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Biochemistry

ISOLATION, PURIFICATION AND IMMOBILIZATION OF SULFIDE OXIDASE FROM ALCALIGENES FAECALIS

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ABSTRACT Sulfide oxidase is the enzyme involved in sulfide pathway which catalyzes the conversion of toxic form of sulfide to non toxic sulfate. In this study, an attempt was made to isolate and purify sulfide oxidase from *Alcaligenes faecalis* and its activity was measured. The isolated enzyme was purified using acetone precipitation and ion exchange chromatography. Column purified extracts registered sulfide oxidase activity of 39 µm/hr/mg of protein and the purification was increased up to 65 fold. The optimum sulfide oxidase activity was found in pH (6- 8), salinity (10-30 ppt) and temperature (30 & 35 ◦C). In addition to this, chitosan beads were prepared for the immobilization of sulfide oxidase. Further, experiments to evaluate the capacity of the immobilized enzyme to remove sulfide with more emphasis on stability in field conditions would provide useful information to mitigate sulfide from aquaculture ponds.

KEYWORDS : Sulfide, Alcaligenes faecalis, sulfide oxidase, chitosan and immobilization

1. Introduction

The export of marine products has reached the remarkable growth of US$ 5511.12 million, in which frozen shrimp accounts for 34.01 % in quantity and 67.19% of the total US$ earnings (MPEDA, 2015). Factors like stocking density, quantity of feeds used for growth, chemical inputs, water logged condition, various microbial processes plays a vital role in governing the water and sediment quality. Any deterioration in water and sediment quality will ultimately result in production of undesirable compounds like ammonia, nitrite and sulfide. These compounds have the potential to invade to aquatic organism, thereby decreasing the production (Moriarty, 1997). In aquaculture, application of soil and water probiotics is one of the general management strategy practiced to maintain physico chemical parameters in optimum range. These soil and water probiotics contains live microorganisms as a single strain or as a consortium of bacteria (Kumar et al., 2016). Off late use of immobilized bacteria or enzymes gained lot of importance especially in the field of bioremediation and food technology (Fernandes, 2010). In aquaculture, toxins such as ammonia, nitrite nitrate and sulfide are mitigated generally by biological methods using microbes involved in nitrogen and sulfur cycle (Avnimelech and Ritvo, 2003). Alter native approach is using enzymes involved in the biogeochemical cycle and sulfide oxidase is one such enzyme used for the removal of sulfide from aquatic system. Sulfide oxidase is the enzyme involved in sulfide pathway which catalyzes the conversion of toxic form of sulfide to non toxic sulfate. In this study, we aimed to isolate and purify enzyme sulfide oxidase from *Alcaligenes faecalis*. In addition to this, effect of pH, salinity and temperature and immobilization of sulfide oxidase in chitosan was also studied.

2. Materials and Methods

2.1. Cultivation of *Alcaligenes faecalis*

Alcaligenes faecalis (MTCC No-9780) was grown in 1L Kings medium B (Bacteriological Analytical manual, 1998) for 24 h, respectively at 32℃.

2.2. Enzyme isolation and purification

To isolate sulfide oxidase enzyme, *Alcaligenes faecalis* was grown in 500 ml of autoclaved media and incubated for 2 days in 32℃. Further, 1 ml of 100 µm of sodium sulfide solution was added and incubated further for 2 days and cells were collected by centrifugation at 10,000 rpm for 30 min at 4℃ (Mohapatra et al, 2006). The pellet was washed twice with 50 mM Tris-HCl, pH 7.5, centrifuged again to collect the cells. The bacterial cells thus collected were sonicated under ice cold condition, centrifuged as mentioned above and this was used as crude enzyme extract (CEC). The crude extracts were further subjected to acetone precipitation and used as partially purified enzyme extract (PPE). The acetone precipitated extracts were subjected to column purification using anion exchange containing DEAE cellulose column. For this, DEAE cellulose was packed in glass column (10 cm length) and it washed thoroughly with elution buffer. To this column, 0.5 ml of acetone precipitated enzyme extracts were added and eluted with increasing concentration of sodium hydroxide (0- 0.5 M). Each fraction was collected separately and absorbance was measured in UV spectrophotometer at 280 nm. Active fractions were pooled together and used for the sulfide oxidase assay and immobilization in chitosan beads.

2.3. FTIR sample preparation

Then, the chitosan beads were pressed to thin plates and the FTIR spectra was determined by recording the transmittance. After baseline correction, spectra were recorded in the spectral range of 370–4000 cm^{-1} and beads were scanned at 16 scans/min.

