https://doi.org/10.48047/AFJBS.6.13.2024. 3553-3586

African Journal of Biological Sciences

ENHANCED PRODUCTION AND PURIFICATION OF BACTERIAL POLYPHENOL OXIDASE FROM *BACILLUS SUBTILIS VK3*: OPTIMIZATION AND CHARACTERIZATION

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Article History
Volume 6, Issue 13, 2024
Received: 18June 2024
Accepted: 02July 2024
doi:*10.48047/AFJBS.6.13.2024*. 3553-3586

ABSTRACT

Bacterial polyphenol oxidase (PPO), also known as bacterial tyrosinase, is an enzyme present in some bacteria. This enzyme is similar in function to polyphenol oxidases found in plants and other organisms that oxidize phenolic compounds and is responsible for food browning, defensive mechanisms, and significant industrial applications. This study presents the optimized microbial production, extraction, and purification of PPO from Bacillus subtilis vk3, a strain isolated from Tirupur, India's wetlands. The enhanced PPO production was optimized through controlled conditions, considering factors like incubation time, pH, temperature, carbon source, nitrogen source, and substrate concentration through sequential experimentation. Enzyme production reached its peak at an incubation time of 42 h, a neutral pH of 7.0, and a temperature of 40°C. The enhanced PPO production was optimized through controlled conditions, considering factors like incubation time, pH, temperature, carbon source, nitrogen source, and substrate concentration through sequential experimentation. The enzyme yield reached its peak at an incubation time of 42 h, a neutral pH of 7.0 (111.52 U/ml), and a temperature of 40°C (114.69U/ml). Glucose (216.33 ± 2.08) and yeast extract (177 μg/ml) were identified as the most effective carbon and nitrogen sources, respectively. The optimal point for enzyme activity was determined to be a substrate concentration of 600mg/L. For purification, a combination of ammonium sulfate precipitation, dialysis, and gel filtration chromatography (Sephadex G-50) was employed. This process achieved a substantial increase in enzyme purity, with a noteworthy yield and specific activity. The molecular weight of the purified PPO was identified as 35 kDa. The study's findings offer valuable insights for industrial-scale PPO production from *Bacillus subtilis vk3*, highlighting its potential in various sectors. The research underscores the importance of optimizing production conditions to maximize yield and efficacy, setting a foundation for future applications and advancements in enzyme technology.

Keywords: *Bacillus* enzyme, PPO, production yield optimization, enhancement enzyme dialysis, gel filtration

Highlights:

1. **A novel *Bacillus subtilis vk3* strain isolated from wetlands of Tirupur, Tamil Nadu, India and the strain produced polyphenol oxidase (PPO) enzyme.**
2. **The PPO optimization revealed that this strain able to produce maximum PPO at an incubation of 48hrs, neutral pH, and 40°C utilizing glucose and yeast extract as carbon and nitrogen sources respectively.**
3. **The PPO enzyme was purified (35K Da) through ammonium sulfate precipitation, dialysis, and gel filtration chromatography methods (Sephadex G-50).**
4. **The PPO enzyme from Bacillus subtilis vk3 strain is promising with many industrial applications.**

1. Introduction

Soil microbes constitute a significant component of the terrestrial ecosystem with myriad ecological functioning through extracellular enzyme production including; 1) primary decomposition of organic matter; 2) nutrient cycling; 3) maintenance of soil structure and stability; 4) carbon sequestration and mitigating climate changes; 5) symbiosis with plants, and 6) ecosystem resilience [1]. There are a variety of extracellular enzyme that soil microbes can produce including but not limited to oxidases, hydrolyses, reductases and mineralizing enzymes and are widely appreciated in many industrial processing [2]. Polyphenol oxidase (PPO) is a copper-containing oxidative metalloenzymes that has been widely found in animals, plants, bacteria, and fungi [3]. PPO enzyme is known for its browning effects due to the catalysis of polyphenol oxidation leading to quinones/ melanoid production.

Its mechanism of action involves two reactions: the catalysis the hydroxylation of monophenol in the presence of oxygen (that resuls in the formation of diphenol), which is followed by an oxidation, that removes a hydrogen molecule from diphenol to yield quinine. During catalysis, enzyme active sites undergo met-, oxy-, and deoxy- transactions in a cyclic manner.[4] This enzyme is having two copper binding and one proteolytic site in its active site and proximity of C-terminus respectively [5]. Each copper ion is coordinated by three histidine residues and the valence state are mostly responsible for PPO activity and are

classified into three types; *met*-PPO, *deoxy*-PPO, and *oxy*-PPO [6]. PPO produces catechol, which are key intermediates in synthesis of many chemicals and pharmaceuticals [7].

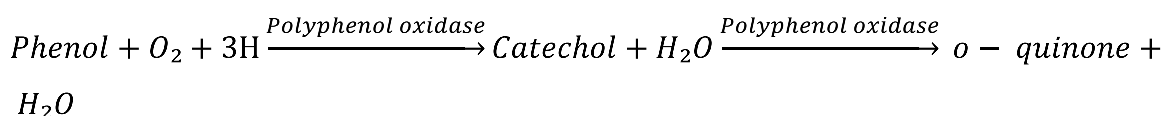
Molecular oxygen induces phenol oxidases to oxidize various phenolic compounds. A polyphenol oxidase [EC class 1.10.3] can be categorized into three types based on substrate specificity and carbon monoxide inhibition: laccases (EC 1.10.3.2, p-benzenediol: oxygen oxidoreductase), catechol oxidases (EC 1.10.3.1, o-diphenol oxidoreductase), and tyrosinases (EC 1.14.18.1, monophenol monooxygenase). As a result of subsequent enzymatic and non-enzymatic reactions, a heterogeneous undesirable dark brown group of melanin is formed. The catechol oxidases (EC. 1.10.3.1) and the laccases (EC. 1.10.3.2) are copper-containing enzymes that oxidize phenol into quinones. In nature, catalase and phenol oxidase activities may exist in tandem, suggesting the dual existence of catalase and phenol oxidase activity may have important implications for antioxidant function. The phenolic compounds are known to be effective antioxidants since catalases handle reactive oxygen species (ROS) generated by oxidative stress.

