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Production, Isolation and Purification of Peroxidase Using Bacillus Subtilis

P.Raja Rao [1]and P. Kavya[2]

[1,2]University College of Technology,Osamania University, Hyderabad-500007

Abstract. Peroxidases are classified as oxido reductases which are used for catalyzing various oxidative reactions. They are isolated from various sources like plants, animals and microbes. Peroxidases have wide applications in many areas like industrial, medical and food processing. In the present study, Bacillus subtilis was employed for the production of peroxidases. B.subtilis was isolated from soil using the Serial dilution method. Identification of B.subtilis was done by performing various staining techniques and biochemical assays. Pure cultures of B.subtilis were obtained and screened for the production of peroxidases and those cultures which produced the same were selected for further study. B.subtilis produced 0.00045 units of peroxidase per ml of fermentation media. Optimization studies were performed and it was found that the optimum conditions for the production of peroxidase are pH-6, Temperature-370C. Purification of peroxidase enzyme was done using Salt precipitation, dialysis and Ion exchange chromatographic techniques. Quantification of the resultant peroxidase was done by Lowry's method. Kinetics of the peroxidase enzyme were also studied .Enzyme immobilization was done and was found that the peroxidase activity increased after immobilization.

Keywords: Peroxidase, Bacillus Subtilis, Enzyme Activity, Immobilization

1. Introduction

An enzyme is a globular protein with an active site which bind to substrate molecules and helps to catalyse a reaction by holding molecules in the correct spatial conformation for the reaction to take place. The activities of the enzymes are determined by their 3-dimensional structure. Most enzymes are much larger than the substrates they act on and only a small portion of the enzyme around 3-4 aminoacids is directly involved in catalysis. Enzymes carry out transformation of molecules and also mediate transformation of energy. The term peroxidase in the widest sense includes a group of specific enzymes such as NAD peroxidase, fatty acid peroxidases, glutathione peroxidase as well as a group of very non-specific enzymes from different sources simply known as peroxidases. Most, if not all, haeme proteins may be converted to forms which show some peroxidase activity. For example metmyoglobin and methaemoglobin and fragments of non-peroxidase haem proteins such as microperoxidase from cytochrome c are catalytically effective. Even protein free haeme complexes show significant peroxidase and catalase-like activity. Peroxidases are a group of enzymes that catalyses oxidation reduction reactions. As such they are classified as oxidoreductases. Toxic molecules such as super oxide and hydroxide radicals can be found in cells due to the presence of oxygen. Peroxidases are oxidoreductases which use H2O2 as electron acceptor for catalyzing different oxidative reactions. Peroxidase is an enzyme, which may contain haeme that catalyze the transfer of oxygen from hydrogen peroxidase to a suitable substrate and thus brings about oxidation of the substrate. Its specificity, biological functions vary with sources of the enzyme. Extracellular peroxidase is the most important component of the extracellular lignin degrading system which is responsible for the initial attack of lignin by a non specific oxidation mechanism. The production of peroxidase which was detected in trace amounts in dispersed carrot cell cultures was found in high amounts (5.0–14.5 Ug−1 FW) in CCA cultures by John Evans (1963). The peroxidase treatment of waste streams is much less sensitive than

bacterial degradation to variations in pH, other toxic constituents, concentration of phenol or aromatic amine and temperature.

2. Materials and Methods

2.1. Isolation and Identification of Bacteria

The isolation of microorganisms from soil was performed by serial dilution method. Standard procedure is followed using solidification and incubation. A thin bacterial smear was prepared on a clean glass slide and air dried and heat fixed. The smear was flooded with crystal violet (30 sec. to 2 min).The excess stain was removed by rinsing it under tap water. After that, the smear was fixed with Gram's iodine (1 minute).Then it was decolorized with alcohol. Again it was rinsed under running tap water slowly. Finally, the smear was counterstained with Safranin (30 sec to 2 min.) The excess stain was removed by rinsing it under tap water. The slide was blot dried with bibulous paper and observed under 10x, 40x and 100x objectives of Microscope.

