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Ultrasound assisted three phase partitioning of peroxidase from waste orange peels

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Abstract: Ultrasound assisted three phase partitioning (UATPP) has been explored to intensify the extraction and purification of peroxidase from orange peel (*Citrus sinensis*). UATPP of peroxidase was studied by varying different process parameters such as ammonium sulfate concentration, pH, broth to tertiary butanol (t-butanol) ratio, temperature, ultrasound frequency, ultrasound power and duty cycle. Under optimized conditions, i.e. 50% ammonium sulfate saturation, pH 6, temperature 30°C, broth to t-butanol ratio 1:1.5 (v/v), at 25 kHz frequency and 150 W ultrasonication power with 40% duty cycle, 6 min irradiation time, 24.28 fold purity of peroxidase with 91.84% recovery was been obtained. UATPP resulted in higher purification fold of peroxidase due to enhanced mass transfer with reduced operation time from 80 min to 6 min as compared to conventional three phase partitioning (TPP). SDS PAGE analysis of the partially purified enzyme indicated a molecular weight of 26 kDa. The integration of ultrasound with TPP enhances the utility of both processes. Thus, UATPP has been proved as an efficient alternative to partial purification of peroxidase.

Keywords: *Citrus sinensis*; extraction; peroxidase; ultrasound assisted three phase partitioning.

1 Introduction

Peroxidase (EC 1.11.1.7) is a widely spread heme containing enzyme. Due to broad substrate specificity and multifunctional properties, it has diverse applications in analytical and biochemical processes. It has been used for quantification of uric acid, glucose and cholesterol. Several reports have been published on decolorization and removal of textile dyes from polluted water using soluble and immobilized peroxidases [1]. It has also found application in the treatment of wastewater containing phenolic compounds and has been used as a raw material for the synthesis of aromatic chemicals [2]. It has also proved to be an efficient catalyst in biotransformation reactions [3].

Several reports have been published on the extraction and purification of horseradish peroxidase which represents a commercial source of peroxidase, but availability and cost of the commercially available horseradish peroxidase restricts its applications [4–6]. Various sources have been explored as a source of peroxidase, such as soybean [7], black gram [8] and apples [9].

Fermentative production of peroxidase has been reported [10] but stirred tank fermenter is not appropriate option due to the shear sensitivity of microbes and lower yields. Lower yield and high production cost are the major obstacles in the exploration of peroxidase as a biocatalyst. Developing a cost-effective purification procedure and cheap sources of peroxidase could be an attractive alternative for minimizing higher production cost. The extraction of enzymes from agro industrial waste could be a better substitute for the reduction of overall production cost. Thus, we have used orange peel as a peroxidase source which is waste of orange processing industry. Further, an innovative bioseparation technique called three phase partitioning (TPP) was explored for the separation in combination with the ultrasound. The protocol of TPP is the addition of salt (ammonium sulfate) to the crude protein/enzyme, followed by addition of tertiary butanol (t-butanol) to obtain three distinct phases. Typically, t-butanol is completely miscible with water, but on the addition of ammonium sulfate at adequate concentration, the solution separates into a lower aqueous phase and an upper t-butanol phase. In the resulting three phases, the upper t-butanol phase contains non-polar compounds which are separated from the lower aqueous phase (containing polar compounds) by an interfacial protein precipitate. The desired enzymes are selectively partitioned to one phase and other contaminant proteins to the other phase. This causes partial purification and concentration of the enzyme. The extraction process is an amalgamation of kosmotropic, salting out, isotonic

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cosolvent and osmolytic precipitation of proteins. TPP is a simple and inexpensive technique which can be directly operated with crude suspensions [11, 12].

TPP has been used for extraction and partial purification of various enzymes such as invertase from baker's yeast and tomato [12, 13], α-galactosidase from pepino (*Solanum muricatum*) [14] and protease [15]. It is also employed for the extraction of natural products. Kulkarni et al. reported TPP as an extraction technique for the extraction of mangiferin from the leaves of *Mangifera indica* [16]. Although TPP has been reported as an effective extraction technique, it has a long operation time which may be attributed to mass transfer limitation. Earlier work on TPP of peroxidase from orange peel at optimized conditions reports 80 min extraction time [17]. Thus, there is scope to reduce the time and increase the recovery with the help of ultrasound.

