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Development of an Efficient Cell Disruption Method for Release of Lipoic Acid From *Saccharomyces cerevisiae*

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Abstract: Production of lipoic acid (LA) is undergoing a paradigm shift with significant preference for fermentative production over conventional chemical synthesis. However, low product yield triggers the importance of the development of an efficient cell disruption method as a critical step in its recovery. In this paper, we report screening of various physical, mechanical and chemical methods of cell disruption for high release of LA from *S. cerevisiae*. Among these methods, ultra sonication and EDTA were found to be most effective. The optimized ultra sonication process with respect to cell density (10g/L), time of sonication (3 min), acoustic power (50 W) and duty cycle (70%) resulted in maximum release of 11.4 mg/g DCW of LA in one cycle. Although batch release of LA gave a maximum yield of 42.5 mg/g DCW in six cycles of ultra sonication, the high energy consumption during ultra sonication (5400 kJ/ m^3) was discouraging during scale-up. Hence, incubation of the cell mass with 50 mM EDTA for 2 h which released 25.59 mg/g DCW of LA was preferred as the method of choice for subsequent work. Assuming LA release during ultra sonication and EDTA permeabilization to follow first order kinetics, the rate constants obtained were 0.011 /min and 0.074 min respectively.

Key words: Lipoic Acid · *Saccharomyces cerevisiae* · Cell Disruption · Ultra sonication · High Pressure Homogenization

INTRODUCTION

Lipoic acid (LA), originally found as a microbial growth factor, is ubiquitously present in living organisms and plays an essential role in energy metabolism [1]. LA is claimed to enhance the effects of other antioxidants such as glutathione and vitamins C and E [2] and forms a part of several multi enzyme complexes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain α-keto acid dehydrogenase and glycine cleavage enzyme complexes in eukaryotes and bacteria [1]. Currently, industrial production of LA is carried out exclusively by chemical synthesis which gives a racemic mixture of the R-form and S- form [3]. The R-isomer functions as a cofactor of several enzymes in the body, whereas the S-isomer acts as a xenobiotic to the body. Due to complicated synthesis steps, low yields and high material costs, all the known methods for producing enantiomerically pure lipoic acid are currently not economical [3]. This has resulted in a paradigm shift in LA synthesis with preference for fermentative production that results in production of only the R-isomer. A critical factor in the production of biomolecules by fermentation is its separation and subsequent purification.

Intracellular LA acid exists as protein-bound form [4]. Previous reports on the release of protein-bound LA are based on the use of potassium hydroxide and further hydrolysis by autoclaving at 110°C for 3 h [1]. Pratt *et al*. [5] have also reported the release of LA by first refluxing the *E. coli* and *H. halobium* cells with hydrochloric acid followed by constant purging with nitrogen and then further extraction using benzene. Tremendous loss of LA observed during hydrolysis and subsequent solvent extraction limits the practical use of these methods [6]. This necessitates the development of an efficient cell disruption method for maximum extraction of LA from microbial cells. The choice of the cell disruption method and its efficacy depends on the intracellular location of the product and the type of microorganism involved [7].

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The cell wall of microorganisms is a semi-rigid structure which imparts intrinsic strength to the cell and protects the cell from osmotic lysis [8]. The cell wall of *S. cerevisiae* is composed primarily of glucan, mannoprotein and chitin. Complete disruption of the cell wall and the release of all intracellular components require destruction of the rigid components of the wall, namely peptidoglycan in Gram-negative bacteria and glucan in yeast. Complete non-specific destruction of the wall is usually achieved by mechanical means such as ultra sonication, agitation with glass beads and high pressure homogenization [9]. However, they have several disadvantages, the most important being high energy requirement leading to increased cost, degradation of biomolecules during the process due to high heat generation and development of a very fine debris that may interfere during subsequent downstream processing [8, 9]. Moreover some methods like ultra sonication that are efficient on lab-scale pose several problems during scale-up. Physical and chemical methods that are gentler in their cell disruption mechanism can be used as alternatives to these mechanical methods. Physical methods rely on disruption of the wall structure without tearing it entirely apart. Chemical methods causing permeabilization of the microbial cell wall by interaction with certain components of the wall or membrane, allow leakage of periplasmic and cytoplasmic constituents from the cell without complete disintegration of the cell wall or membrane [9].

The objective of this study was to develop an efficient method of cell disruption for the release of LA from *Saccharomyces cerevisiae*. Various mechanical, physical and chemical methods were screened and the best method was further optimized for enhancing the release and recovery of LA.

MATERIALS AND METHODS

Media Components and Culture: Media components and chemicals like glucose, proteose peptone, yeast extract, malt extract, papain and agar were procured from Himedia Labs, Mumbai. Buffer components such as sodium dihydrogen phosphate and disodium hydrogen phosphate were procured from Merck India Ltd, Mumbai. Lipoic acid was obtained as a gift sample from Biocon Ltd., Bangalore. All the chemicals used were of analytical grade. *Saccharomyces cerevisiae* NCIM 3287 was procured from National Chemical Laboratory, Pune, India.

