32	89
2.5	58	42	94
5.0	46	49	93
10.0	31	61	95

200 mM AOT; $w_o = 50$.
FE buffer: 10 mM phosphate buffer, pH = 7.0; Contact time: 1 hour.
BE buffer: 0.5 mM NaCl in phosphate buffer, pH = 7.0.
Sonication time: 10 minutes.

cell dispersion in the RM solutions. Table 3 shows no significant effect of the speed of agitation (250 to 1000 rpm), on the total protein recovery. If the intracellular migration of the species is the controlling factor in the extraction rate then the external conditions can be unimportant as observed in the present studies. After three extractions (i.e. three contacts) of cells, each time with a fresh RM solution, ~90% proteins were recovered from the RM

Table 3. Effect of agitation speed on total protein release (%) by multiple extractions in a stirred vessel

	Protein recovery from RM solutions			Total catalase reovery in cell wash and after ultrasonication of RM treated cells	
RPM	1st extraction	2nd extraction	Cell wash	% catalase activity recovered	Purification factor
Shake flask				88	1.8
250	46.6	73	92	60	4.55
500	48.3	70	91	46	4.81
1000	42.2	63	93	28	4.37

200 mM AOT; Cell loading: 5%; $w_o = 50$.
FE buffer: 10 mM phosphate buffer, pH = 7.0; Contact time: 1 hour.
BE buffer: 0.5 mM NaCl in phosphate buffer, pH= 7.0.
Sonication time: 10 minutes.

solutions. However, after sonicating the cells, the catalase recovery was found to be severely affected (Table 3) as seen by the drop in activity from 60% (~2810 IU/gm) at 250 rpm to 28% (~1300 IU/gm) at 1000 rpm. Moreover, the purification fold also decreased, though slightly from 5.2 to 4.4. The above results varied from those obtained in the shake-flask studies, wherein the catalase recovery was up to 88%. Thus it was observed that the agitation speed might help in increasing the recovery rate of the proteins but on the other hand may possibly affect the stability of the desired and other enzymes.

Release Rate of Catalase from the RM-treated Cells

The rate of release of catalase was checked during sonication of the RM-treated cells and the results were compared with those of untreated cells. After two extractions with RMS the thick mycelial network of the *A. niger* cells was visibly loosened. During the sonication of the RM-treated cells, the rate of release of catalase was faster as compared to its release from the untreated cells. The catalase activity from the untreated cells was maximum (4700 IU/gm cells) after 40 minutes of sonication while after the RM-treatment, 80% catalase was recovered in just 10 minutes of sonication with a 5 fold increased purity (Figures 3 & 4). The loss of catalase activity in subsequent 10 minutes can be attributed to the shear sensitivity of the enzyme. It is possible, therefore, that the enzyme may not get destabilized by the reverse micellar treatment but gets denatured by the high shear conditions during ultrasonication. A short time of ultrasonication should be industrially advantageous to recover the maximum activity of the enzyme.

A precipitate was formed when the cells were kept in contact with the RMS overnight for a period of 14 hr at 25°C. The precipitate was soluble only in boiling H_2SO_4 (98%) and also tested positive for the common Phenol-sulfuric acid test for polysaccharides (28). This indicates that the protective polysaccharide layers of the fungal cells were affected by the RM treatment.

DISCUSSION

Detergents and organic solvents have been used to permeabilize microbial cells, but their exact mechanism of action is still under investigation (4–6). The treatment of fungal cells with ether and of *E.coli* cells with toluene caused permeabilization by disrupting their plasma membranes, followed by internal membrane disorganization (5). DMSO influences the membrane fluidity in yeast cells by extracting sterols (3). Various surfactants like Triton X-100, SDS, Tween 80, and CTAB have been used to permeabilize fungus and mainly yeast cells (3–7).