Besides PPO, tyrosinase, catechol oxidase, and laccase may also be exhibiting similar such activities and are generally classified based on their structure and substrate specificity [4]. Based on the physical separation of enzyme located in the chloroplast from the substrates located in the vacuole, the most proposed function of PPO in plants has been defence against herbivores and pathogens. The various functions of PPO in plant and fungal are not well understood, however, there are evidence support that the melanin by fungal PPO supports their defense against various stress factors and boosts the spore formation which indirectly supporting its virulence factors [8]. A few of the culture conditions helps to optimize the higher PPO production, for instance, lignocellulosic and aromatic compound induced their production in *Thermoascus aurantiacus* [9]. A novel bifunctional catalase with phenol oxidase is extracted from Szytalidium thermophilum [10]. Toxic phenolic compounds present in wastewater can be effectively removed with immobilized tyrosinase, PPO and chitosan, for instance, PPO producing fungal strains were reported to be suitable for the bioremediation aspects [11]. Enzyme catecholase belongs to the class of oxidoreductases mainly involved in catechol degradation into benzoquinone and oxygen. Tyrosinase, also called polyphenol oxidase, is a multifunctional copper-containing metalloenzyme present in a broad variety of organisms. It catalyses the ortho-hydroxylation of monophenol molecules and can also catalyse the oxidation of ortho-diphenolic to produce ortho-quinones.

Tyrrosinases are essential in the melanogenesis process and plays critical roles in the browning of food and in the development of insects. In addition to potentially lowering the nutritional value of the tissue and decreasing predation, the o-quinone–protein complexes that result from cell destruction can also help establish structural barriers that prevent the entry of pathogens. PPO has the capacity to alter proteins through reactions with amino, phenolic, and mercapto groups. This process results in alkylation, which lowers the bioavailability of cellular proteins and inhibits insects' and microbes' ability to digest and absorb nutrients.

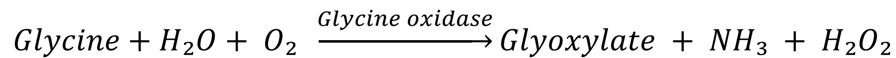
The application and detailed study of these enzymes in various catalytic processes is increasing due to their ecofriendly nature, substrate specificity, and biological origin [12]. Purified PPOs has a wide range of applications, including nutrition, environment, biomedical and pharmaceutical industries. They are employed in several processing such as removing phenols from wastewater generated in various industries and maintaining the quality of bread, dry fruits, tea, cocoa, canola meal and coffee. PPO enzymes being one of the most widely used methods to increase nutrient bioavailability in millets. In recent years polyphenol oxidases have garnered significant interest because of their high capacity for oxidizing aromatic compounds. They can be used in beverage processing for the elimination of phenolics which are responsible for browning, haze formation and turbidity development in beer, wine, and fruit juice. In environmental technology, the presence of hazardous phenolic compounds and their derivatives in industrial wastewaters from coal conversion, petroleum refining, wood preservation, textile, paper, food, and chemical industries. In addition, PPO's high substrate specificity makes it ideal for the development of sensitive biosensors that can function even at very low substrate concentrations [13]. The medicinal application of PPO has great potential for treating diseases such as Parkinson's disease, vitiligo cancer and oral infections caused by *Streptococcus sorbrius* [14]. It has been observed that enzyme is useful in the manufacture of hair dyes and skin lightening products [15].

Bacillus subtilis has emerged as the gram-positive model organism of choice largely because endospore formation is a highly tractable system for studying fundamental aspects of cellular development. *Bacillus subtilis* produces many types of enzymes that can be involved in oxidation reactions, for instance, polyphenol oxidase, an enzyme helps in the oxidation of phenol to catechol and further to o-quinone.



$$Phenol + O_2 + 3\mathrm{H} \xrightarrow{Polyphenol\ oxidase} Catechol + H_2O \xrightarrow{Polyphenol\ oxidase} o - \ quinone + H_2O$$

Not just phenol oxidase, there are other similar enzymes can well involve in oxidation, for eg. glycine oxidase, an enzyme that catalyses the oxidative deamination of primary, secondary amines, and D-amino acids.



$$Glycine + H_2O + O_2 \xrightarrow{Glycine\ oxidase} Glyoxylate + NH_3 + H_2O_2$$

There are many reports suggested their potency in producing several different extracellular enzymes which are industrially important that includes but not limited to carbohydrases (amylase, cellulase, chitinase, glucanase, xylanases, pullulanase, etc), proteases, nucleases, oxidases, dehydrogenases, phosphatases, lipases, and ureases [16];[17]; [18]; [19]; [20];[21] Cell fate determination, cell morphogenesis, and the decision to initiate sporulation were all researched in molecular detail when it was very difficult to dissect these processes in higher organisms. PPO enzyme is industrially important but not much literature focuses on detailed research. For instance, some reviews focussed related enzyme peroxidase [14]; [22];[23]. Besides, the PPO production optimization, extraction and purification methods are not described extensively as many reviews focus mainly on sources, analysis, mechanism, physiochemical properties, molecular characterization, and genotype [24]. Maximising the yield is the prerequisite for any substances for reaching commercial potentials.

But several of these advanced strategies were found to be very expensive which divert us to focus on traditional methods of optimizing the production strategies. In general, the major concern in polyphenol oxidase production is its quantity which restricts their usage on a commercial scale. As well known the efficient production relies on the various factors on microbial growth including nutrient availability, media components, temperature, pH and other conducible environmental factors and hence optimization of these factors may credit the production process in an efficient manner [25]. The sequential optimization procedure involves in addressing one problem at a time rather than focussing all problems collectively making it difficult to draw a notable suggestion [26]. So, in this study the optimization involves a sequential optimization procedure that optimizes one parameter at a time while keeping all the parameters constant [27];[28]. Very next step to the production optimization is the purification, most of the industrial processing focusses a lumpsum on the purification protocols as the purity determines enzyme activity. Thus, this piece of work is aimed at explicating the microbial production, extraction, and purification of PPO, including supportive quantitative data that will ultimately help in process designing at pilot and commercial scale of using polyphenol oxidase extracted form *Bacillus subtilis vk3*.