Media Preparation: *Saccharomyces cerevisiae* NCIM 3287 was maintained on potato dextrose agar (PDA) slants by preparing fresh slants every 2 weeks. Seed culture was prepared by inoculating 20 ml of GPYM broth (1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract, pH 6.5) with a loopful of 24 h old culture from the slants and incubating on a Remi incubator shaker (180 rpm) at 25°C for 20 h. The broth was centrifuged in a Beckman J2-MC Centrifuge, USA, at 12100 x g at 15°C for 15 min and the resulting cell pellet was suspended in saline so as to obtain a seed cell suspension having an optical density of 1.8-2.0 at 660 nm (Hitachi UV-Vis Spectrophotometer). 50 ml of the growth medium (GPYM broth) was inoculated with 1 ml of seed suspension and incubated for 24 h at 25°C and 180 rpm on a Remi incubator shaker.

Preparation of Cell Suspension: The cells were harvested by centrifugation at 12100 x g at 15°C for 15 min and the harvested cells were washed twice by suspending them in distilled water followed by centrifugation at 12100 x g at 15°C for 10 min. The washed cell pellet was weighed and 50 g/l cell suspension was prepared by re suspending them in appropriate amount of 100 mM phosphate buffer, pH 7.0 for all experiments.

Papain Treatment: Since LA is bound to the protein in the mitochondria, proteolytic treatment was optimized using several enzymes at various concentrations (data not given). All the samples obtained after lysis of *S. cerevisiae* were treated with 405 TU/ml of papain (One unit of potency is that unit which, while acting on the specified casein substrate at the specified conditions, will produce one microgram of Tyrosine per minute), prepared in phosphate buffer, for 20 h at 35°C. The papain reaction was stopped by addition of 10% TCA and the cell-free supernatant obtained by centrifugation at 16500 x g at 15°C for 20 min using Plastocraft Superspin R-V/FA, India, was analyzed for LA content.

Physical Methods of Cell Lysis: pH pretreatment: The harvested cells, as previously mentioned, were suspended in buffer solutions of different pH (2-12) using Brittonson - Robinson buffer solutions. These cell suspensions (1 ml each) were incubated for 2 h at 35°C on the Neolab Incubator Rocker and then centrifuged to remove the treatment buffer. The resulting cell pellet was resuspended in 1 ml of 100 mM phosphate buffer, pH 7.0 and treated with papain as mentioned earlier.

Freeze-Thaw: The process was carried out by freezing 5 ml of 50 g/l cell suspension at -20°C for 2 h followed by thawing at 4°C. This cycle was repeated 7 times, with aliquots being removed after each cycle. The aliquots were further treated with papain and analyzed for the LA content as described previously.

Mechanical Methods for Cell Disruption

Cell Disruption Using Glass Beads: The release of LA using glass beads was studied by mixing 10 ml of the cell suspension (50 g/l) with glass beads for 3 min on a vortex mixer. The ratio of the cell suspension volume and bead volume was varied by keeping a constant suspension volume, while varying the bead volume from 1:0.5 to 1:2. The samples were finally treated with papain as described previously after which they were analyzed for LA.

Cell Disruption by Homogenization: Cell disruption by homogenization was carried out in a two stage pressure homogenizer (APV-Gaulin Lab 60 TBS, Germany) consisting of basically a high-pressure positive displacement pump. The homogenizer was operated at varying pressures of 13.78, 27.57, 41.3, 55.14 and 68.92 MPa for a maximum of 10 passes. The pressure was achieved by adjusting the hand wheel for both the first stage and the second stage, such that the second stage pressure was adjusted to one third of the final pressure in the beginning followed by adjusting the remaining pressure by using the first stage hand wheel. The pressure was adjusted by using water followed by addition of 300 ml of pre-cooled cell suspension followed by its re-circulation through the two-stage valves for the disruption process. The flow rate of the cell suspension was measured at all operating pressures. Aliquots were collected after calculating the time required for the entire 300 ml to complete one entire pass and was continued for 10 such passes. The aliquots were further treated with papain and analyzed for the LA content as described previously.

Cell Disruption by Ultra Sonication: Time of sonication: 10 ml of 50 g/l cell suspension was subjected to ultra sonication (Branson Sonifier S 450A, Danbury, USA) with a ½" diameter tapped biohorn that delivers ultrasound at a constant frequency of 20 kHz. The height of the probe from the base of the container was kept constant at 1 cm and the samples were kept in an ice bath to prevent over heating of the cell suspension during lysis. The time of sonication was varied for 3, 6, 9 and 12 min at a constant acoustic power (50W) and duty cycle (70%). The aliquots were further treated with papain and analyzed for the released LA content.