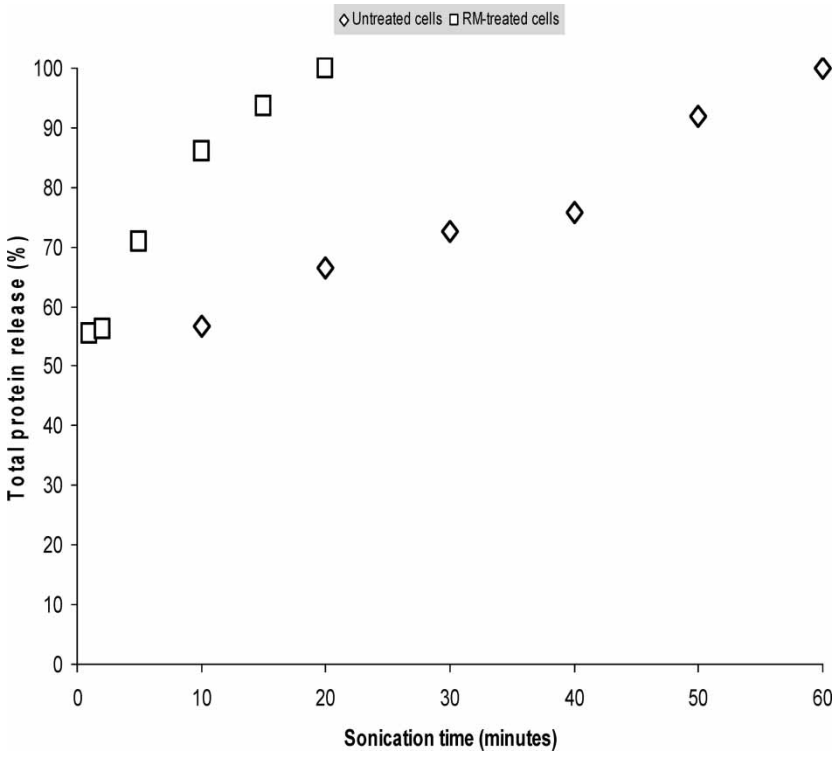

Figure 3. Comparison of release rate profiles of total proteins from untreated and RM-treated cells.

Surfactants and hydrotropes probably act by interacting with polysaccharide chains on the cell wall surface, leading to the cell permeabilization. The lower protein recovery with hydrophobic AOT than that with hydrophilic SDS suggests that the hydrophilicity of a surfactant plays a role in the cell permeabilization. Fungal cells when contacted with pure organic solvents—hexane, heptane, and isooctane were not at all dispersed. These apolar solvents, probably, are unable to wet the cells owing to their hydrophilic cell surface. The adsorption of AOT on the cell surface, however, facilitates the cell dispersion and the contact of organic solvent with the cellular surface. The AOT/organic solvent reverse micellar systems showed 45–50% total protein recovery as compared to ~25% recovery with the aqueous solution of AOT of the same concentration. This increased protein recovery means that AOT alone is not responsible for the cell permeabilization but the solvent augments its action.

The solvent along with the surfactant should form reverse micelles of dimensions suitable for specific protein solubilization. The relation between surfactant and solvent that governs the size and shape of the micellar

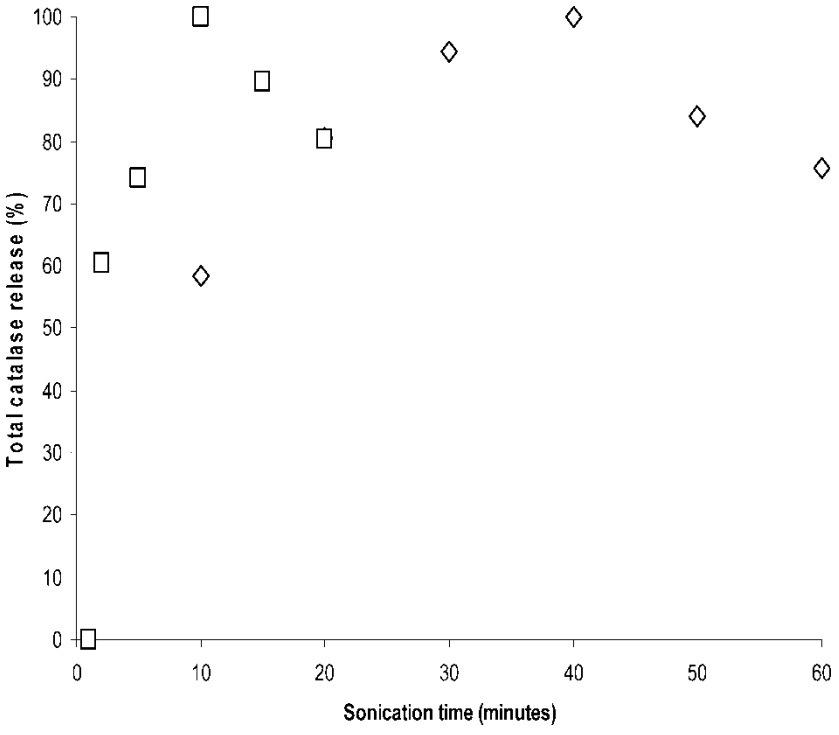

Figure 4. Comparison of release rate profiles of catalase from untreated and RM-treated cells.