Endospore Staining : A thin bacterial smear was prepared on a clean glass slide, air dried and gently heat fixed. Slide was placed on a boiling water bath. Malachite green was added drop wise. Steam was applied for five minutes. Stain was added continuously to prevent the drying. Allowed the slide to cool and then rinsed with deionized water until water runs clear. The counter stain safranin was added and allowed to act for two minutes. Then examined under the microscope in which the vegetative cells appear pink and endospores appear green.

Pure culture preparation: Pure cultures of the isolated bacteria were prepared by quadrant streaking method. Nutrient agar media were prepared and kept for sterilization. It was later poured into the sterile Petri plates and allowed to solidify. After solidification, the Colony no. 1 from the dilution 10-4 was streaked onto the nutrient agar medium.

BioChemical Tests: Mannitol Fermentation test, Methyl Red and Voges Proskauer Test (MR-VP) ,Starch Hydrolysis, Gelatin Hydrolysis and Casein Hydrolysis are performed.

Screening of B Subtilis for Production of Peroxidase : Nutrient broth was prepared and inoculated with bacterial inoculums. It was incubated for 24 hours.1 ml of the screening media and 1 ml of the broth was taken in a test tube. Added H2O2 drop by drop until the pink color was seen.

Observation : Appearance of pink color shows positive.

Production media for peroxidase: 50ml of production media was prepared in conical flask and it is autoclaved at 1210c at 15 lbs pressure for 15 minutes. Then a loopful of Bacillus subtilis culture was inoculated into the production media. Finally the bacterial medium was incubated at 370C for 48 hours in shaking incubator. Like above another production medium also prepared.

Enzyme extraction: Taken 50ml of production broth and transferred it into centrifuge tubes. They are centrifuged at 6000 rpm for 10 minutes. Supernatant having crude peroxidase was collected and the pellet was discarded. The supernatant was used for enzyme determination.

Assay of crude peroxidase enzyme: Enzyme assay was performed to check the activity of crude enzyme using Continuous Spectrophotometric Rate Determination method.

Conditions : Temperature --200C, PH ---- 6.0, Wave length -- 420nm

The reagents are taken as per standard procedure. Enzyme was added to only test marked tube. After the addition of enzyme, the OD values were taken immediately at 420nm in spectrophotometer. In this process freshly prepared pyrogallol was used.

Purification of crude peroxidase enzyme from Bacillus subtilis : Purification of peroxidase enzyme is done by three methods: Salt precipitation method, Dialysis method and Ion exchange (Table 1) chromatographic method. The protein content was estimated by Lowry's method (Table.2)

Effect of pH on Enzyme Activity: Enzyme activity varies with changes in pH (fig.11a) and at extreme pH values, tertiary structure of protein may be disturbed. Exact value of pH also play important role in enzyme activity.

Effect of activator on enzyme activity: Activator is the substance which increases the enzyme activity. Phosphate buffer, H2O2, Pyrogallol, Manganese sulphate(MgSO4) are used as activators(Table.4)

SDS-PAGE : Standard procedure was followed by preparing the buffers, adding the gel, degassing, addition of TEMED, solidification followed by observing the changes.

Observation: Bands were observed.

Table 1: Enzyme Assay of Ion Exchange Elute Samples

TT no	Vol of D/W(ml)	Vol of Buffer(ml)	Vol of $H_2O_{2(/ml)}$	Vol of Pyrogallol(ml)	Vol of D/W(ml)	Vol of Enzyme(ml)	OD at 420nm
Blank	2.10	0.32	0.16	0.32	0.10	_	0.000
1	2.10	0.32	0.16	0.32	_	0.10	0.040
2	2.10	0.32	0.16	0.32	_	0.10	0.060
3	2.10	0.32	0.16	0.32	_	0.10	0.018
4	2.10	0.32	0.16	0.32	_	0.10	0.070
5	2.10	0.32	0.16	0.32	_	0.10	0.046
6	2.10	0.32	0.16	0.32	_	0.10	0.032