Ultrasound is an efficient way to increase mass transfer of solid-liquid extraction processes which leads to higher yield. It has several advantages and has been employed for the extraction of different phytochemicals like cinchonine, rauwolfine, digitalin, atropine and berberine [18]. Vetal et al. [19] reported the process intensification of TPP using ultrasound for the extraction of ursolic acid and oleanolic acid from *Ocimum sanctum*.

Enzyme partitioning is driven by the mass transfer, and subsequently mass transfer enhancement will improve the partitioning of enzyme which results in higher purity. Increase in the extraction rate and yield in ultrasound assisted extraction is mainly attributed to the acoustic cavitation. An ultrasonic wave results in the formation of gas filled bubbles which collapse, causing a shock wave and mechanical shear to the surrounding environment. These mechanical shears which occurred due to the cavitational phenomenon accelerate the mass transfer across the different phases [20].

To the best of our knowledge, no research has been reported on the ultrasound assisted three phase partitioning (UATPP) of peroxidase. Thus, we carried out the ultrasound mediated process intensification of TPP. The major emphasis of the present study was to achieve a maximum purity and activity of peroxidase from orange peel in a lower time using UATPP. Various process parameters such as ammonium sulfate concentration, broth to t-butanol ratio, pH, temperature, ultrasonic frequency, ultrasonic power and duty cycle were optimized. Enzyme purification fold and activity recovery obtained through UATPP were compared with conventional TPP. SDS PAGE analysis was performed to evaluate purity of the partially purified enzyme.

2 Materials and methods

2.1 Materials

Orange peels (*Citrus sinensis* [L.]) were procured from the local juice center at Matunga, Mumbai. t-Butanol and ammonium sulfate (analytical grade) were purchased from S. D. Fine-Chem Ltd., Mumbai, Maharashtra, India.

2.2 Preparation of peroxidase extract

Orange peels were first washed thoroughly with distilled water. Some 10 g of peels were cut into small pieces and homogenized in 100 ml of 100 mM phosphate buffer of pH 7.0 using a blender. The homogenate was filtered through Whatman filter paper No. 1 and filtrate was centrifuged at 10,000×*g* for 20 min. After centrifugation, supernatant was collected and assayed for peroxidase activity and protein content.

2.3 Ultrasound set up and treatment

UATPP of peroxidase was carried out in an ultrasonic water bath of 6.5 l tank capacity with dimensions of 0.23 m×0.15 m×0.15 m (Model No. 6.51200 H, Dakshin, India Pvt. Ltd, Mumbai, Maharashtra, India). This can be operated at a maximum rated power of 200 W and frequencies of 25 kHz and 40 kHz. It was also equipped with a heater and digital temperature controller/indicator. The temperature of the bath can be controlled with the recirculation water system. Four transducers were arranged in a zigzag position at the bottom of the ultrasonic bath. A selector switch was provided for frequency selection and AC voltage was varied to change irradiated power modulation through an autotransformer. A flat bottom glass vessel was used and placed above 0.02 m from the bottom of ultrasonic bath. The position and shape of the vessel had been optimized earlier [21]. UATPP was executed with 10 ml of crude broth with 4 g of ammonium sulfate (40% W/V) and 10 ml of t-butanol at pH 7. Ultrasound was irradiated at 25 kHz ultrasonic frequency with 150 W rated power and 20% duty cycle at 30°C. The irradiated mixture was then allowed to separate for 1 h in a separating funnel and the separated bottom phase was analyzed for enzyme activity and protein content. Different process factors such as irradiation time, ammonium sulfate, pH, broth to t-butanol ratio, temperature, ultrasonic frequency, power and duty cycle were optimized by one factor at a time approach. All experiments were performed in triplicates and results were displayed with standard deviation. Purification factor, specific activity and % recovery were calculated using the following equations [Eqs. (1)–(3)].

$$\text{Purification factor} = \frac{\text{Specific activity of purified sample (U/mg)}}{\text{Specific activity of intial sample (U/mg)}} \quad (1)$$

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme concentration (U/ml)}}{\text{Protein concentration (mg/ml)}} \quad (2)$$

$$\% \text{ Recovery} = \frac{\text{Total activity of purified sample} * 100}{\text{Total activity of initial sample}} \quad (3)$$

The purification factor describes the purity of enzyme after purification process with respect to initial sample.