aggregates is explained by the packing factor, $(p = v/a.l)$, which depends directly on the volume (v) of hydrocarbon chain (of length l) and effective area per polar head group (a) of the surfactant. The tail length l_c is fixed for a particular solvent, while a is determined by a balance of the opposing forces, chiefly minimization of hydrocarbon-water contact versus the head group repulsion, and is mainly affected by the type and concentration of ions in the reverse micellar water pool. In case of AOT, the two alkyl chains increase the effective v of the hydrocarbon chain of surfactant, keeping l_c and head group area a more or less constant. Thus the larger p value of AOT facilitates the formation of reverse micelles and hence AOT is the surfactant of choice in the present study. The short chain hydrocarbon solvents, such as hexane, penetrate the surfactant layer more easily than the larger ones and swell the surfactant tail regions. This solvent penetration increases the effective 'v' of the hydrocarbon chain of surfactant and reverse micelles are formed more easily as $p > 1$. The penetration depth corresponds to alkanes of similar chain length as that of surfactant (20).

However, in the present studies, no effect on the protein recovery was observed, of the hydrocarbon chain length. The protein recovery was

slightly higher with AOT/hexane RMS at $w_o = 20$ as compared to the other three systems. The small size of hexane was expected to help in its penetration into the cell-surface-adsorbed surfactant layer easily. However, very compact reverse micelles are formed in hexane up to $w_o = 30$. As known from our previous studies on the recovery of penicillin acylase from *E. coli* using AOT/hexane system (20), that beyond $w_o = 30$, the enzyme favored the excess water phase of the macroemulsion instead of the RM water pools and the protein/enzyme recovery showed an increasing trend up to $w_o = 80$. Thus we expected that catalase, which is adhering to the cell surface but is unable to get solubilized in RM, might favor the excess aqueous phase obtained at higher w_o. But the results showed a decrease in the total protein recovery at $w_o > 30$. Fungal cells, being very hydrophilic owing to the very low lipid content, favor the excess aqueous phase obtained at higher w_o. Hence their contact with RMS was poor, causing a lower degree of cell permeabilization and hence poor protein recovery.

The presence of the catalase activity in the cell wash of the RM-treated cells, indicates the ability of the RM solutions to permeabilize the cells. Catalase is, therefore, translocated from cytoplasm to the cell surface. Since it is a large tetrameric protein with a molecular weight of approximately 244 *k*D and having an equivalent diameter of 102 Å (29–33), reverse micelles with an average size ~10 Å are not big enough to accommodate the enzyme. This probably is the reason for no solubilization of catalase in any AOT/hydrocarbon RMS, and it remains bound to the cell surface. It is detached when washed with bulk water phase.

The water solubilization capacity of RM is mainly governed by the type of solvent and the water content (w_o). For a given solvent, increasing w_o increases the solubilization capacity, which is decided by the size of the reverse micelle. RM should have an adequate aqueous micropool to allow the proper distribution of cells and their better contact with RMS. As explained earlier, above a certain w_o value, RMS forms a biphasic system, and the cells dispersed in such a system remain in the excess aqueous phase and the total protein recovery decreases. In case of AOT/iso-octane RMS, however, increasing w_o has apparently no effect on the total protein recovery up to $w_o = 70$, beyond which it showed a decrease. The AOT/isooctane RM solution formed biphasic system beyond $w_o = 70$, with poor protein recovery (Fig. 3).

The cell loading in the RM solution has a significant effect on the total protein release (Table 2). Pfammatter et al. (31) have reported that dehydrated yeast cells were not at all dispersed by RMs even at higher water content. This indicates that the surfactant can disperse the cells in an organic solvent only if the cells are coated with sufficient amount of water. Thus there is a possibility of mass transfer between the aqueous coating on cells and aqueous pools of RMs. At a constant water content of the RM phase, the lower cell loading renders effective dispersion of fungal biomass and thus a better contact of the cells with RM solutions. As seen in our previous work (21, 22), more

surfactant can gain access to the cellular micellial mesh and adsorb onto the polysaccharide layer of the cells. Thus permeabilization increases as indicated by higher protein recoveries. Higher cell loading may lead to mass transfer limitations between cells and RMS. Upon reducing the cell loading to half (i.e 5%), the protein recovery increased by 50%, which suggests that a proper contact with the RM solution is necessary for the cell permeabilization. Reducing the cell loading further increased the total protein release. However, the handling of the biomass at very low cell loading became difficult. There was ~20% loss in the biomass during the transfer and filtration. At low cell loading, some catalase was lost upon washing the RM-treated cells and the remaining (~70%) could be recovered by sonication. The catalase recovery (after sonication) was high at higher cell loading which may be due to the lesser permeabilization of cells, hence less enzyme loss in the washings.