2.4. Sulfide oxidase assay

Sulfide oxidase assay was determined in crude extract, precipitated and column purified extracts. Briefly, 50 µl of enzyme extracts were transferred to 1 ml sterile tubes and to this 100 µm of sodium sulfide solution was added and incubated for the period of 1 h. After the incubation period, the absorbance of remaining sulfide was measured in a spectrophotometer (UV 1700, Shimazdu) at 670 nm. One unit (U) of sulfide oxidase activity was expressed as the micromoles of Na_2S oxidized per hour per milligram-protein of enzyme preparation under the assay conditions

2.5. Determination of protein

Protein concentration in enzyme extracts were estimated using Lowry's method using Bovine serum albumin as standard

2.6. Preparation of chitosan beads

For the preparation of chitosan beads and for the immobilization of sulfide oxidase, method of Zhang et al (2009) was used with little modification. Briefly, 2.5 g of chitosan powder was dissolved in 100 mL of 1% (v/v) acetic acid and kept at 4°C overnight. This solution was passed through a syringe into 200 ml of NaOH solution containing formaldehyde (3:1) under constant stirring. After 3 h, chitosan beads were transferred to clean container and rinsed several times until it reached pH 7. Beads thus formed were dried at 80 °C for 8 h and used for immobilization. 100 ml of enzyme was added to 1 g of dry chitosan beads and incubated at room temperature and further incubation in 0.05 % glutaraldehyde for about 6 h at 40 °C. The chitosan beads were washed several times until the pH was neutral.

3. Results and Discussion

3.1. Purification of sulfide oxidase

Sulfide oxidase was extracted from *Alcaligenes faecalis* and sulfide oxidase activity was measured. Prior to measuring enzyme activity, absorbance was measured in broad wavelength range of 400-260 nm. In case of crude enzyme extracts, highest absorbance of 0.641 was

found in 280 nm (Fig 1). Similarly, highest absorbance of 1.662 was found in 280 nm for acetone precipitated extract.

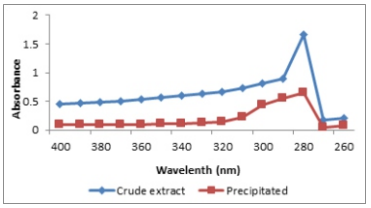

Fig 1: UV absorption spectra of sulfide oxidase

Further, precipitated enzyme extracts were applied to Ion exchange column containing anionic exchanger. Enzyme extracts were eluted with increasing eluent concentration of 0.1 to 0.5 M NaOH. Each fraction was collected separately in sterile tubes and marked as fraction 1 to 6. Spectral scan was performed using UV visible spectroscopy and sulfide oxidase activity was measured in each fraction. Out of five fractions, fraction 2, 3 and 4 exhibited highest sulfide oxidase activity and they were pooled together (Fig 2). Protein concentration, specific activity, purification and yield were calculated and the results are summarized in table 1. When crude enzyme extracts were subjected to acetone precipitation and column purification, it was purified up to 2.5 and 65 fold, respectively. Purification of enzyme is necessary because, crude enzyme extracts may contain interfering substances which may hinder its activity during downstream process. Acetone precipitation is proven to be rapid and efficient way to concentrate proteins and enzymes. Similarly, Ion exchange chromatography was performed using DEAE cellulose, an anionic ion exchange resin with positive charge which retains all the negatively charged compounds. In this study, column purified extracts showed sulfide oxidase activity of 39 µm/hr/mg of protein and the purification was increased up to 65 fold from the previous step. Previous studies have described the usefulness of ion exchange chromatography for the purification of various enzymes. Absar et al. (2009) isolated intracellular enzyme from *Aspergillus niger* and purified using anionic ion exchange column with the enzyme activity 43 U/mg.

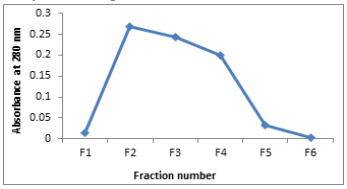

Fig 2: Purification of sulfide oxidase by Ion exchange chromatography.
F1-F6 indicates various fractions collected

Table 1: Purification of sulfide oxidase

Method of purification	Volume of culture (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (µm/hr/mg of protein)	Purification (fold)	Yield %
Cell free extract	250	15.12	3780	0.6	1	100
Acetone precipitation	80	6.19	495	1.5	2.5	38
Ion exchange chromatography	2	2.169	4.338	39	65	5.3

3.2. Characterization of sulfide oxidase by FTIR spectroscopy

Acetone precipitated (PPT) and column purified (CP) enzyme extracts were characterized using FTIR spectroscopy (Fig 3). Stretching vibrations at 3366 cm-1 in PPT is indicates inter molecular bonding and peak at 2884 in both extracts indicates C-H stretching. Regions stretching from 1390-1662 indicates the presence of C=O group while peak at 606 indicated the presence of ether group (Vodnar, 2012).

