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Research Article

Isolation and molecular identification of polyphenol oxidase and associated enzyme production from *Bacillus spp.*

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Abstract
Polyphenol oxidase (PPO) causes browning in food by converting phenolics to quinone, rendering unwanted organoleptic and nutritional changes. As a result, it represents a major problem in the food industry which must be addressed to reduce food waste and maximize food quality and shelf life despite PPOs importance in various industrial processes. The present study aimed to isolate Bacillus sp., evaluate their PPO production and related enzymes, and identify them through 16S rRNA sequencing. The selected strains isolated from soil were induced, and the cultures were grown OD_{600} of 1.0 ($1x10^8$ CFU) for evaluation. The study identified various types of *Bacillus* sp. as the source of PPO, tyrosinase and catecholase production. The data revealed that the strains *Bacillus cereus*, *Bacillus albus*, *Bacillus subtilis*, *Bacillus thuringenesis* and *Bacillus tropicus* exhibited PPO, tyrosinase and catecholase enzyme production with a maximum of 104.2 IU/ml, 68.61 IU/ml and 61.59 IU/ml respectively. Enzyme activity differed between organisms and substrate activities, which must be standardized. Despite the importance of PPO and related enzymes to browning reactions and food processing, there has been limited progress in this area. Therefore, taking a different approach to provoke more concerns is probably necessary.

Keywords: Polyphenol oxidase, Tyrosinase, Catecholase *Bacillus spp,* Enzyme Activity, Polymerase Chain Reaction amplification, bacterial 16S rRNA gene sequencing

INTRODUCTION

Polyphenol oxidase (PPO) (E.C 1.14.18.1), which is also categorized as tyrosinase (E.C 1.14.18.1), catecholase (E.C 1.10.3.1) and laccase (E.C 1.10.3.2) enzymes are essential for various biological processes. For example, in plants, PPO is involved in defense mechanisms against herbivores and pathogens by producing toxic compounds upon substrate oxidation. In animals, tyrosinase is essential for melanin synthesis and, therefore, plays a role in determining skin and hair color (Whitaker, 1994). In both cases, these enzymes are part of complex metabolic pathways crucial for the organisms' survival and function (Gurunathan *et al*., 2021). These enzymes catalyse two different types of reactions while oxidising a broad array of monophenolic and o-diphenolic substances. It involves hydroxylating a monophenol to generate a diphenol; the other entails taking the hydrogens out of a diphenol to obtain quinone. In the current study, the organisms that produce polyphenols are isolated from the soil sample and molecularly identified using 16S rRNA sequencing. Enzymes catalysing polyphenol oxidation have numerous important uses in the food, pharmaceutical, and indus-

trial sectors (Raveendran *et al*., 2018).

PPO has mostly been employed in food processing to enhance the flavour of tea, coffee, and cocoa (Shahidi and Ambigaipalan, 2015; Taranto *et al*., 2017). However, in some food processing, role of PPO is not accountable and is detrimental to determining the food quality. Since the action of PPO causes the nutritional value of many fruits, such as apples, peaches, grapes, apricots, bananas, and strawberries, as well as the vegetable lettuce, potatoes, eggplants, and mushrooms, to be unviable because mechanically defective food activities result in oxidation and reduction in the presence of O_2, which makes the deprivation of nutrients and is intolerable for the consumers (Moon *et al*., 2020). Controlled PPO activity is suggested to be essential to preserving the quality in such aspects (Can *et al*., 2014). Apart from their undesirable activities, PPOs are a very distinguished class of enzymes benefiting various industrial processes, including removing phenols from the waste stream (Mukherjee *et al*., 2013; Wang *et al*., 2020), quality maintenance of food products (Jukanti and Jukanti, 2017) including dry fruits, canola meal (Lacki and Duvnjak, 1998), bread (Martin *et al*., 2005), tea (Tang *et al*., 2023), cocoa products (Misnawi *et al*., 2003), especially coffee (Mathur *et al*., 2015).

The higher the substrate specificity, the more widely PPOs are employed as sensitive biosensors in many fabrications (Raymundo-Pereira *et al*., 2020). Being an immediate intermediates in many valuable pharmaceutical components, it is even widely employed in the pharmaceutical industries treating a range of diseases, including parkinson's, cancers, oral infections, and vitiligo etc (Panadare and Rathod, 2018). This enzyme was generally preferred in skin care cosmetics and hair dyes even (Dana *et al*., 2017). So, it is obvious that this enzyme has a great capacity for industrial applications, thus, many researchers focus on maximizing PPOs production and attention to the cost-effective sources may be credited importance (Mayer, 2006). Among the sources, microbes are generally the optimal preference (Zaidi *et al*., 2014).