Effect of Cell Density: 10 ml of cell suspension having varying cell densities, viz. 10, 30, 50, 70 and 90 g/l was ultra sonicated for 3 min at a constant acoustic power and duty cycle of 50 W and 70% respectively. One ml of each of these samples was further treated with papain and used for analysis of the released LA.

Effect of Acoustic Power: Ultrasound waves at varying acoustic power of 10, 30 and 50 W were applied to 10 ml of 10 g/l cell suspension for 3 min. The samples were processed with papain to estimate the release of LA.

Effect of Duty Cycle: The effect of duty cycle on the release of LA was studied by sonicating 10 ml of a 1% cell suspension at varying duty cycle of 10, 30, 50 and 70% for a period of 3 min. LA in the sonicated samples was determined by treating the samples with papain, as described previously.

Batch Release: 10 ml of 10 g/l cell suspension was sonicated for 3 min with a duty cycle of 70% and acoustic power of 50 W. The suspension obtained was centrifuged at 12100 x g at 15°C for 15 min. The cell pellet obtained was resuspended in 10 ml of 100 mM phosphate buffer, pH 7.0 and further sonicated under the same conditions. This process was carried out for 5 cycles and all the samples were assayed for LA content after papain treatment as detailed earlier.

Chemical Permeabilization: Chemical permeabilization of *S. cerevisiae* for release of LA was carried out as follows: 0.9 ml of 50 g/l cell suspension was incubated with 0.1 ml of varying concentrations of toluene (0.5 - 2.0% v/v), triton X-100 (0.5 - 2.0% v/v), ethyl acetate (0.5 - 2.0% v/v), petroleum ether (0.5 - 2.0% v/v), guanidine HCl (25-100 mM) and EDTA (0.025-100 mM). The treatment was carried out for 2 h at 35°C. The samples were further treated with papain and the cell-free supernatant was analyzed for its LA content.

Assay of LA: LA was quantified spectrophotometrically by modification of the method reported by Koricanac *et al*. [10] based on the complexation reaction between palladium (II) chloride and LA. To 0.5 ml of cell-free supernatant, 1.5 ml of 100 mM phosphate buffer solution,

pH 7.0 was added and the reaction was initiated by addition of 0.2 ml of 0.02 mM palladium (II) chloride solution, prepared in 0.1 mM phosphate buffer, pH 7.0. The ionic strength of the final solution was kept constant at 0.2 M by addition of 2M KCl solution. The mixture was incubated in a water bath at 25°C for 10 min and the absorbance was measured at 365 nm against a reagent blank in a spectrophotometer (Hitachi UV-Vis Spectrophotometer). A standard graph prepared using LA in the range of 0-60 µg gave a regression equation y = 0.0152 x (where y is the absorbance at 365 nm and x is the concentration of LA, µg).

Determination of DCW: The dry cell weight was determined by centrifuging 1 ml of the cell suspension in previously weighed Eppendorf tubes and further drying at 70°C for 24 h [11].

RESULTS AND DISCUSSION

Physical Lysis for Release of LA from *S. cerevisiae*

Effect of pH Pretreatment: The use of pH extremes is an extremely harsh but an effective technique for cell disruption, especially if the product of interest is resistant to degradation at very high or low pH. Also when growth of the microorganism is carried out at a neutral pH, the cell walls may be damaged more readily in acidic and basic pH extremes, thereby becoming amenable to permeabilization that allows the release of intracellular components as a response to this changed pH environment [12]. The release of LA from *S. cerevisiae* (Fig. 1) was found maximum at pH 7 (5.35 mg/g DCW). A release of only 1.91 and 3.35 mg/g DCW of LA was found at pH extremes of 2 and 12 respectively. PH extremes have been used in various cases for cell disruption. The use of 6N HC1 has been reported to hydrolyze *Candida lipolytica* cells. However, the process is slow, requiring 6 to 12 h and also resulted in concomitant hydrolysis of proteins to amino acids [8]. The use of acidic pH is mostly undesirable since it has a tendency to precipitate many macromolecules [8]. In our study, low release of LA was obtained in the acidic pH as compared to alkaline pH. Alkaline lysis, on the other hand, works by saponification of the lipids in the cell wall and has been used by many researchers [9]. Farkade *et al.* [12] showed that alkaline pre-treatment assisted in effective release of β-galactosidase from *Kluveromyces lactis*. A process improvement was also reported in which mechanical cell disruption for the extraction of