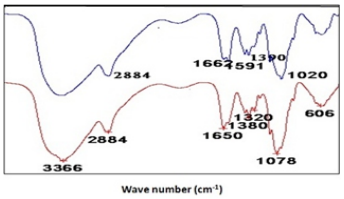

Fig 3: FTIR spectroscopy for enzyme extracts. Red line indicates acetone precipitated enzyme extract and blue line indicates column purified enzyme extracts

3.3. Effect of pH, salinity and temperature

The effect of pH, salinity and temperature on sulfide oxidase enzyme activity was given in fig 4 to 6. The sulfide oxidase activity was greatly affected in acidic and alkaline pH ranges and the optimum activity was achieved on pH 6, 7 and 8. Our results are in agreement with previous studies where the optimum of sulfide oxidase activity isolated from *Arthobacter* sp was found in the range of 6 to 7.5. (Mohaptra et al, 2006). Similar reports of sulfide oxidase enzyme activity were reported in heterotrophic and autotrophic microorganism. (Nakada and Ohta, 1999). The optimum sulfide oxidase isolated from *Serratia* sp was achieved in pH range of 5 to 7 and beyond this range, SO activity was severely inhibited (Zakaria, 2009).

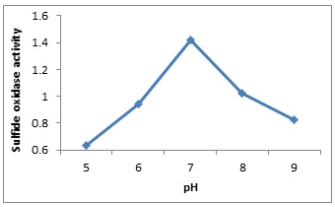

Fig 4: Effect of pH (5-9) on sulfide oxidase activity

Salinity is one of the important water quality variable in aquaculture, because shrimp is cultured in varies salinity ranges from 0 to 40 ppt. It is important to study the effect of salinity on sulfide oxidase activity. The optimum activity was achieved in 10, 20 and 30 ppt saline range. The sulfide oxidase activity was much lesser in 5 ppt and 40 ppt salinity range when compared to other salinity (10, 20 and 30 ppt). Similarly, when effect of temperature is taken into consideration, the optimum enzyme activity was achieved in 20, 30 and 35 ◦C. The optimal temperature for the enzyme activity was found to 30-37 ◦C beyond which the enzymes undergo denaturation which initiates the decrease in enzyme activity (Hogg, 2005).

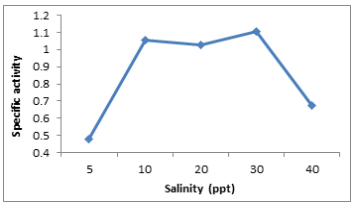

Fig 5: Effect of salinity (5-40 ppt) on sulfide oxidase activity

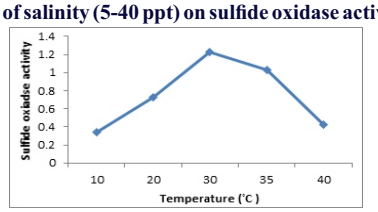

Fig 6: Effect of temperature (10-40 ◦C) on sulfide oxidase activity

3.4. Immobilization of sulfide oxidase in chitosan beads

The sulfide oxidase immobilized chitosan beads are shown in fig . During the process of sulfide oxidase immobilization with glutaraldehyde was used to increase the resistance against sheer forces and increases stability against various environmental conditions (Zhang et al, 2009). Prepared chitosan beads were observed to be rigid and strong. Zhang et al (2009) used sulfide oxidase isolated from Streptomyces species LD048 to immobilize on chitosan beads. Further it was tested for the sulfide its removal capacity and 100 % sulfide removal was achieved in sulfide oxidase bioreactor.

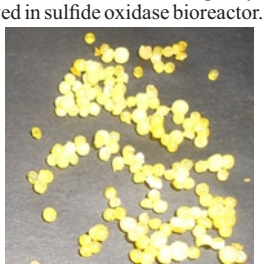

Fig 7: Sulfide oxidase immobilized chitosan beads

Many studies have reported the usefullness of immobilized enzymes for various environmental applications like waste water treatment and biosynthesis of various compounds. For instance, urease entrapped in sol gel matrix and glucose oxidase cross linked with glutaraldehyde was used for the detection of mercury and chromium respectively (Tsai and Doong, 2005 and Zeng et al, 2004)

4. Conclusion

Immobilized bacterial and enzyme products are considered as viable alternative for the treatment of various pollutants and undesirable compounds from the environment. Generally immobilized bacterial cells and enzymes are preferred over the free cells since they are protected by matrices which would ultimately protect the enzymes from harsh environment. In this study, sulfide oxidase was isolated from *Alcaligenes feacalis* and purified using acetone precipitation and anionic ion exchange. Effect of pH, salinity and temperature on sulfide oxidase activity was studied to determine the optimum range. In addition to this, immobilization of sulfide oxidase onto chitosan beads was carried out sucessfully. Experiments to determine the capacity of the immobilized enzyme to effectively remove sulfide with more emphasis on stability in field conditions would provide useful information to mitigate sulfide from aquaculture ponds.

Acknowledgements

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