The wetlands are ecosystems regarded as significant reservoirs of diverse microbes, notably for their higher productivity, nutrient recycling, and greenhouse gas emissions (Bodelier and Dedysh, 2013). These microbes are actively involved in various ecosystem functions with an array of useful extracellular enzymes (Fu *et al*., 2023). However, not many studies focussed on characterizing the polyphenol oxidase-producing microbes from the wetlands of Tirupur, especially the genera *Bacillus,* was given less attention. Hence, the present study hypothesis that the wetland microbes of Tirupur are metabolically active, producing various extracellular enzymes, particularly the PPO and characterizing the multifaceted polyphenol oxidase enzymes from the wetland bacteria will be an advantage for industrial optimization. To achieve the hypothesis, the present study framed objectives in i) Isolation and screening of PPO and associated enzymes-producing bacteria from the wetland ecosystem, ii) Quantification of the enzymes and, iii) Identification and characterization of the wetland soil bacteria. With this background, the present study aimed to exploit Tirupur's natural wetland soils to identify the source of enzyme-producing industrially important potent strains.

MATERIALS AND METHODS

Chemicals

Gallic acid and catechol were procured from Sigma Chem Ltd. (USA). In addition, only molecular biology-grade chemicals and medium from HiMedia were used (India).

Isolation and identification of microorganisms

In polystyrene bags, soil samples were randomly taken from wetlands (Kulathupalayam: Fig.S1) in Tirupur, Tamil Nadu, India, 15 cm below the soil's surface. The soil sample (10g) was suspended in 90 ml of sterile distilled water and diluted to a serial plate on agar 1.5% medium containing Yeast extract 0.15, Tryptone 0.15, Sodium chloride 0.5, and Syringadazine 0.01%. Incubation was carried out for 24 hours at 37°C. Brown-pigmented colonies subjected to Gram staining were identified, and colonies showing Gram-positive reactions were picked and maintained on LB agar slants. Biochemicals (IMViC) and physiological parameters (pH and salt tolerance) were examined (Fig.S2) and isolates were identified using Bergey's Manual of Determinative Bacteriology (Bay and Wodard, 1994).

Secondary screening of copper-containing enzyme-producing organisms

The selected colony were inoculated in a liquid medium that contained Yeast extract 0.15%, tryptone 0.15%, NaCl 0.5% and syringadazine 0.01% and incubated at 37^{o}C for 24 hrs (Sura *et al*., 2022). If observed in the liquid medium, brown pigmentation indicated that the selected microorganism(s) in the culture will likely produce copper-containing enzymes (PPO, Tyrosinase and Catacholase) capable of oxidizing Syringaldazine. Additional biochemical assays and tests were performed to identify these enzymes further and characterise them.

Production of polyphenol oxidase

At 45°C and 155 rpm in a shaker incubator, fermentation was carried out in shake flasks (250 ml) of fermentation process media (pH 7.0) containing 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 1.5% glucose with 10 ml of preculture medium (Khan *et al*.,

2017). Before PPO activities started to decrease, samples of the fermentation medium were taken daily to measure the enzyme's activity.

PPO assay

A Spectrophotometer was used to quantify the activity of PPO. The culture supernatant was used as a crude enzyme source. As a substrate, 100 mM catechol was dissolved in 0.2 M phosphate buffer at pH 7.0. The substrate was made by combining 0.11g catechol with 10ml of 0.2M phosphate buffer (pH 7.0), which had previously been heated to 50°C, and vortexed the mixture to dissolve the catechol (Qian *et al*., 2021; Rashid *et al*., 2022). 1 ml of 0.2 M phosphate buffer (pH 7.0), 0.5 ml of culture supernatant, and 0.5 ml of substrate solution (100 mM) were added to the reaction mixture and incubated at 50°C for 3 minutes. The initial reaction rate was used to calculate the enzyme activity in the reference cuvette, which had a buffer in place of the enzyme and monitored changes in absorbance at 410 nm. Enzyme of one unit (U) is expounded as the portion of the enzyme required to obtain 0.01 O.D (Yan *et al*., 2022) and results were expressed in IU/ml. The modified Bradford method was used to measure protein concentration using Bovine Serum Albumin (BSA) as a standard (Bradford, 1976).

Production of tyrosinase

The selected strain was inoculated with a production medium containing yeast extract 1.5 gm/l, NaCl 5.0 gm/l, tryptone 1.5 gm/l, and L-Tyrosine 0.1%. The culture was inoculated and kept in a Shaking incubator 37°C for 48 hrs (Dalfard *et al*., 2006).