2. Materials and Methods

2.1. Source strain and chemicals used

The strain Bacillus subtilis vk3 (ON564692) isolated from the wetlands of Tirupur, India is used in this study. Our earlier reports documented that the PPO production is maximum in this isolate among the rest of the other isolates (data not included). All the media and chemicals used in our experimentations are analytical grade purchased from HiMedia and Merck, India respectively.

2.2. Preparation of crude enzyme

The fermentation process was performed in shake flasks (250ml) containing 0.4% yeast extract, 0.1% KH_2PO4, 0.5% MgSO4, 1.5% glucose and 10ml of overnight seed culture of *Bacillus subtilis* at 37°C and 155 rpm in a shaker incubator (NeoLab OSI-262). The polyphenol oxidase activity of the fermentation medium was determined from samples taken till the polyphenol oxidase activity had reached a certain level [29].

2.3. Polyphenol Oxidase Assay

The activity of polyphenol oxidase was measured spectrophotometrically. Crude enzymes were extracted from culture supernatant [30]. 2M phosphate buffer at pH 7.0 was used as substrate to dissolve 100 mM catechol [31];[32]. The substrate was prepared by mixing 0.1g catechol with 10ml 0.2M phosphate buffer (pH 7.0) that was previously heated to 50°C and then vertexing it to dissolve it. The reaction mixture contained 1 ml 0.2 M phosphate buffer (pH 7.0), 0.5 ml culture supernatant and 0.5 ml substrate solution (100 mM) incubated at 50°C for 3 minutes (NeoLab OSI-262). The reference cuvette contained buffer instead of enzyme and change in absorbance was followed at 410nm (Labman LMSP-UV1200) and initial reaction rate was used to determine enzyme activity [33]. One unit of enzyme (U) is defined as the amount of enzyme required to obtain 0.01 O.D [34] Protein concentration was

measured according to the modified Bradford method by using bovine serum albumin (BSA) as a standard[35].

2.4 Specific activity of PPO enzyme

Specific activity of an enzyme is expressed as the number of units per mg of protein.

Specific Activity=Enzyme activity/ Protein Concentration

Enzyme unit is defined as the amount of enzyme that will catalyze the transformation of 1µmol of substrate /min under specified conditions of pH and temperature. Specific activity is the relative measure of the purity of an enzyme preparation. Protein concentration can be estimated by measuring the UV absorbance at 410nm. The activity of PPO can be calculated indirectly by measuring the OD value at 410 nm.

2.5 Enzyme kinetics

The 600mg/L substrate concentration was found to be optimal for enzyme activity (Fig 1). At low substrate concentrations, the reaction rate increases rapidly as the substrate concentration rises. Enzyme's catalytic site is often empty, waiting for substrate binding, and the rate of product formation is limited by the available substrate concentration. As substrate concentration increases, enzyme becomes saturated with the substrate. The rate of product formation is now dependent on enzyme’s activity, and adding more substrate will not significantly affect the reaction rate. V_{max} is the maximum reaction rate when enzyme is saturated with substrate. This interaction depends on enzyme's affinity for the substrate. Km (inverse measure of affinity) is the Michaelis constant of the enzyme, representing the substrate concentration that allows enzyme to reach half of V_{max}. A low K_m value indicates a substrate-poor enzyme with low affinity and needing a higher substrate concentration to reach V_{max}.

2.5.1 METHODOLOGY

2.5.2 Enzyme Assay

Enzyme activity of PPO was measured spectrophotometrically using the initial reaction rate.

2.5.3 Determination of Kinetic Parameters

In order to obtain the K_m and V_{max} values separately for catechol at optimum pH and temperature, enzyme activity was measured at five different substrate cuvette concentrations of. catechol. (Table 1). The K_m and V_{max} values were determined by means of Lineweaver-Burk graphs, allowing for a more comprehensive understanding of the enzymatic kinetics.

2.6 Effects of metal ions and inhibitors on PPO activity

Different metals have unique characteristics and can act as effectors. Metallic coenzymes are important because they regulate the activity of enzymes by being present or absent in enzymatic reactions.

2.6.1 METHODOLOGY

2.6.2 Effect of metal ions on PPO Activity

1% (w/v) of different metal ions, including Mg2+, Cu2+, and Al3+, were added to the production broth medium. Enzyme activity was subsequently by adding 100 mM catechol measured under standard assay conditions.

2.6.3 Extraction of Crude Enzyme

3ml of the production media culture was transferred to centrifuge tubes and spun for 20 minutes at 5000 rpm. The supernatant portion was then decanted to obtain the crude enzyme extract.

2.6.4 Enzyme Assay

Enzyme activity is the measure of the catalytic efficiency of an enzyme, often expressed as the rate of conversion of substrate to product per unit time. Determining enzyme activity can be achieved through spectrophotometrically at 410 nm.

The presence of a specific metallic ion, along with essential nutrients, can either inhibit or increase PPO activity. Hence, the concentration of metal ions such as Mg2+, Cu2+ and Al3+ was measured in the production broth media in this study. Confluent bacterial growth was observed when these metal ions were present.

2.6.5 Effect of Inhibitors on PPO Activity

The effects of different inhibitors on PPO enzyme were measured using an inhibitory Inhibitory study was conducted to demonstrate the effect of various chemicals on PPO enzyme. The inhibitors used included Sodium aside, Sodium chloride, Polyethylene glycol, Citric acid and Ascorbic acid at varying concentrations (0.05 mol/L, 0.10 mol/L, 0.15 mol/L 0.20 mol/L and 0.25 mol/L). PPO activity was assessed using catechol as the substrate at a concentration of 100 mM, while potential inhibitors were tested by monitoring PPO activity during catechol oxidation [36].

2.7.5. Effect of different carbon sources on PPO enzyme production

The effect of carbon sources on enzyme production was examined by incorporating starch, glucose, glycerol, maltose, and fructose into the medium (with optimum nitrogen and other parameters) [46];[47].