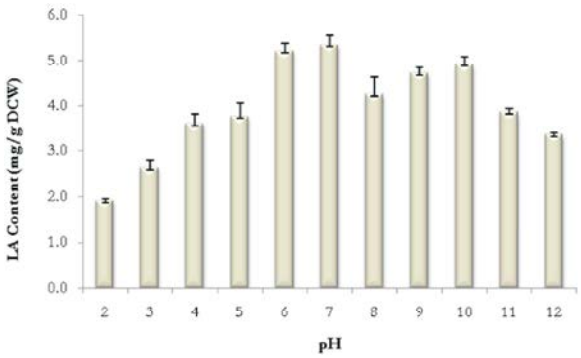

Fig. 1: Effect of pH pretreatment on the release of LA from *S. cerevisiae*

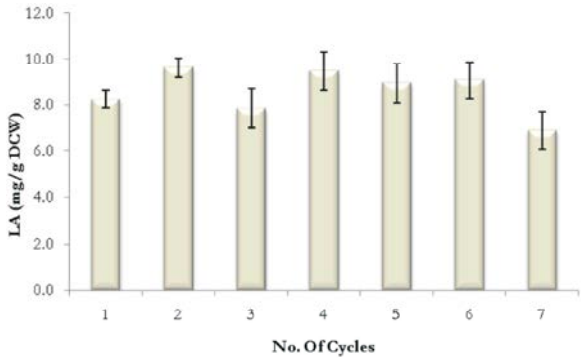

Fig. 2: Effect of Freeze and thaw on the release of LA from *S. cerevisiae*

L-asparaginase from *Erwinia* was replaced by alkaline treatment at pH 9.0 [9]. Contrary to the above reports, pH pretreatment was not found to be effective in release of LA.

Effect of Freeze-Thaw: The results of disruption of *S. cerevisiae* for release of LA using freeze-thaw were illustrated in Fig. 2. It can be seen that a maximum release of 9 mg/g DCW of LA was obtained through physical disruption in 2 freeze-thaw cycles. However, no substantial difference in the release of LA was seen when the number of cycles was increased. Freezing and thawing of a cell paste causes cell disruption through the formation and subsequent melting of ice crystals. Gradual freezing results in larger crystals thereby causing more extensive damage to the cells compared to flash freezing and thawing that gives smaller crystals [7]. Mayerhoff *et al*. [13] have shown that among the various lysis methods used for release of xylose reductase from *Candida mogii*, highest specific activity was achieved when cells were disrupted by freezing and thawing. The high efficiency of freeze-thaw for release of enzymes

has also been reported by Burns *et al.* [14] who adopted this method to release aspartate carbamoyltransferase from *Helicobacter pylori*. Freeze-thaw is an efficient and relatively gentle process which permeabilizes the cell envelope and causes minimal effects on the overall cell-integrity, little shearing of membrane-bound proteins and good recovery of soluble proteins [13]. However, it was not very effective in the present study for the release of mitochondrial LA from the cells of *S. cerevisiae*.

Mechanical Methods of Lysis for Release of LA from *S. cerevisiae*

Disruption by Glass Beads: Cell disruption in bead mills is regarded as one of the most efficient techniques for physical cell disruption, with many studies being reported on yeast and bacterial cells [8, 15]. Glass beads disrupt microbial cells by compaction or shearing action that causes energy transfer from beads to cells. This process of disruption by the tearing apart of the cell wall, as with all the mechanical methods, is highly non-specific [9] and is affected by a number of parameters like bead density (volume), cell concentration, nature of the cell envelope and size of the organisms [8, 9]. Asenjo [16] reported that bead volume to cell suspension ratio is very important for good grinding and low wear of the stirrer system and consequently the extent of cell disruption. In the present work, different ratios of suspension volume to the bead volume were used (1:0.5, 1:1 and 1:2). A ratio of 1:0.5 released 8.21 mg/g DCW of LA. The release decreased with further increase in the volume of beads showing that varying ratio of the sample volume to glass beads volume did not have any significant effect on the release of LA from *S. cerevisiae*. The low yield obtained could also be due to rise in temperature during the process leading to heat dissipation within the system resulting in product degradation [9].

Pressure Homogenization: High pressure homogenization remains the method of choice for the breakage of microbial cells in large scale processing. It operates by raising the pressure of a cell suspension and then releasing it through a specially designed valve assembly. The cells experience a range of forces and disrupt through their interaction with the fluid and solid walls of the valve assembly [17]. Several early studies have examined the disruption of organisms, particularly yeasts, by high pressure homogenization. One of the studies, dating from 1932, examined the release of vitamins from beer during fermentation. In the 1960s and 1970s, yeast disruption by homogenization was examined by