2.4 Calorimetric study

Efficiency of the ultrasonic bath was evaluated by calorimetric study. Dissipated power was calculated from the temperature rise of the solvent (water) after a specific time at 25 kHz and 40 kHz frequency with varying irradiated power (50–200 W) [22].

2.5 Analytical method

2.5.1 Measurement of peroxidase activity: Peroxidase activity was determined calorimetrically by a spectrophotometer (Spectroscan UV 2700, Double Beam UV-Vis spectrophotometer, Chemito, India) following the formation of tetraguaiacol (Amax=470 nm, ε=26.6 $mM^{-1}cm^{-1}$). The reaction mixture contained a final concentration of 0.05% H_2O_2 and 18 mM guaiacol in 100 mM phosphate buffer (pH 7). To measure the peroxidase activity, 50 µl of the sample containing enzyme was added to 2.95 ml of substrate solution and increase in the absorbance rate was measured at 470 nm for 3 min. One unit of peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min [23].

2.5.2 Measurement of protein content: Concentration of the protein was determined by the Bradford method using Coomassie Blue G250 [24].

2.5.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: UATPP bottom phase protein sample was analyzed by the Laemmli method of SDS PAGE with 12% gel for determination of its homogeneity and molecular weight using Biorad Mini Protean II electrophoresis unit [25]. Biorad broad range molecular marker mixture consisting of standard enzymes ranging from 10 kDa to 170 kDa was used. The silver staining method was used to visualize the protein band.

2.6 Statistical analysis

Statistical analysis is very important to summarize and interpret the obtained data. All experimental results were performed in triplicate (nP3) and the data are expressed as means±SD. The statistical significances of process parameters were evaluated by analysis of variance (ANOVA) using Microsoft Excel. Single factor ANOVA was used for statistical analysis. A p value <0.05 was considered to be statistically significant.

3 Results and discussion

3.1 UATPP

UATPP was studied by evaluating the effects of ammonium sulfate saturation, pH, temperature, t-butanol volume (broth to t-butanol ratio), ultrasound frequency and power, duty cycle and irradiation time on activity recovery and purification fold of peroxidase. Ultrasound helps to improve the partitioning of target molecules through mass transfer enhancement across three phases.

3.2 Effect of ultrasonication frequency and irradiation time

There should be a minimum possible time for any industrial process to make it economically feasible and sustainable. Thus, time has been optimized to get maximum purification fold and activity recovery. Ultrasonication frequency decides the magnitude of power dissipation to the medium and irradiation time estimates the dose of ultrasound. Ultrasonication frequency is an important parameter that needs to be critically optimized. In UATPP, the effects of 25 kHz and 40 kHz ultrasonic frequencies were studied at 150 W rated power with 20% duty cycle for different time intervals. Figure 1 demonstrates the noteworthy increase in the purity of enzyme along with irradiation time for both frequencies ($p<0.05$). However, irradiation at 25 kHz for 6 min gave a maximum purity of 15.10 fold and a recovery of 74%. This could be attributed to higher

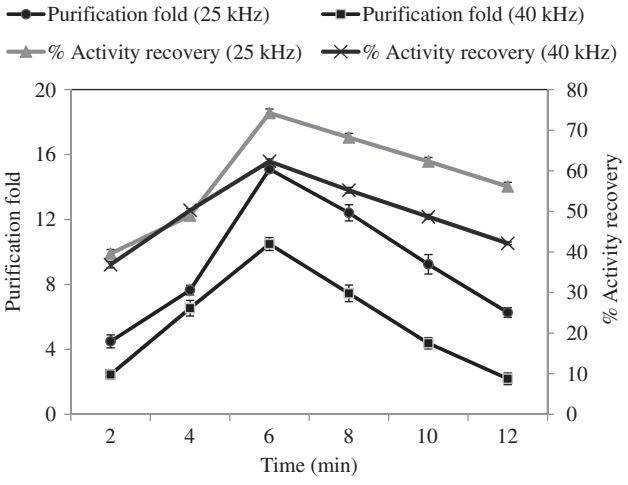

Figure 1: Effect of ultrasonication frequency (25 kHz and 40 kHz) and irradiation time on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