2.7.6. Effect of Incubation time on PPO enzyme production

The isolated strain was cultured in screening medium at 37°C in 175 rpm for 4 days, followed by spectrophotometric measurements (Labman LMSP-UV1200) at 6-hour intervals to estimate the amount of polyphenol oxidase enzyme in the culture [48];49].

2.8. Purification of PPO enzyme from the crude extract

The purification of the PPO was carried out with modified protocols of previous research work [50];[51]. Enzyme extracts derived from crude sources have been purified by salt

precipitation, dialysis, gel filtration chromatography, etc., so that enzyme is of the highest purity [52];[53];[54]. Further analysis can be performed using the pure enzyme that has been purified.

2.8.1. Ammonium sulphate precipitation and dialysis

Precipitation of ammonium sulfate was carried out in an ice bath using finely ground ammonium sulphate [55]. In order to ensure complete solubility, the powder was weighed and added slowly to the extract while constantly stirring, followed by centrifugation (REMI C24 plus) at 5000Xg for 30 minutes at 4°C [56];[57]. Several precipitation steps were performed for PPO's enzyme precipitation (45–80%) and precipitates were collected [58];[59];[60]. By changing the buffer three times per day, the precipitate was dialyzed against 100 mM potassium phosphate buffer (pH 7.0) for 24 hours [61];[62]. Dialyzed fractions were used for measurements of PPO activity and protein content [63];[64]. The cutoff range is 60 for the dialysis tube used in this study (HiMedia).

2.8.2 Sephadex G-50 gel filtration

Sephadex G-50 column (Merck, IN) pre-equilibrated with 100 mM phosphate buffer pH 7.0 was used to dilate the dialyzed ammonium sulfate fraction [65];[66]. Protein elution was carried out at a flow rate of 5 mL/min using the same buffer [67];[68]. The fractions were collected at 4°C. The protein content and enzyme activity were analyzed at 280 nm [69];[70]. Dialysis was performed against a pH 7.0 phosphate buffer to remove the active fractions and then concentration was performed [71];[72].

2.8.3 Determination of molecular weight

The molecular mass of purified polyphenol oxidase was estimated with slight modification [73];[5]. The protein solution was heated for 5 minutes at 100 °C [74];[75]. A voltage supply of 70 V was run on the stacking gel for 45 minutes and a voltage supply of 100 V on the separating gel for 90 minutes [76] in a SDS PAGE unit (Medox). Protein bands were

visualized using Coomassie blue staining, and the molecular weight was calculated by using proteins of known molecular weights ranging from 14-66 KDa [77];[78]

3. Results and Discussion

Enzyme polyphenol oxidase, also called catecholase, oxidoreductase, catechol oxidase, or tyrosinase is the main endogenous enzyme playing major role in the food industries. PPO enzyme catalysis the hydroxylation of monophenols followed by oxidation to form O-quinones. The reactive O-quinones can condense or irreversibly react with variety of functional groups including amines and sulfhydryl resulting in cross-linking and browning of food products, a major issue depreciating the economic value of foods [6]. But in general, this browning in plants or animals indicate the first line defense system against the pathogenic or insect attack upon disruption of plant tissue, it means that these are biologically active substances [79]. Besides PPO enzyme is widely applicable in many industrial processing; it has been reported that the substrate affinities of PPOs widely differed from the different sources of its extraction and mostly depends on the type and abundance of the substrates and/or mono- di- phenolase activity [80].

In our recent study, bacterial strain S13 exhibits a strong positive result in the polyphenol oxidase assay. By in-depth observation of the specific activity exhibited by strain 104.2 IU/ml The strain produced an average of 195±4.29 units of activity with a standard deviation of 1.87±0.020, indicating its significant potential role in polyphenol oxidation processes (Fig.1). The kinetic parameters for the synthesis of PPOs enzyme were determined through calculation. according to the Lineweaver-Burk plot method [81] with Catechol as the substrate (Fig.2). The concentrations varied between 9.1mM and 91mM.

Values of maximum rate; Vmax (mU/mg/min) and Michaelis-Menten constant Km(mg/mL) were measured and all the results were recorded at a temperature of 40℃ and a pH level of 7. PPO enzyme's activity increased gradually as the substrate concentration was increased from 0.1 % to 1 percent, according to research on how the concentration of substrate affects the enzyme's activity. Based on the Lineweaver Burk plot, it was determined that the maximum rate (Vmax) is 85.88U/ml.

The addition of metal ions will influence the activity of PPO enzyme These observations are consistent with a previous study which showed that maximum activity of PPO enzyme and bacterial growth in Mg^{2+} (81.54 IU/ml/min) (Table 2), was observed [82].

Addition of Cu^{2+} (65.46 IU/ml/min) and Al 3+ (76.22 IU/ml/min) ions to the broth culture had a significant effect on the physiology and metabolism of bacteria It was reported in the study that Mg^{2+}, Cu^{2+}, and Al^{3+} ions were found to inhibit the activity of PPO enzyme, which is beneficial for achieving an anti-browning effect in the food industry. The inhibitory mechanisms of these metal ions varied depending on their chemical properties and interactions with PPO enzyme.

Inhibitors are important for controlling PPO activity, which is crucial for preserving the quality of food products. Ascorbic acid shows maximum inhibitory effect (85± 0.02 U/ml) on PPO enzyme whereas the other inhibitors such as Citric acid (79± 1.04) Sodium chloride (73 ± 1.12 U/ml), shows moderate inhibitory effect on PPO enzyme. Sodium azide (65± 1.23) and Polyethylene glycol (53± 0.76) [36] and shows minimum inhibitory effect on PPO enzyme.

The study may provide new insight into natural PPO inhibitors in the goal of replacing the chemical anti-browning agents such as sulphites with natural ones for protection human health against toxics and may contribute to increased knowledge of the natural inhibitor of PPO. Thus, ascorbic acid enhances the stability of PPO enzyme. It will be also used as a natural inhibitor to prevent browning mechanism and increase the shelf life and consistency in fruits and food products.