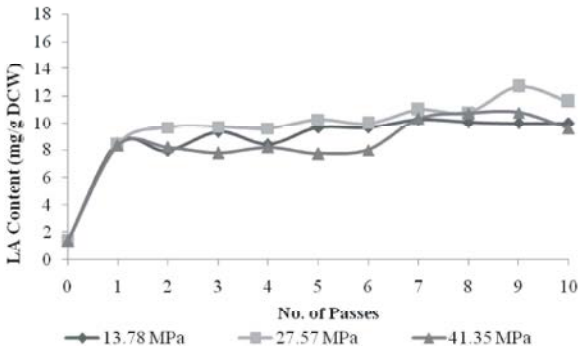

Fig. 3: Effect of high pressure homogenization on the release of LA from *S. cerevisiae*

various research groups as a part of the attempts to establish economical processes for the manufacture of single cell protein [9]. Follows *et al.* [18] observed the rate of release of enzymes to depend on their location within the cell. Soluble enzymes were released at the same rate as total soluble protein. Enzymes located outside the cell membrane were released faster, while enzymes contained within cellular components were released at a slower rate [9]. Different operating pressures are required to disrupt different micro-organisms. Gram-negative bacteria are easier to disrupt than Gram-positive bacteria and filamentous fungi which in turn are easier to disrupt than unicellular yeasts. Thus, the ease of disruption depends on the cell wall composition, size and shape and growth phase of the micro-organism in consideration [8]. The release of LA at different pressures was depicted in Fig. 3. It was seen that maximum LA (12.67 mg/ g DCW) was released at a pressure of 27.57 MPa with 9 passes. In a study carried out by Shirgaonkar *et al*. [19], it was found that high pressure homogenization up to 27.57 MPa was not very effective in disrupting yeast cells and substantial cell disruption was obtained only at discharge pressures above 34.46 MPa. This is contrary to the results obtained in our study where lesser yields of LA were obtained at pressures above 41.35 MPa. This could possibly be attributed to the degradation of LA caused by heat generation at very high pressures.

Effect of Ultra Sonication on Cell Disruption: The effect of ultra sonication on cell disruption for the release of LA from *S. cerevisiae* was studied by varying the time of sonication, cell density, acoustic power and duty cycle. When the time of sonication was varied from 3 min to 12 min, it was observed that the release was maximum (8.45 mg/g DCW of LA) when the cells were exposed to just 3 min of ultra sonication and LA yield was decreased

to almost half (4.24 mg/g DCW) in 6 min. This is in accordance with the report by Lateef *et al.* [19] who studied the effect of ultra sonication on the release of fructosyltransferase (FTase) from *Aureobasidium pullulans* CFR 77. The FTase activity of the lysate increased rapidly with increase in time of exposure to ultra sonication till 9 min after which it decreased. Ultra sonication for 12 min decreased the release of LA drastically to 0.87 mg/g DCW. This sudden loss could be attributed to thermal degradation of the liberated LA when the sonication time exceeded 9 min. These findings can be supported by the findings of Chisti and Moo-Young [15], who have stated that most of the ultrasound energy absorbed by cell suspension ultimately appears as heat. Prolong sonication is reported to cause significant degradation of antigens due to thermal denaturation in the proximity of the sonication probe [21]. Cell density is also an important factor for complete release of intracellular biomolecules. Maximum LA (11.40 mg/g DCW) was released when the cell density was 10 g/l. The release gradually decreased and almost remained constant with an increase in the cell concentration. Lovitt [22] investigated the effect of cell concentration on protein release from baker's yeast using a hydraulically operated cell disrupter and found a linear relationship between protein release and disruption operating pressure. Further, they observed a 15% lower release of protein at maximum cell concentration as compared to more dilute cell suspensions. The effect of acoustic power on the release of LA was investigated between 10W-50W. It was observed that the LA release increased with an increase in the acoustic power and maximum release (11.01 mg/g DCW of LA) was achieved at a higher acoustic power. Doulah [23] proposed the theory of cell disruption associated with shear stresses developed by dissipative eddies generated by imploding cavitating bubbles within the ultra sonication medium. An increase in acoustic power resulted in higher degree of cellular disruption with higher product release. Similar results were shown by both Choonia and Lele [24] and Kapucu *et al.* [25] where increasing acoustic power enhanced cell disruption. Duty cycle is the fraction of time for which a system is in an active state. It is generally expressed in percentage or ratio. In the pulsed mode used in ultra sonication, the sound waves are pulsed at a fixed repetition rate of one pulse per second. This control varies the duration of the ultrasonic pulse. So, at a duty cycle of 10%, the ultrasound energy input remains on for 10% of each second. In our study, varying duty cycle had no substantial effect on the release of LA from *S. cerevisiae*.