Table 2: Estimation of protein by Lowry's method

TT no	Vol of BSA	Vol of Dist.water	Vol of Alkaline $CuSO_4$		Vol of Fc		OD at 660nm
BLANK	0 ml	1 .0ml	5 ml		0.5 ml		0.00
1	0.2 ml	0.8 ml	5 ml		0.5 ml		0.110
2	0.4 ml	0.6 ml	5 ml		0.5 ml		0.225
3	0.6mlL	0.4 ml	5 ml		0.5 ml		0.300
4	0.8ml	0.2 ml	5 ml		0.5 ml		0.465
5	1 .0ml	0ml	5 ml		0.5 ml		0.525
CRUDE	0.1ml	0.9ml	5ml	INCUBATE AT ROOM TEMP FOR 10 min	0.5ml	INCUBATE AT DARK	0.174
DIALYSIS	0.1ml	0.9ml	5ml		0.5ml		0.163
ELUTE 2	0.1ml	0.9ml	5ml		0.5ml		0.150
ELUTE 4	0.1ml	0.9ml	5ml		0.5ml		0.100

(from the above , Elute 2 is used for further processes like Enzyme kinetics, Immobilization)

Table 3: Specific activity of peroxidase at different concentration of substrate

TT no	Vol of D/W	Vol of Buffer	Vol of H_2O_2	Vol of Pyrogallol	Vol of D/W	Vol of Enzyme	OD at 420nm
Blank	2.10	0.32	0.16	0.32	0.10	_	0.00
2	2.21	0.32	0.05	0.32	-	0.10	0.015
3	2.16	0.32	0.1	0.32	_	0.10	0.029
4	2.06	0.32	0.2	0.32	_	0.10	0.042
5	1.96	0.32	0.3	0.32	_	0.10	0.066
6	1.86	0.32	0.4	0.32	_	0.10	0.086
7	1.76	0.32	0.5	0.32	_	0.10	0.086

Table 4: Specific activity of peroxidase at different concentration of activator

TT no	Vol of D/W (ml)	Vol of Activator (ml)	Vol of Buffer (ml)	Vol of H_2O_2 (ml)	Vol of Pyrogallol (ml)	Vol of D/W (ml)	Vol of Enzyme (ml)	OD at 420nm

Blank	3.10	0.0	0.32	0.16	0.32	0.10	_	0.00
2	2.90	0.2	0.32	0.16	0.32	_	0.10	0.048
3	2.70	0.4	0.32	0.16	0.32	_	0.10	0.060
4	2.50	0.6	0.32	0.16	0.32	_	0.10	0.076
5	2.30	0.8	0.32	0.16	0.32	_	0.10	0.093
6	2.10	1.0	0.32	0.16	0.32	_	0.10	0.115

3. Result and Discussion

3.1. Isolation of bacteria from soil by serial dilution

After incubation, different types of colonies were observed on the inoculated Nutrient agar plates.

Morphological characteristics of Bacillus subtilis Gram staining: Examined the slide microscopically (100x) using oil immersion objectives. Bacterial colony, 10-4(1st), has taken up the crystal violet colour and appeared in purple colour. So, 10-4(1st) colony was positive for Grams staining (fig.1)

Endorspore staining: Examined the slide microscopically under oil immersion objective.

10-4(1st) - bacterial colony has taken up malachite green and appeared in green colour.

So, 10-4(1st) colony was positive for endospores (fig.2)

Pure culture of Bacillus subtilis on nutrient agar medium

Colony no. 1 from the dilution 10-4 was streaked onto the nutrient agar medium (fig.3)

Mannitol Fermentation Test: Colour change from red to yellow was observed in the mannitol fermentation tube inoculated with the colony from 10-4(1st) .Thus from the above observation it was confirmed that 10-4(1st) colony was found to be Bacillus subtilis.(fig.4)

MR-VP Test: Ruby pink color was obtained when VP-1 and VP-2 reagents were added.

The organism was positive for VP test. Thus confirmed that 10-4(1st) colony was found to be Bacillus subtilis (fig.5).

Starch Hydrolysis: A clear zone was observed around the organism which indicates that the organism has utilized starch. Therefore, the organism is positive for Starch hydrolysis (fig.7)

Gelatin Hydrolysis: After the incubation period the gelatin tube was not turned from solid state to liquid state. Therefore, the organism is negative for Gelatin hydrolysis (fig.6)

Casein Hydrolysis: A clear zone was observed around the organism which indicates that the organism has utilized casein. Therefore, the organism is positive for Casein hydrolysis (fig.9)

Screening of B Subtilis for the production of peroxidase : Pink colour was observed.