In general, an enzyme's surface charge changes with changes in the pH of the media, which affects its solubility, binding capacity to different substrates or inhibitors, and conformation [83]. Not only enzyme's activity, but the enzymes production is also guided by various input factors, medium components, and environmental conditions. Without optimizing the conditions for maximum production, it is merely possible for further processing, for instance industrial scale production. Hence, optimization is the major criteria, else it is requiring to meet out the unnecessary expenses on the various production related inputs [84]. The initial selection of various parameters that affect the enzyme production are to be considered and optimized in order to increase the yield. In general, there are several sequential optimization procedures using different statistical models that do concentrate on optimizing single factor at a time to know about the most influential range of that factor, and then multitudes of their combinations to be studied to know enhance the production [85]. But for this experimentation was planned to evaluate few factors that influences the enzyme production.

Some of the major impacting factors with respect the optimal enzyme production are the medium components including the different carbon and nitrogen sources, the temperature and incubation time, the pH of the medium as well as the substrate availability. The sequential optimization for single factors is considered for each experimentation. Different pH showed different effects on the Bacillus growth as well as the PPO yield. (Fig 3 A) shows an optimal pH for PPO that ranges from 5 to 10. The maximum activity of 111.52 U/ml was recorded in the cultures at neutral pH (pH-7) when compared to acidic and alkaline pH ranges. According to the researchers, the pH optimization was carried out on specific substrates in order to determine the optimal pH. The optimum pH value for a given PPO varies with different substrate types [23], the optimal incubation time of the maximum growth and production of PPO was found to be pH7 with the total protein concentration of 188 ± 0.047 and enzyme activity of 209.66 ± 2.51 U/ml. After 42 hours of incubation the enzyme production was observed to decline at every 6 hours up to 72 hours, indicating the declining phase of the microbial growth and enzyme production.

. It is important to study temperature because of its kinetic control and its effect on oxygen solubility in solution [86]. It was observed that the optimum temperature varied from 20 to 80°C depending on the source and substrate [87]. Where, PPO degraded beyond 80°C. The maximum production was observed at 40°C with PPO 114.69 U/ml (Fig.3B) which is in alignment with the study by Toro et al., [88]. Enzyme reactions rate will increase as the temperature rises, however, as the enzyme becomes denatured after passing the optimal limit, its activity will decrease. Enzymes that have been denatured lose their active part. It results in a decrease in enzymatic reactions. The substrate concentration of 600mg/L was found to be very optimal in enzyme activity (Fig 3C). When substrate concentration is low, the reaction rate increases rapidly as substrate concentration increases. For most of the time, the enzyme's catalytic site is empty, awaiting substrate binding, and the rate of product formation is limited by the available substrate concentration. As substrate concentration increases, enzyme saturates with substrate. Once the catalyst site is closed, more substrates are available for binding and reaction. The rate of product formation is now dependent on the enzyme's activity, and the rate of reaction will not be significantly affected by the addition of additional substrate. Vmax is the maximum reaction rate at which enzyme is saturate with substrate. The interaction between reaction rate and substrate concentration is dependent on the enzyme's substrate affinity. This is typically expressed as Km (inverse measure of affinity), which is the Michaelis constant of the enzyme. For simplicity, Km represents the substrate

concentration that allows the enzyme to reach half of Vmax. A substrate-poor enzyme with a Km value has a substrate with a low affinity and needs a higher substrate concentration to reach Vmax. A substrate activity concentration of 100mg/l to 1000mg/l at pH7.0 was followed by 40°C incubation [89]. It has been found that the cells utilized the substrate catechol 215.66 ± 3.05 U/ml enzyme activity present in 600 mg/l concentration for cellular metabolism.

There are many factors that affect the purification of enzyme such as the kind of the buffer to be used, the buffer pH and the environmental factors; these may either assists or obstructs the yield of enzymes and quality [90].

The nitrogen source of yeast extract was observed to be at a maximum activity of 185.24 ± 0.37 U/ml (Fig.3 D). Nitrogen is a nutrient that plays an important role in growth and metabolism. It is the base for ribonucleic acids, amino acids, and proteins, as well as other micronutrients used by bacteria. It is also necessary for the production of energy during anaerobic fermentation. The effects of nitrogen enhancement were different for the ion forms. As the world's fermentation industry continues to grow, the demand has increased for natural, uniform, sustainable and varied nitrogen sources. Nitrogen can be found in a variety of organic and inorganic sources, but yeast-based nutrients have emerged as the preferred nitrogen source due to their numerous benefits. Yeast-based nutrients include protein (more than 50% of yeast dry weight is protein) as well as complete amino acids. They are also high in vitamins and minerals (B-vitamins), making them a complex and rich food source for all microorganisms. Because they can be tailored to various probiotic strain cultures, yeast-derived nutrients are widely used in a variety of industries. Yeast provides a stable source of nutrients, as opposed to plant peptones and animal peptones or hydrolysates, which are susceptible to changes in climate, soil, and even the cultivars used. Yeast is 100% natural, which makes it a great option for products that end up in the hands of eco-conscious consumers who appreciate natural ingredients. Yeast-based nutrients can also be produced in a way that minimizes environmental impact. Some of the market leaders have structured their manufacturing processes in a way that is as sustainable as possible. In addition, yeast-derived nutrients are entirely non-animal-based, making them even more eco-friendly. Yeast derived nutrients are now the preferred nitrogen source for producers in the fermentation sector. They can be applied in a variety of ways and tailored to specific needs, they are safe and derived from natural sources, and they promote the growth of strong, healthy microorganisms.