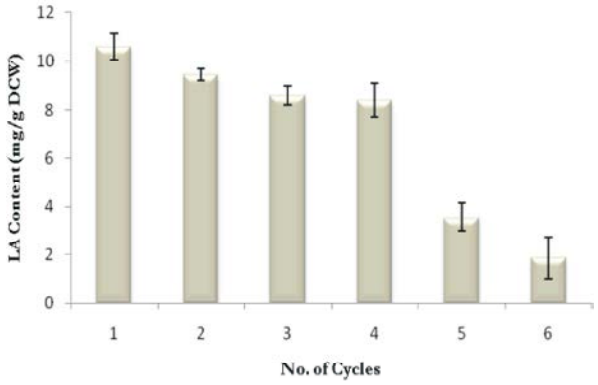

Fig. 4: Batch release of LA from *S. cerevisiae* using ultra sonication

When the cells were exposed to varying times of ultra sonication, a maximum release of LA was achieved at 3 min. From Fig. 4, it can be seen that the release was almost half when the time was increased to 6 min and the yield dropped drastically to 0.87mg/g DCW when the time was increased to 12 min. As explained earlier, this loss could be attributed to localized heat generation during ultra sonication [21]. To prevent this loss, the process was carried out in cycles. The release of LA in the first 3 min of ultra sonication was 10.60 mg/g DCW and in the second cycle of sonication was 9.45 mg/g DCW. The release remained constant for the next 2 cycles and then decreased. A cumulative release of about 42.5 mg/g DCW of LA was obtained from *S. cerevisiae* when the cell suspension was sonicated in batches. Thus, disruption of the cell suspension for shorter time periods but with more cycles proved to be a better and efficient method as compared to prolonged periods of continuous sonication. This method by far was the most efficient in achieving maximum recovery of LA.

Energy Efficiency: Among all the methods evaluated as above, ultra sonication in batch mode proved to be the best. For large scale processing, the method needs to be economical and easy to operate. Due to drawbacks associated with scale up, high pressure homogenization is more suited to large scale processing compared to ultra sonication. Here, we calculated the energy input required for the release of LA from *S. cerevisiae* using both high pressure homogenization and ultra sonication. Energy requirement for LA release by high pressure homogenization was calculated using the following equation [26].

$$E = PQT \quad (1)$$

Table 1: Energy efficiency for maximum LA release from *S. cerevisiae* by ultra sonication and high pressure homogenization

Disruption methods	Pressure/ Power	Time of operation	Energy efficiency for maximum LA released	Maximum LA released per unit of energy
Ultra sonication[a]	50 W	3 min	900 kJ/m^3	1.17 mg/kJ
Ultra sonication[b]	50 W	21 min	5400 kJ/ m^3	0.78 mg/kJ
High pressure homogenization	27.57 MPa	9 passes	247.3 MJ/ m^3	0.16 mg/MJ

[a]Continuous ultra sonication

[b]Batch release

where, E is the energy input during homogenization (MJ), P is the operating pressure of the homogenizer (Pa), Q is the volumetric flow rate (l/s) and t is the time in sec. The energy efficiency for maximum LA released using various disruption methods was shown in Table 1. In high pressure homogenization at operating pressure of 27.57 MPa, the total energy consumption was 247.3 MJ/ m^3 with an LA release per unit energy being only 0.16 mg/ MJ.

In case of ultra sonication being carried out at 50 W acoustic power, the total input energy was 900 kJ/ m^3 in one cycle with an overall consumption of 5400 kJ/ m^3 in batch sonication process. The energy efficiency of ultra sonication process for LA release in batch mode was found to be much better than high pressure homogenization with an LA release of 0.78 mg/kJ. It can be observed here that although high pressure homogenization is more suited for large scale operations, it is highly uneconomical due to high energy requirements. Ultra sonication which is more energy efficient and gives higher yield has issues related to scale up. This necessitates exploration of alternative lysis methods that are easily scalable, economical and energy efficient.

Chemical Pretreatment for the Release of La from *S. cerevisiae*

Effect of Solvents: The use of solvents for the disintegration of microbial cells has long been recognized. Although solvents act by dissolving hydrophobic components in the wall or membrane in Gram-negative bacteria and yeast [9], the success of the permeabilization depends on the composition of the cell wall and the cell membrane, as well as on the location of the molecule of interest. Membrane associated molecules can be released more easily than those located inside the cell. In yeasts, organic solvents have been known to result only in permeabilization without the release of any enzymes or proteins [9, 27]. Toluene is frequently employed in chemical permeabilization [9]. When added to biomass, toluene is absorbed in the cell wall lipids which results in swelling and further ruptures the cell [8]. However, some reports indicate low efficiency of disruption of *E. coli* cells by toluene treatment with a recovery of only 25% of the protein [9]. Toluene has also been employed for permeabilization of *Kluveromyces lactis* cells for in situ β-galactosidase activity [9]. Use of petroleum ether as a solvent for permeabilization of *E. coli* was not very effective. However, in some studies complete disorganization of the cytoplasm and disruption of the cell membrane of fungi was observed [9]. Ether has also been shown to release nucleotides from *E. coli* with only low levels of release for higher molecular-weight components. Ethyl acetate was found to be very effective at 50% to cause complete disintegration of *Alcaligenes eutrophus* cells [8]. Table 2 shows the effect of different solvents at varying concentrations on the release of LA from *S. cerevisiae*.