Molecular Weight Determination of Peroxidase by SDS – PAGE: The molecular weight of the enzyme was found to be 44 KDa approximately (fig10).

DS=Dialysis sample CE= Crude enzyme EL=Elute after ion exchange chromatography

Observation: Clear bands were observed on the gel.

Based on sample migration the molecular weight was determined by comparing with BSA. The molecular wt (in kilo Daltons) of the samples as follows:

B S A - 66 KDa, Crude enzyme - 44 KDa, Dialysis sample - 44 KDa

Ion exchange sample-1 - 44 KDa Ion exchange sample-2 - 44 KDa

Result: By comparing above molecular weight of the samples, purified elute sample molecular weight is similar to the enzyme peroxidase molecular weight i.e., 44 K.Da. It indicates that the purified elute sample was determined as peroxidase.

Immobilization and Observation: When sodium alginate with enzyme sample was transferred drop by drop wise into the calcium chloride solution beads were formed indicates the enzyme was immobilized.

Result: 196 sodium alginate beads were obtained. An amount of 0.03 ml of enzyme is required for 6 beads for immobilization. After immobilization the concentration of enzyme sample is similar to purified elute sample i.e. 0.00175 Units/ml.

4. Discussion

Isolation of peroxidase from Bacillus subtilis was done successfully under favorable conditions. It was produced using different production media and was purified by the ammonium sulphate precipitation, dialysis and ion exchange chromatography (Table 1). The purified enzyme was characterized by considering parameters like pH, temperature, substrate, activator and inhibitor (fig 11a,b,c,d,e).

The effect of pH on enzyme activity was studied by taking buffers with difference like 4, 5,6,7,8 and it is observed that enzyme shows maximum activity at pH 6.For optimum temperature estimation the enzyme was incubated at different temperatures like 40 C, room temperature 370 C, 550 C, 1000C.

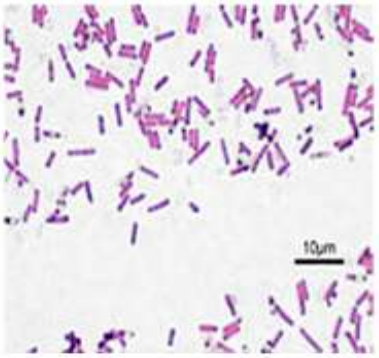

Figure 1: Gram's staining of *Bacillus subtilis*


Figure 2: Endospore Staining of *Bacillus subtilis*


Figure3: Pure culture of *Bacillus subtilis*


Figure 4: Mannitol fermentation test

Figure 5: Voges-Proskauer test

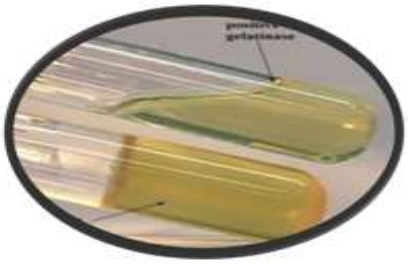
Figure 6: gelatin hydrolysis test

PVP decreases the activity of peroxidase and acts as inhibitor. Manganese sulphate increase activity peroxidase acts as activator this enzyme has potential or industrial uses. The purity of the enzyme was confirmed by running the purified enzyme on SDS-PAGE(fig.10) and proteins are separated based on their size by this method. Immobilization was done with elute sample containing high amount of peroxidase. In this work 196 sodium alginate beads were obtained. This indicates enzyme was immobilized.


Figure7: starch hydrolysis test


Figure8: immobilization beeds


Figure 9: Casein Hydrolysis test

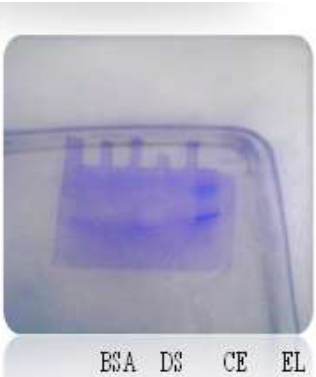

Figure 10: SDS-PAGE Of Enzyme

5. Conclusions

The purified enzyme shows maximum activity at pH 6.0

The optimum temperature of the enzyme is at room temperature (fig.11b).