Carbon sources are essential form of abiotic factor for the growth of the microorganisms and their metabolic potentials [91]. The utilization of carbon sources and expression of the enzyme activity was found that the production ranges employing starch found to be 130.61± 4.16 U/ml, for Glucose it was 169.24 ± 3.05 U/ml, for Glycerol it was 48.68 ± 4.04 U/ml, for Maltose we found it was 75.35 ± 3.51 U/ml, and for Fructose it was 64.28 ± 4.21 U/ml (Fig. 3E). In glucose, the maximum activity has been observed. In general, the microbes tend to naturally consume the carbon source that is readily available (monosaccharides) than that of the disaccharides and polysaccharides used [91]. Glucose is the simplest form of the monosaccharides and the easiest available energy source thus it became the indispensable carbohydrate for the optimal production of PPOs in this study. The optimal incubation time of the maximum growth and production of PPO was found to be 42hrs with the total protein concentration of 177 µg/ml and enzyme activity of 96.98 U/ml (Fig.3 F). After 42 hours of incubation enzyme production was observed to decline at every 6 hours up to 72 hours, indicating the declining phase of the microbial growth and enzyme production. The more time an enzyme spends in contact with its substrate, the more product will be produced. Nevertheless, the speed at which the product is produced does not follow a straight forward, linear relationship with the amount of time it is incubated. As the incubation period continues, the speed at which the product is formed decreases. If the incubation time is excessively long, enzyme's activity will be inaccurately low when measured. Typically, the incubation period should be sufficient for a modest amount of product to be produced and for timing errors to be negligible, but not so long that a plateau in the curve becomes noticeable.

The substrate concentration of 300mg/L was found to be very optimal in enzyme activity There are many factors that affect the purification of the enzyme such as the kind of the buffer to be used, the buffer pH and the environmental factors; these may either assists or obstructs the yield of the enzymes and quality [90]. The summary of all the purification steps undertaken for Bacillus PPO is given in the table (Table 3). There are many methods for the purification of the enzymes, out of all the precipitation methods are economical with minimal experimental setup and are mostly preferable for the industrial scale production by cutting down the unnecessary expenses [92]. In most of the research the combinations of salt precipitation, temperature induced phase separation and chromatographic methods were employed for purification [7]. The ammonium sulfate precipitation is the most preliminary methods that was proposed for the protein purification with several advantages including easy operations, higher yield, structural integrity, lesser impact on enzyme activity as well as

process selectivity. Purification fold is a measure of how much the sample has been purified during the purification process. It is calculated by dividing the specific activity of purified enzyme by the specific activity of the original crude sample. The purification fold indicates how many times the sample has been purified and can give an idea of the effectiveness of the purification process in increasing the purity of enzyme if the specific activity of the crude sample was 1.53 units/mg and the specific activity of the purified sample was 12 units/mg, the purification fold would be 7.84%.

Yield % is a measure of how much of enzyme of interest was recovered after the purification process compared to the initial amount present in the starting material. It is calculated by dividing the final amount of enzyme obtained after purification by the initial amount of enzyme present, then multiplying by 100 to get a percentage if the analysis is started with 100 units of enzyme and obtained 8.38 units after purification, the yield would be 7.84% (12/1.53 x 100 = 7.84%). Purification Fold = Specific Activity (Purified)/Specific Activity (Crude)×100. These calculations provide crucial information about the efficiency of the purification process and the quality of the final product.

The results revealed the 7.84% yield with the higher total activity of 14.6 which is quite higher than the dialysis and gel filtration methods. In a similar study by Bibhuti B. Mishra, [4] in eggplant showed that the yield was 45% with the total activity of 38000 and a purification fold of 3.42 which is higher than that of our experimental results, this may be due to the effectiveness of the employed protocols.

Dialysis involves in removing and exchanging small impure molecules from the solution using semipermeable membrane made of cellulose acetate [93]. A purification fold of 4.95 with total activity of 56.7 U was observed when the PPO was purified with dialysis. The chromatographic purification methods yield products of higher purity however the methods are complicated and costlier, hence selection of appropriate chromatographic technique based on the type of enzyme, the possible impurities, charges, and molecular sizes are to be considered [7]. Previous reports documented the employment of size exclusion [6] gel filtration [22], hydrophobic interaction [94] and ion exchange chromatography [95] for purification of PPO. The results showed that 7.84-fold purification with the total yield of 8.38% with the specific activity of 12 U/mg (Fig.4) after eluting from ion exchange chromatography using Sephadex- G50. A similar kind of results were obtained by Catherine Joke Adeseko *et al*., [96] employing Sephadex G 200, the PPO obtained from the African

bush mango showed an exemplified 685-fold purification with 20% yield and specific activity of 411 U/mg. In general, the loss of activity and reduction in recovery in purification is attributed to the due course in removal of the associated enzymes or proteins.

Loss of activity and reduction in recovery of enzyme cannot be avoided as other proteins accompanied enzyme of interest (polyphenol oxidase) will be removed during purification process; thereby resulting in decreased activity and percentage yield [5]. A low yield and increased purification folds indicated that the PPO enzyme is highly purified with lower contaminations as aligned with that of Catherine Joke [96] that when there is purified enzyme or protein product with lower yield leaves lesser protein contamination than that with the higher yield. The molecular weight of PPO varies between different sources and according to the earlier reports, it varied between 27 – 144 kDa and the very active PPO enzyme ranged between 35 – 70 kDa [60]. The native PAGE and SDS-PAGE are mostly employed for estimating the molecular weight of the PPOs, in this our study we employed SDS PAGE was employed for determining the purified PPO form Bacillus subtilis. The molecular weight of the purified PPO was found to be 35kDa. These results are corroborated with the results by other researchers, PPOs extracted from water yam [64] and snow pears [97]. The present study depicts the boundaries and possible future implications by incorporating the following elements such as utilizing creatively crafted optimization techniques rooted in statistics, commonly employed in a range of biotechnological procedures like fractional factorial design and response surface methodology. By employing these strategies, one can delve into the interplay among multiple factors, a dimension commonly ignored in the traditional one-variable-at-a-time approach, potentially preventing misinterpretation of findings. In general, there were no adverse health effects have been reported to use of PPOs in living being. However, their safety can be ensured by good work practices, engineering controls and appropriate protective equipment. There are no ethical issues involved while using PPOs for analysis. However, in the production unit the air monitoring techniques can be employed to measure the level of enzymes in the dust or mist in the air and assisting engineering controls may help to reduce their exposure level in employees.