In the present study, we used toluene, ethyl acetate and petroleum ether as solvents to effect release of LA through permeabilization of *S. cerevisiae* culture and the results were compiled in Table 2. In all cases, it was observed that there was no substantial release of LA at all the varying concentrations of the solvents used. LA release was approximately 7 mg/g DCW at all concentrations of toluene used, 8 mg/g DCW using petroleum ether at different concentrations and a maximum release of 7.15 mg/g DCW with 0.5% ethyl acetate. All the results showed that changes in solvent concentration did not result in any marked increase in the release of LA.

Effect of Detergents: Detergents are amphipathic molecules having a hydrophilic portion, which is often ionic and a hydrophobic region, usually a hydrocarbon such that they are able to interact both with water and lipid molecules [9]. Detergents have primarily been employed to affect the recovery of membrane proteins from pretreated Gram-negative bacteria or from yeast. Only few reports mention the use of whole cell disruption using detergent alone, primarily because extraction solely with detergent requires high concentrations which further results in added process cost and complications in downstream processing. Triton X-100 is a non-ionic detergent with a high binding affinity for hydrophobic species and is thus effective in binding to and solubilizing phospholipids from both inner and outer membrane fragments [8]. From Table 2, it can be seen that a maximum

Table 2: Effect of solvents and Triton X-100 on the release of LA from *S. cerevisiae*

	LA (mg/g DCW)[a]			
Conc. (% v/v)	Toluene	Ethyl acetate	Pet. ether	Triton X-100
0.5	7.39±0.589	7.15±0.277	7.70±0.806	11.85±0.970
1.0	7.96±0.635	5.72±0.972	7.96±0.866	13.95±0.744
1.5	7.81±0.276	5.78±0.470	8.02±0.971	12.93±0.635
2.0	7.37±0.416	5.78±0.214	7.13±0.852	11.51±0.838

[a]Results are mean ± SD of three determinations

Table 3: Effect of guanidine hydrochloride and EDTA on the release of LA from *S. cerevisiae*

	LA (mg/g DCW)[a]	
Concentration (mM)	Guanidine hydrochloride	EDTA
25	2.29±0.276	21.82±0.264
50	4.30±0.513	25.59±0.894
75	4.15±0.432	20.78±0.788
100	4.04±0.132	16.36±0.975

[a]Results are mean ± SD of three determinations

release of 13.95 mg/g DCW of LA was obtained at a triton concentration of 1%. Similar reports by Galabova *et al.* [27] have shown that maximum phosphatase activities were obtained when the yeasts cells were permeabilized with triton concentrations in the range of 0.1-0.2%. Further the electron microscopic observations proved no change in the cell integrity but a structural alteration in the cell wall and membrane by permeabilization with triton. This in turn could be due to weak cohesion of proteins and lipids subjected to detergent action [27].

Effect of Chaotropes: Chaotropic agents disorganize the structure of water making it less hydrophilic and thus weakening the solute-solute interactions [9]. They are also known to inhibit cross linking of peptidoglycan and cell wall synthesis thereby solubilizing protein from membrane fragments and altering hydrophobic interactions [26]. Chaotropes are generally used in very high concentrations. This sometimes leads to unacceptable expenses and handling problems. Some reports have suggested effective protein release even at low chaotrope concentrations (e.g. 0.1M guanidine hydrochloride) [9]. Hence, in our study the range of guanidine hydrochloride was varied from 25 to 100 mM. The release increased gradually with an increase in the concentration of guanidine hydrochloride and a maximum of only 4.15 mg/g DCW of LA was released at 75 mM (Table 3).

Effect of Chelating Agents: Chelating agents such as EDTA act by disrupting the outer membrane by binding to the divalent cations such as Mg^{2+} and Ca^{2+} that cross-bridge adjacent lipopolysaccharide molecule. The release using EDTA is reported to be rapid but strain specific. The effectiveness of EDTA treatment depends on the polysaccharide chain lengths of the strains. EDTA is clearly effective at disrupting the outer membrane and is therefore employed to recover periplasmic proteins [26]. Table 3 shows the effect of pretreatment with varying concentrations of EDTA on the release of LA from *S. cerevisiae*. It is evident that the release was maximum (25.59 mg/g DCW) at 50 mM EDTA beyond which the release gradually decreased (Table 3). Similar results were obtained by Anand *et al*. [25] where the release of acid phosphatase showed a small increase with increase in EDTA concentration but the activity reduced with further increase in concentration. As a pretreatment prior to high pressure homogenization, Anand *et al*.[25] showed a higher release of β-galactosidase at low operating pressures (13.8 MPA) vis-à-vis the release achieved from untreated cells at higher pressures of 34.5 MPa. EDTA does not have any effect on peptidoglycan and hence it results only in permeabilization of bacterial cells [9]. The high release obtained in our study could be due to the composition of the yeast cell wall thereby resulting in effective cell disruption and better release of LA.