The substrate concentration of the enzyme is increased up to certain concentration after that the effect is constant (Table.3). The activity of the enzyme is increased by increasing the concentration of manganese sulphate.The activity of the enzyme is decreased by the increasing the concentration of inhibitor like PVP.

Purity of the enzyme was confirmed by running the purified enzyme on SDS-PAGE and proteins are separated based on their size. The size was determined approximately 44kDa. The purity of the enzyme was immobilized by using sodium alginate method. Finally it is concluded that Bacillus subtilis is a good source for the production of peroxidase enzyme and has potential industrial application.

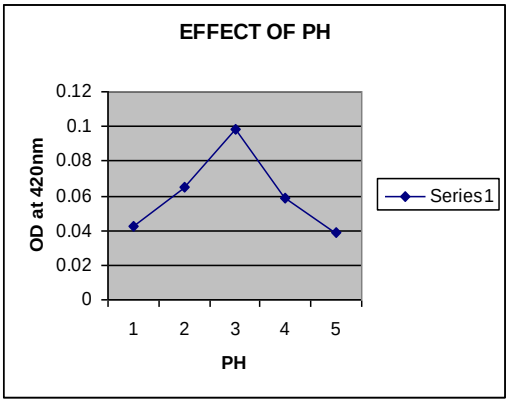

Figure 11(a)

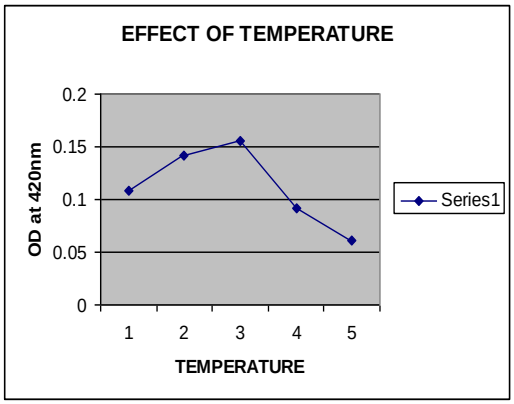

Figure 11 (b)

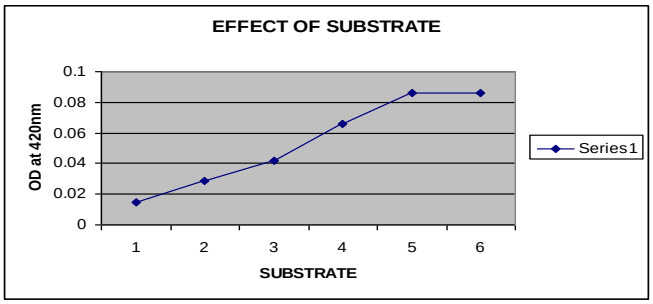

Figure 11 (c)

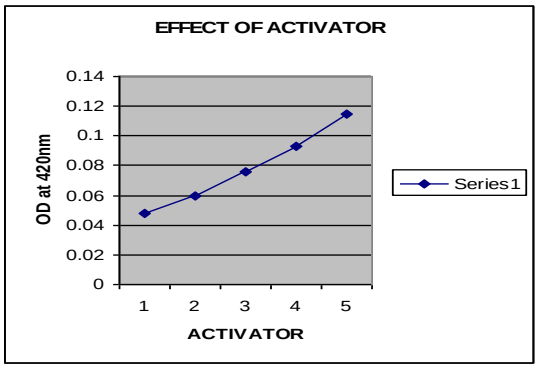

Figure 11(d)

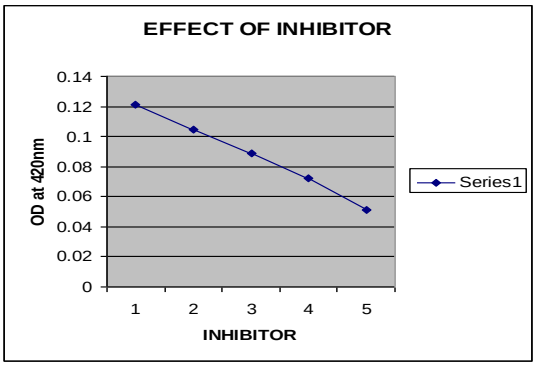

Figure 11(e)

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