power dissipated at 25 kHz as compared to 40 kHz which has been confirmed by calorimetric study. The dissipated power for 25 kHz and 40 kHz frequency at 150 W rated power was found to be 61.6 W and 49 W, respectively, thus higher power dissipation at 25 kHz designated the higher fold purity. Apart from this, normally at lower frequencies of irradiation, e.g. 20 kHz, the physical effects due to cavitation are dominant. These physical effects, i.e. liquid circulation currents and turbulence, are the governing factors that decide the extent of extraction. It has been generally recommended to employ a lower operating frequency for extraction [26].

Excessive irradiation beyond 6 min causes loss of enzymatic activity and protein degradation. Similar results have been reported by Avhad et al. [22]. Higher purity and activity recovery were achieved in UATPP (15.10% and 74.23%) as compared to TPP (12.14% and 71.43%) [17], with significant reduction of time from 80 min to 6 min at 25 kHz. Thus, 6 min and 25 kHz were considered as optimum parameters for the next set of experiments.

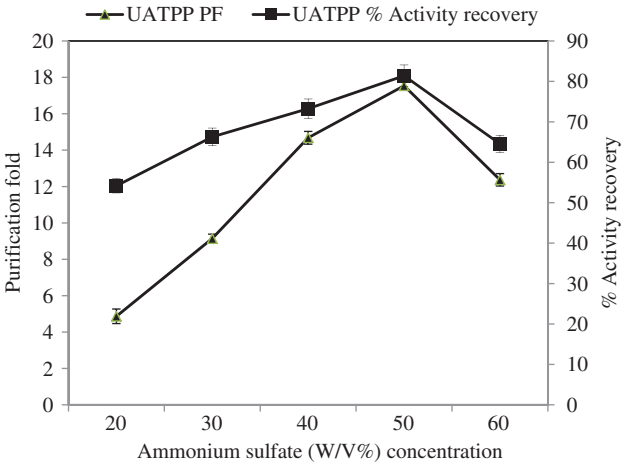

Figure 2: Effect of ammonium sulfate saturation on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

3.3 Effect of ammonium sulfate saturation

Ammonium sulfate concentration has significant effects on the TPP system as it helps in protein precipitation by a salting out mechanism. Protein solubility is influenced by the type of salt and its concentration. It has been reported that at higher salt concentration, water molecules are attracted by salt ions which results in stronger protein-protein interactions, and the protein molecules coagulate through hydrophobic interactions. The effect of ammonium sulfate saturation was studied by varying the concentration from 20% to 60% in UATPP, while other experimental parameters were as follows: pH 7, temperature 30°C and feed to t-butanol ratio 1:1 (v/v). In UATPP, the ultrasonic frequency was 25 kHz, irradiation time 6 min with 20% duty cycle.

Figure 2 revels that with increased $(NH_4)_2SO_4$ concentration, the purity of peroxidase was significantly increased up to 17.54 fold ($p<0.05$) with activity recovery of 81%. Further increase in $(NH_4)_2SO_4$ concentration results in reduced fold purity and % recovery, which may be due to decreased selective partitioning of peroxidase in the bottom phase. Lower ammonium sulfate concentration (20% and 40%) may not be able to change the hydrophobic surface of peroxidase. Akardere et al. [12] reported a similar trend in three-phase partitioning of invertase from baker's yeast, where invertase purity was increased at 50% $(NH_4)_2SO_4$ concentration, and further increase in % $(NH_4)_2SO_4$ concentration led to reduced purity. Higher purification fold and % recovery in UATPP as compared to that of TPP confirmed substantial contribution of ultrasonication in enhancement of partitioning of peroxidase [17]. Maximum purity and recovery were achieved at 50% $(NH_4)_2SO_4$ concentration, thus this was selected as the optimum value for the next experimentation.