4. Conclusion

In recent years polyphenol oxidases have garnered significant interest because of their high capacity for oxidizing aromatic compounds. This feature makes the use of polyphenol oxidases, a very suitable for some biotechnological applications in food industry, pulp and

paper industry, textile industry, medicine, and environmental technology. In food industry, although polyphenol oxidases are undesirable for their browning effects, they can be applied for various beneficial purposes. They can be used in beverage processing for the elimination of phenolics which are responsible for browning, haze formation and turbidity development in beer, wine, and fruit juice. In environmental technology, the presence of hazardous phenolic compounds and their derivatives in industrial wastewaters from coal conversion, petroleum refining, wood preservation, textile, paper, food, and chemical industries constitutes a major problem. Bacillus subtilis an aerobic, spore-forming, and able to grow at 70ºC, was exploited for their PPO production in this study. These organisms have gained significant biotechnological and industrial interest due to their ability to synthesize thermostable enzymes such as lipases, proteases, amylases, pullulanase, xylanases, glucose-isomerases, and DNA restriction endonucleases. The characterization studies indicated that polyphenol oxidase from thermophilic Bacillus subtilis had highest activity at pH 7.0 and 40ºC. Enzyme was stable at temperatures between 40ºC and retained approximately 85% of its activity between pH 7.0 and 8.0. PPO yield and tailoring some additional features for making this enzyme wide applicable for other industrial processes including cosmetics. Further, the gene responsible for the PPO enzyme can be cloned and expressed in suitable hosts for crediting maximum yield.

Acknowledgement

The authors are gratefully thanking the management and faculties of Department of Biotechnology, Rathnavel Subramaniam College of Arts and Science, Sulur, Tamil Nadu, India Coimbatore for providing amenities and support throughout the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