Release Rate Kinetics: The release of intracellular products generally follows first order kinetics and in our study release of LA could be described by the following equation:

$$ln\left(\frac{R_m}{R_m - R}\right) = Kt \quad (1)$$

where R_m is the maximum LA available for release, R is the amount of LA released and k is the release rate constant (/min).

Anand *et al*. [26] modified Eq. (1) to describe the release of protein during permeabilisation using pre treatment. The same concept of pre-treatment was used in this manuscript to determine the release rate constants. All the methods described in the manuscript would be considered as pre-treatment steps followed by protease treatment for final and maximum LA release. Since ultra sonication and EDTA permeabilization were found to be most promising in maximum LA release, kinetic rate constants were determined for both the methods. The equation modified by Anand *et al*. [26] was given as follows:

$$ln\left(\frac{R_m - R_o}{R_m - R}\right) = Kt \quad (2)$$

where R_0 is the amount of LA released during pre-treatment. Hence when the cells were further treated with protease the total LA available for release was R_m - R_0 so that the equation could be rewritten as:

$$ln\left(\frac{R_m - R_o}{R_m - R_o - R}\right) = Kt \quad (3)$$

The release rates of LA using EDTA and ultrasonication were $1x10^{-3}$ /min and $6x10^{-3}$ /min respectively. As seen in Fig. 5 and 6 the correlation coefficients obtained also showed that the model was successful in describing the release of LA from *S. cerevisiae*. As seen in Fig. 7 when protease treatment was used the release rate was $74x10^{-3}$ /min using EDTA and $11x10^{-3}$ /min using ultra sonication. The release rates were much higher when protease treatment was used in combination with chemical permeabilization and ultra sonication resulting in maximum release of LA.

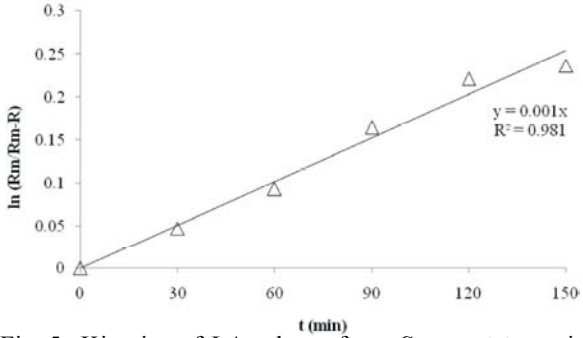

Fig. 5: Kinetics of LA release from *S. cerevisiae* using EDTA

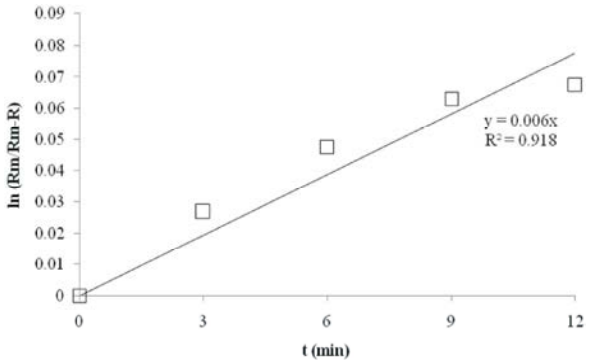

Fig. 6: Kinetics of LA release from *S. cerevisiae* by sonication

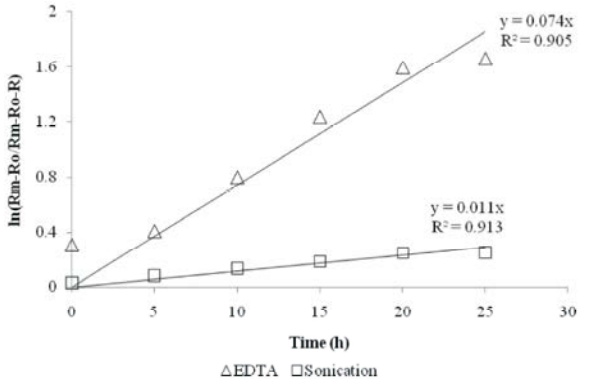

Fig. 7: Kinetics of LA release from *S. cerevisiae* using protease treatment

Disruption of the cell mass by ultra sonication for shorter time periods but with more cycles proved to be a better and efficient method as compared to prolonged periods of continuous sonication. The high energy requirements and problems during scale up for ultra sonication necessitate looking for alternatives such as chemical permeabilization. Permeabilization with EDTA was found to be the method of choice due to its cost effectiveness and also for its ease of operation and scale up.

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