3.4 Effect of broth to t-butanol ratio

t-Butanol was selected as a co-solvent from the reference of available literature and due to its advantages over other alcohols (methanol and ethanol). It is a higher molecular size branched chain alcohol which is unable to permeate inside the folded three dimensional structure of protein and incompetent to denature partitioned enzyme [27]. Also at 20–30°C, t-butanol imparts significant kosmotropic and crowding effects which enhance the partitioning. The effect of broth to t-butanol ratio was varied from 2 to 0.4 (v/v) in UATPP at 50% $(NH_4)_2SO_4$ concentration, pH 7, temperature 30°C, ultrasonic irradiation at 25 kHz and irradiation time of 6 min with 20% duty cycle. Purity and % yield of partitioned enzyme were increased along with a decrease in broth to t-butanol ratio from 2 to 0.5 (v/v); afterwards, it was decreased ($p<0.05$) as shown in Figure 3. Higher values of purification factor and % recovery in UATPP as compared to TPP confirmed the substantial role of ultrasonication in enhancement of peroxidase partitioning. A higher quantity of t-butanol may lead to denaturation of enzyme and also attracts more water from the aqueous phase, which subsequently increases salt concentration in the aqueous phase, which leads to precipitation of enzyme at the interface. Moreover, at

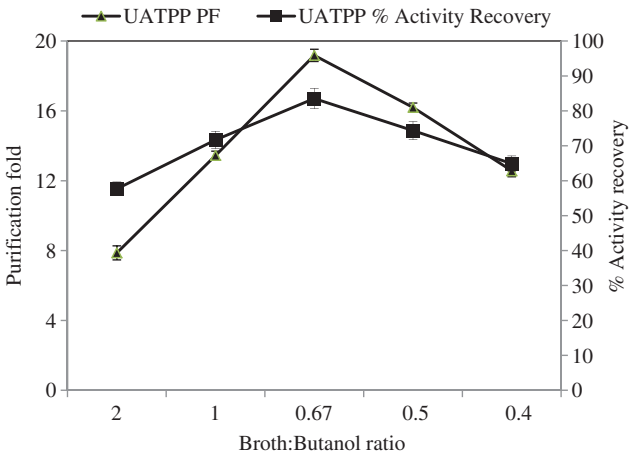

Figure 3: Effect of broth to t-butanol ratio on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

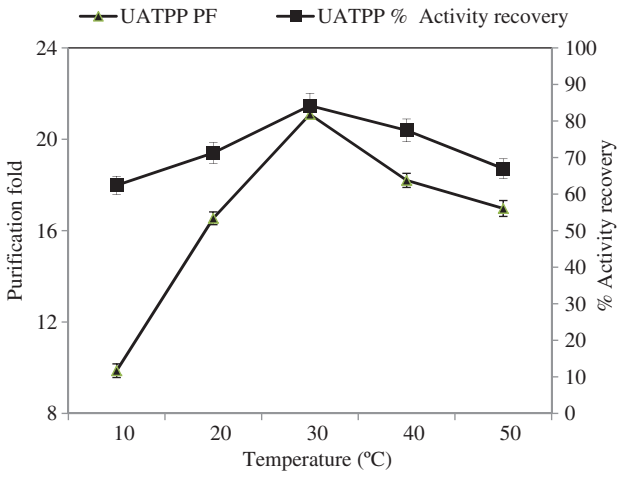

Figure 4: Effect of temperature on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

lower broth to t-butanol ratio, surface tension and vapor pressure of the mixture were higher due to a higher amount of t-butanol. At higher vapor pressure, more vapor entered in to the bubbles, imparting a cushioning effect to the bubbles and these bubbles collapsed less intensely resulting in a decreased cavitation effect [28]. At 1:1.5 ratio (0.67), purity and recovery of peroxidase were found to be 19.18 and 84%, respectively, which is maximum ($p<0.05$), thus further experiments were carried out at 1:1.5 ratio of feed to t-butanol.