Multiple contacts with fresh RM solutions each time in a stirred vessel at high agitation speeds enhanced the permeabilization as seen by the increase in the total protein recovery after every extraction step (Table 3). Washing the cells followed by sonication for 15 minutes gave ~100% recovery of the total proteins. But there was apparently no effect of the speed of agitation on the cell permeabilization, as the protein recovery remained nearly constant for all the three speeds of agitation. However, higher agitation speed seems to affect the catalase recovery, as seen in Table 3. Catalase, that leaches out of the cell, binds to the cell surface and probably gets denatured due to the high agitation speed. However, Thomas and Dunnill (34) in their study on the effect of high shear on different concentrations of bovine liver catalase reported very less to no activity loss in the catalase solutions, thereby ruling out the effect of high agitation on catalase activity. Potapovich et al. (35), on the other hand, have reported catalase inactivation by ultrasonic cavitation at low frequency (20.8 kHz) ultrasound and explained that when the catalase solutions are exposed to low-frequency ultrasound, the free radicals generated in the field of ultrasonic cavitation are mainly responsible for catalase inactivation. However, the degree of catalase inactivation by the radicals is determined by the degree of association between enzyme molecules in the reaction medium and the composition thereof (35).

There was, however, no catalase activity in the cell wash *after* three extractions with the RM solutions under stirred conditions. Moreover, when these cells were sonicated, the total catalase recovery reduced drastically with the increase in agitation speed which gives a clear indication of the catalase's sensitivity to shear. The catalase recovered by sonication of RM solutions treated cells was 4–5 fold pure due to the removal of the contaminating proteins during the multiple RM extractions. A low shear impeller may increase the enzyme purification factor, if the shear is affecting the purification factor.

The rate of release of the enzyme from the RM-treated cells (~80% after 10 minutes sonication) also is much faster compared to that from the untreated cells (100% after 40 minutes sonication). The high shear and RM solutions

together are able to loosen the mycelial network of fungal cells to an extent that intracellular components are released at a faster rate during sonication. The RM extraction followed by ultrasonication gives an advantage over the direct cell disruption technique for faster intracellular product recovery and the final product is free from many contaminating proteins. The reduction in the disruption time gives an advantage in terms of reduced power consumption and reduced loss of enzyme activity by exposure to shear.

Based on our previous studies which explained the interaction of RMS with bacterial and yeast cell wall polysaccharide (19–22), the positive polysaccharide test for the precipitate obtained in the RM-treatment of *A. niger* cells in our present study suggests that the polar AOT head-groups binds to the polysaccharide chains on the cell wall, thereby paving the way for the hydrocarbon solvent which probably solubilizes the cell wall lipid and causes disturbances in the fungal cell wall. This still remains, however, a hypothesis and needs independent investigations directly on the cell wall/membranes.

CONCLUSIONS

Reverse micellar-aided permeabilization of filamentous fungi *Aspergillus niger* with simultaneous extraction of proteins and purification of intracellular catalase has been demonstrated. Aqueous solutions of AOT release ~26% of total proteins while the reverse micellar solution of AOT in an organic solvent gives ~50% protein release. Aliphatic hydrocarbon chain length of solvents has apparently no effect on the extent of fungal cell permeabilization. AOT concentration and the water content has no apparent effect on cell permeabilization; however, the water content plays an important role in the efficient dispersion of the cells in reverse micellar solution. Increasing the cell loading decreases total protein recovery, while the catalase recovery, after sonication was high. Multiple contacts with fresh reverse micelles in an agitated vessel at high agitation speeds enhanced cell permeabilization. About a 4 fold purified catalase was recovered after sonication due to the removal of most of the contaminating proteins during multiple extractions. The catalase leached out due to RM treatment remains bound to the cell surface and probably gets denatured by shear. The rate of release of catalase from RM-treated cell by ultrasonication is faster due to the effect on the fungal cell wall of the RM solutions. Leaching of the polysaccharides in the RM solution implies that it strips-off the polysaccharides in the cell wall, thereby giving to reverse micelles access to the cell wall proteins.

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