FIGURES

Fig 1 : GRAPHICAL ABSTRACT

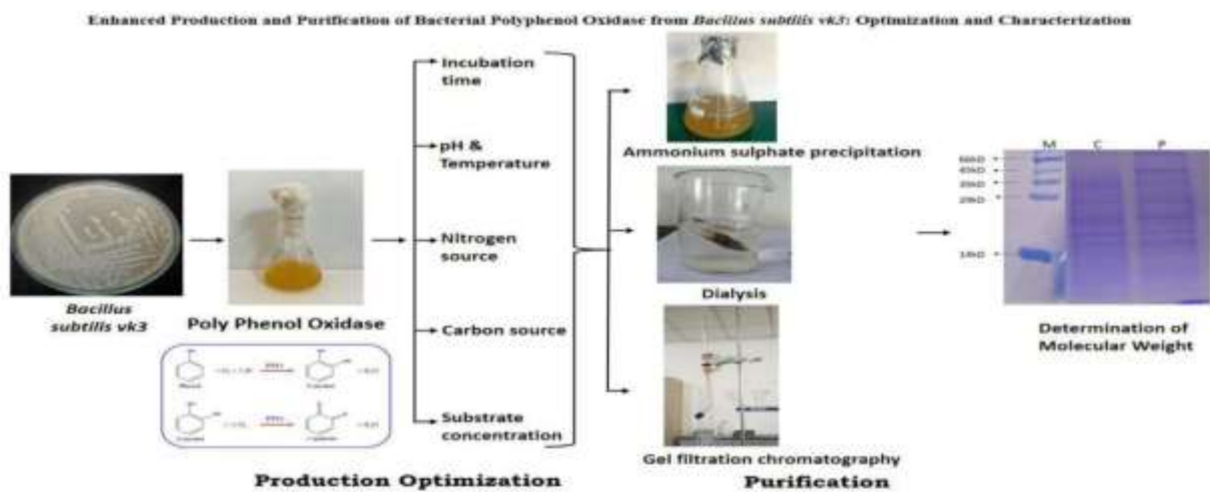


Figure 1: GRAPHICAL ABSTRACT

Fig 2: DETERMINATION OF ENZYME KINETICS

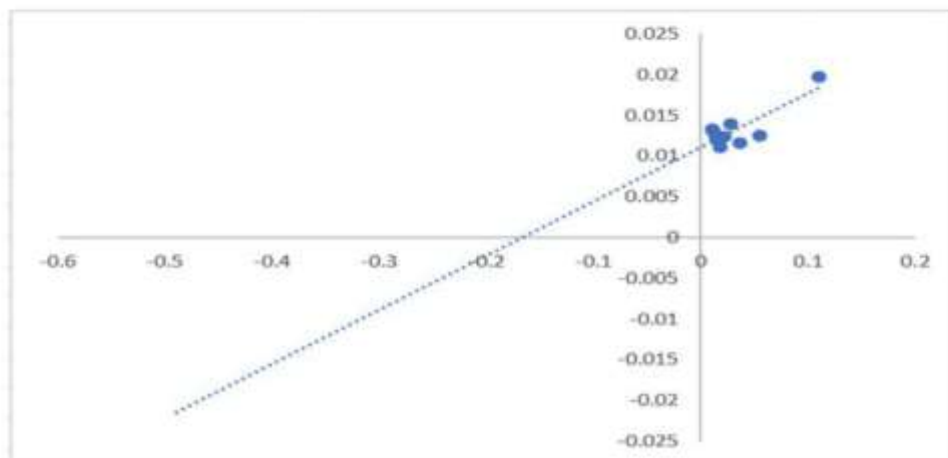


Figure 2: DETERMINATION OF ENZYME KINETICS

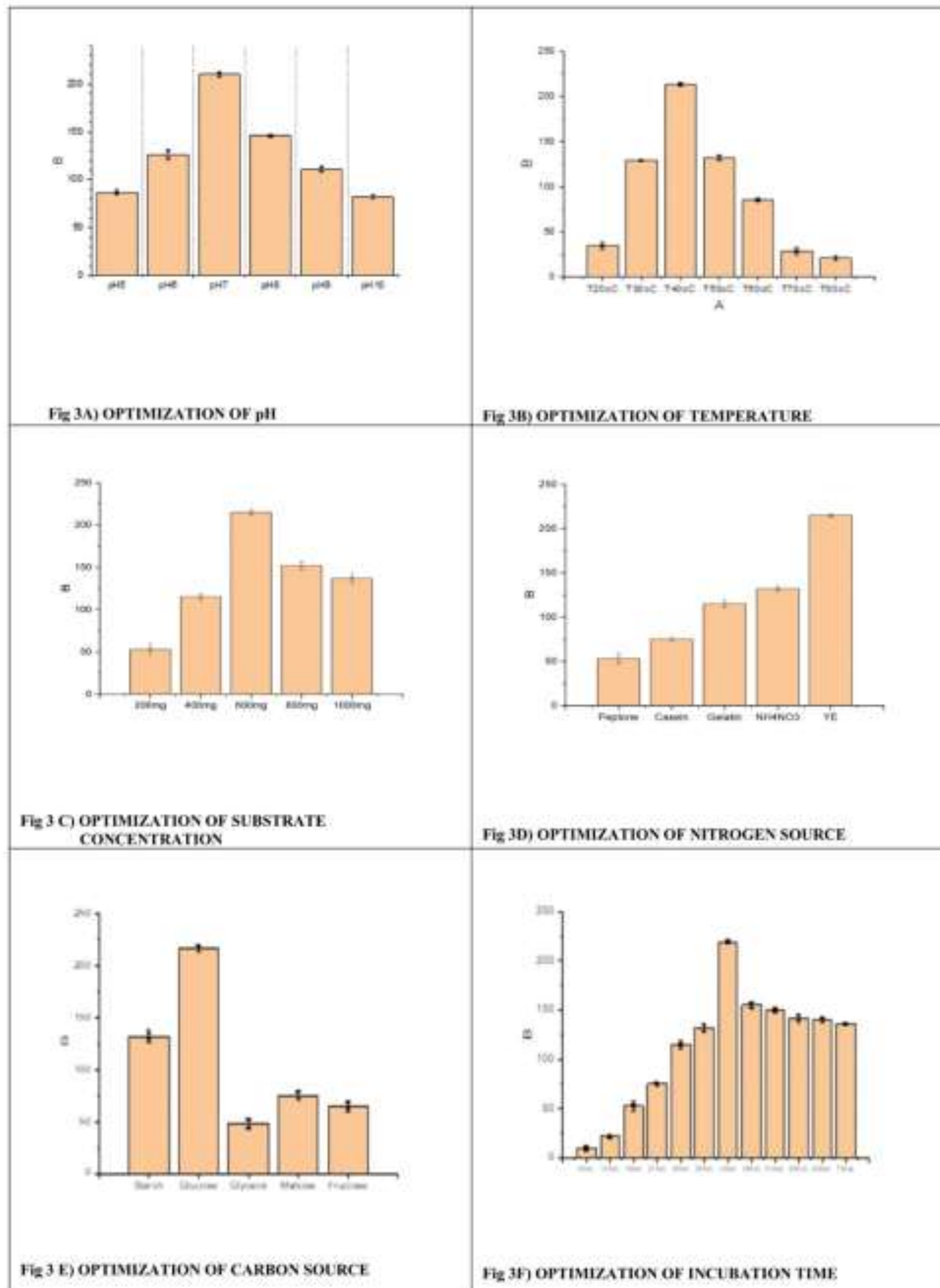


Figure.3: EFFECT OF DIFFERENT PARAMETERS ON POLYPHENOL OXIDASE OPTIMIZATION

Fig.4) PURIFICATION OF PPO ENZYME BY SEPHADEX G-50 CHROMATOGRAM

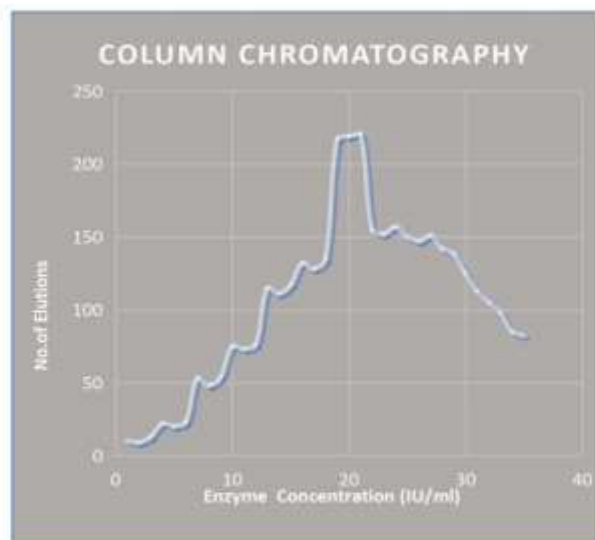


Figure 4: PURICATION OF PPO ENZYME BY SEPHADEX G-50 CHROMATOGRAM

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TABLES

TABLE 1: DETERMINATION OF KINETIC PARAMETERS

S.No	Substrate Concentration (Catechol) (mM)	Km (mM)	Vmax (U/ml)
1	0.2	18/2	79.79
2	0.4	36.4	71.90
3	.0.6	54.6	115.94
4	0.8	72.8	81.25
5	1.0	91.0	75.36

TABLE 2: EFFECTS OF METAL IONS AND INHIBITORS ON PPO ACTIVITY

Metal ions			Inhibitors	
S.No	**Metal ions**	**Enyme Activity (IU/ml/min)**	**Inhibitors**	**Enyme Activity (U/ml)**
1	$MgCl_2$ (Mg^{2+})	81.54	Sodium azide	65± 1.23
2	$CuSO_4$ (Cu^{2+})	65.46	Sodium chloride	73± 1.12
3	$AlCl_3$ (Al^{3+})	76.22	Polyethylene glycol	53± 0.76
4			Citric acid	79± 1.04
5			Ascorbic acid	85 ± 0.02

Table 3. PURIFICATION OF POLYPHENOL OXIDASE

Purification Steps	Volume (ml)	Total Protein (mg)	Activity Units	Total activity U	Specific activity (U/mg Protein)	Yield %	Purification fold
Crude Enzyme	85	115.3	2.07	175.95	1.53	100	1
Ammonium Sulfate	25	11.13	3.13	78.75	7.07	44.75	4.62
Dialysis	15	7.48	3.78	56.7	7.58	32.32	4.95
Sephedex - G50	3	1.23	4.92	14.6	12	8.38	7.84

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