3.5 Effect of temperature

The temperature of the ultrasonic bath affects enzyme partitioning, stability and cavitation phenomenon. All earlier experiments were performed at room temperature (30±2°C). The effect of temperature was studied by changing the temperature in UATPP from 10°C to 50°C, while keeping all other experimental variables constant, such as 50% $(NH_4)_2$ concentration, pH 7, broth to t-butanol ratio 1:1.5 (v/v) and ultrasonic irradiation at 25 kHz for 6 min with 20% duty cycle. Figure 4 demonstrates the effect of temperature on partitioning of enzyme in UATPP ($p<0.05$). Peroxidase purity was increased up to 21.09 fold with the ultrasound irradiation at 30°C temperature.

A decrease in purification factor at a higher temperature (above 30°C) may be due to thermal deactivation of enzyme. Apart from this, it has been reported that lower cavitational dose was imparted to the three phase system at a higher temperature, which could not alter the partitioning of peroxidase. At a higher temperature, bubbles collapses with less intensity, hence compromise between temperature and cavitation must be obtained [28]. Different researchers have extracted enzymes at a temperature range of 20–30°C viz; α galactosidase from pepino at 25°C [14]. Thus, by considering economic, operational and enzymatic stability, the next set of experiments was performed at 30°C.

3.6 Effect of pH

pH affects the state of ionization of amino acids, thus it is necessary to study the effect of pH on the UATPP system. When the pH of the system is maintained above the isoelectric point (pI) of protein, it will attain a net negative charge and will be pushed to the bottom phase. In discrepancy with this, when the system pH is below the pI of the target protein, it is precipitated or accumulated at the interphase. pH enables the change in net charge of the target protein, which causes alteration in partitioning behavior of the protein [27]. The effect of pH was studied by varying the pH in UATPP from 4–8, while keeping all other parameters at a constant value such as, 50% $(NH_4)_2SO_4$ concentration, temperature 30°C, broth to t-butanol ratio 1:1.5 (v/v), and ultrasonic irradiation at 25 kHz for 6 min with 20% duty cycle. Figure 5 designates that as pH increased from 4 to 8, fold purity and activity recovery of peroxidase increased ($p<0.05$) as the pI of the peroxidase was 4.5–5.2 [29]. At pH 6, negative charge on the protein was increased, which resulted in enhanced partitioning to the bottom phase and other contaminating proteins were precipitated at the interphase. Avhad et al. [22] reported enhanced partitioning of fibrinolytic enzyme inhibitors in the bottom phase at pH 9, which was above

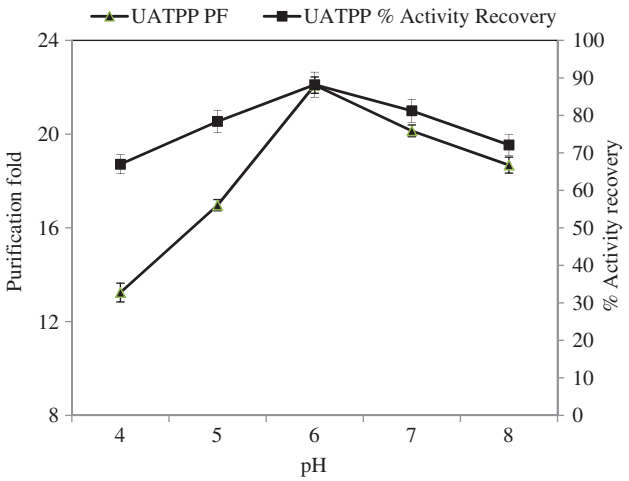

Figure 5: Effect of pH on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

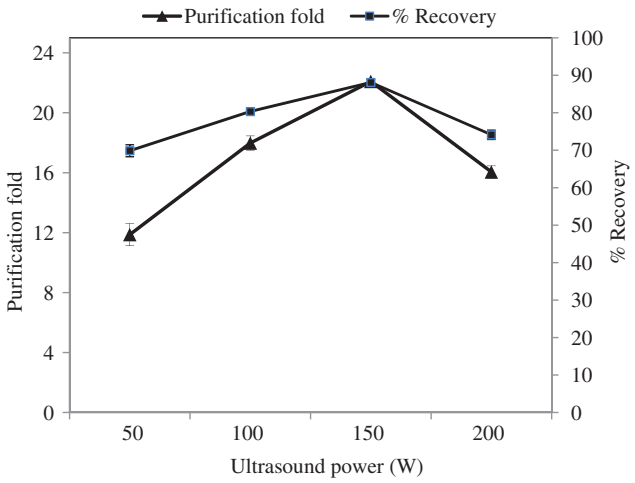

Figure 6: Effect of ultrasound power on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

its pI 6 [22]. Higher purification and activity were obtained in UATPP, as indicated in Figure 5. Enhanced mass transfer in UATPP is responsible for this increase in purity and activity. Maximum purity (22.09) and activity recovery (88.19) were obtained at pH 6, thus the next experiments were carried out at pH 6.

3.7 Effect of ultrasound power

Power is the critically important parameter that affects the overall economy of the process at commercial scale. The effect of power (rated ultrasound power) was studied by varying it from 50 W to 200 W with 20% duty cycle at 25 kHz frequency for 6 min with 50% ammonium sulfate concentration, pH 6, temperature 30°C and broth to t-butanol ratio 1:1.5 (v/v). Calorimetric study was performed to calculate the dissipated power which was found to be 42 W, 46.2 W, 61.6 W and 72.8 W for 50 W, 100 W, 150 W and 200 W rated power at 25 kHz frequency, respectively.

Figure 6 represents the effect of rated power on purification fold and activity recovery of peroxidase in UATPP. Peroxidase enzyme purity was increased up to 150 W rated power (dissipated power 61.6 W) which decreased on further power increment. This may be due to degradation of protein structure at higher power value ($p<0.05$). Large numbers of cavitational bubbles were formed at higher ultrasound power which collapsed more intensely, imparting strong mechanical shear to the three phase system. Conversely, further increase in rated power beyond 150 W (dissipated power 61.6 W) resulted in liquid agitation instead of cavitation and poor propagation of ultrasound through liquid medium [28].

3.8 Effect of duty cycle

An ultrasonicator can be operated in both modes, i.e. pulse mode and continuous. Continuous mode causes temperature rise, which may degrade thermolabile biomolecules. As proteins/enzymes are temperature sensitive biomolecules, the pulse mode is a well suited alternative for operation. The duty cycle was varied by changing the on-off time of ultrasound at 150 W rated power for 6 min at 25 kHz. The purity of peroxidase was increased up to 40% duty cycle (24 s on and 36 s off) and decreased subsequently until 100% duty cycle (60 s on and 0 s off) ($p<0.05$), as shown in Figure 7. A reduction in the peroxidase purity was attributed to the excessive ultrasonication

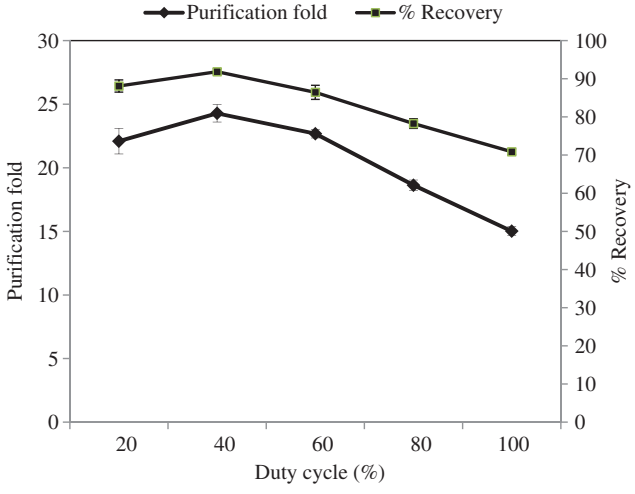

Figure 7: Effect of duty cycle on ultrasound assisted three phase partitioning (UATPP) of peroxidase.

dose. At a higher duty cycle, an extensive cavitation occurs which leads to protein denaturation. In addition to this, higher duty cycle causes temperature elevation which is also one of the factors of enzyme degradation. Several researchers have been suggested intermittent irradiation (pulse mode) for the intensification of various bioprocesses. Pulse mode is an energy efficient approach and it also enhances the lifespan of ultrasonic transducers. Extraction of β carotene from *Spirulina platensis* at different duty cycles has been reported and displayed similar trends of response [30].

3.9 Comparison of conventional TPP and UATPP

TPP displays selective partitioning of peroxidase in the bottom phase and other contaminant proteins to the upper and intermediate phase. Results of UATPP are compared with TPP at optimized conditions [17] as shown in Table 1. At optimum conditions, i.e. 50% ammonium sulfate saturation, pH 6, and broth to t-butanol ratio 1:1.5 (v/v), TPP has shown 19.67 fold purity with 84% recovery of peroxidase after 80 min of conventional stirring [17]. However, ultrasound assisted TPP has shown increase in fold purity of peroxidase up to 24.28 at optimized conditions of irradiation viz; 25 kHz frequency, 40% duty cycle and 150 W rated power for 6 min. Thus, ultrasound irradiation not only decreases the time of operation of TPP, but also increases the fold purity of peroxidase. Thus, UATPP would be an attractive alternative to purification of biomolecules.

3.10 SDS PAGE analysis

Figure 8 represents the SDS PAGE analysis of crude and partially purified peroxidase enzyme after UATPP partitioning on 12% gel. Fewer bands in the UATPP lane demonstrate the enhanced purity of peroxidase enzyme with molecular weight of 26 kDa. It is found that the molecular weight of peroxidase was about 26 kDa, which is similar to the reported results by Clemente [29].

Table 1: Comparison of conventional three phase partitioning (TPP) and ultrasound assisted three phase partitioning (UATPP).

Process	Time (min)	Activity recovery (%)	Purification fold
TPP	80	84±2.3	19.67±1.2
UATPP	6	91.84±1.4	24.28±0.97

Mean±SD of three replicated samples.
Results shows $p<0.05$, it is statistically significant.
TPP, Three phase partitioning; UATPP, ultrasound assisted three phase partitioning.

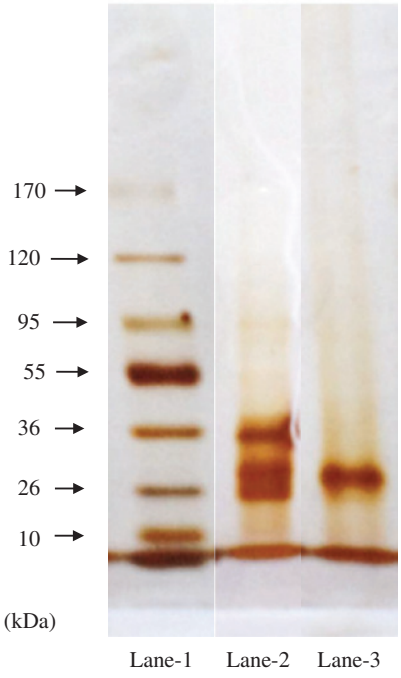

Figure 8: SDS PAGE pattern of peroxidases from orange (*Citrus sinenses*) peel.
Lane 1: molecular weight marker, Lane 2: crude, Lane 3: after UATPP.

4 Conclusion

Purification of peroxidase from orange peel (*Citrus sinensis*) using UATPP has been successfully optimized. Enhanced mass transfer in UATPP results in higher % recovery of peroxidase enzyme as compared to TPP. Higher values of irradiation time, rated power and duty cycle had reported unfavorable effects on peroxidase partitioning. Optimized parameters of UATPP increase fold purity and % recovery of peroxidase, reducing time of operation from 80 min to 6 min as compared to conventional TPP. Partitioned enzyme shows partial purification of peroxidase from crude broth through SDS PAGE analysis. Hence, enhanced purity with reduced time of operation symbolizes UATPP as an attractive contention for a future integrated bioseparation protocol. The combination of ultrasound and TPP enhances the benefits of the both processes and makes this integrated approach an interesting alternative to the downstream purification step.

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