Tyrosinase enzyme assay

L-tyrosine and L-DOPA are used as substrates for measuring tyrosinase activity. To test the enzyme activity, an aliquot of the enzyme solution was added to 0.1M sodium phosphate buffer (pH6.8) containing 1mM L-tyrosine and L-DOPA, and the absorbance at 475nm was measured to determine if dopachrome had been produced. The extinction coefficient (Ɛ) for the product dopachrome is 3600L/mol.cm (Rao *et al*., 2013; Varghese *et al*., 2021). The initial rate was used for the calculation of tyrosinase activity, and the results are expressed in IU/ml. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 µmol of L-tyrosine to dopachrome per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome ($3600M^{-1}cm^{-1}$) by the following equation:

= absorption/min* assay volume (ml)*dilution factor*10000/ Ɛnm ($1*mol^{-1}cm^{-1}$)* enzyme volume (ml) Eq.1

Production of catecholase

The production medium containing catechol (1.0 g/l), yeast extract (0.4 g/l), $MgSO_4$ (2.0 g/l), K_2HPO_4 (6.0 g/l), $CaCl_2$ (0.4 g/l), and NaCl (0.5 g/l) with a pH of 7.0 was inoculated with a 5% (v/v) inoculum. The inoculated production media was incubated at 37 °C for 36 hours at 150 rpm. After completion of production, the cell culture was centrifuged at 5000 rpm for 10 minutes at 4 ° C to separate the cell biomass. The extracellular catecholase activity was estimated by (El-Bayoumi and Frieden, 1957). One unit of enzyme is defined as the amount of enzyme that decomposes one micromole of catechol per minute under standard assay conditions. The results are expressed in IU/ml.

DNA extraction

Genomic DNA was extracted from pure bacterial cultures, 24hours grown in Luria Bertani (LB) media at 37°C, centrifuge for 2min. Proteinase K 20 mg/ml in TE buffer and 10% SDS were used to lyse the bacteria for 1 hour at 37°C (Dewi *et al*., 2022; Huq *et al*., 2012). The DNA was extracted using Phenol/Chloroform/ Isoamyl alcohol (25:24:1). A 0.2 volume of 3M sodium acetate (pH 5.3) and 2 volumes of isopropanol were then precipitated as directed (Lever *et al*., 2015; Sridhar *et al*., 2022). Finally, using 100 µl of TE buffer, DNA was resuspended. The qualitative and quantification of DNA extracted was electrophoresed on 0.8% agarose gel, followed by quantification with UV spectrophotometer at 260nm (Lee *et al*., 2012; Murakami *et al*., 2009).

PCR amplification of Bacterial 16S rDNA

16SrRNA Forward primer (AGAGTTTGATCC TGGCTCAG) and Reverse Primer (AGAGTTTGATCCT GGCTCAG) were amplified using oligonucleotide primers (AAGGAGGTGATCCAGCCGCA). DNA was amplified in a reaction mixture using PCR under the following conditions: 50ul of a final mixture of 10xTaq buffer, 1.25U Tag DNA polymerase, 2mM dNTP mixture, 25mM $MgCl_2$, 50ng of DNA, and double-distilled water (Frank *et al*., 2008; O'Donnell *et al*., 2022). The PCR protocol was 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by an extension at 72°C for 7 min. HiMedia's Prime 96 PCR system was amplified (Lorenz, 2012; Mo *et al*., 2012). Amplicons were determined using electrophoresis on 1% agarose gel after ethidium bromide staining (Hengstmann *et al*., 1999; Tosar *et al*., 2010). ABI Prism BigDyeTM Terminator cycle sequencing ready reaction Kit was used for DNA sequencing in accordance with the manufacturer's instructions (PE Applied Biosystem) Sequencer for ABI PrismTM3736 DNA (Perkin Elmer) (Kieleczawa and Mazaika, 2010; Lee and Chappell, 2008; Nezhad *et al*., 2012). Using BLASTN2.2.31, the resulting bacterial 16S rRNA gene

sequence was compared to other similar sequences published in GenBank (Janda and Abbott, 2007; Sacchi *et al*., 2002). Neighbour Joining (NJ) and the MEGA11.0 1000 bootstrap value analysis were used to infer phylogenetic relationships (Limpiti *et al*., 2014; Tamura *et al*., 2011).

RESULTS AND DISCUSSION

The isolated organisms were identified as *Bacillus sp.* by studying their morphological, biochemical and molecular identificaitons. These potent strains produced the PPO and related enzymes. The results are summarized below:

Isolation of Gram-positive bacteria

A total of 400 different distinct colonies were screened from the soil samples collected from the four sampling sites of Kulathupalayam, wetland of Tirupur (Fig. 1). Among these strains, 25 isolates based on their morphological and biochemical characteristics were as being of the genus *Bacillus* (Łubkowska *et al*., 2021; Mohammad *et al*., 2017; Ulucay *et al*., 2022).

Polyphenol oxidase activity

Different organisms are exposed to diversity in the PPO source (Malviya *et al*., 2011; Sullivan, 2015). The study mainly focused on isolating and screening *Bacillus spp.* from soil samples. Top 5 *Bacillus spp*. strains (S2- ON564688, S4- ON564690, S13- ON564692, S17- ON586859, and S20- ON586861) were selected out of 25 strains for PPO production, tyrosinase and catecholase enzymes maximum activity (Table 1). Maximum cell concentration support for the production of enzymes was used in the investigation. A significant production of PPOs was found in the 27-104 IU/ml range with a minimum of 27.30 IU/ml by strain S7 and a maximum of 104.2 IU/ml by strain S13; a significant production of tyrosinase in the 25-69 IU/ml range with the minimum of 25.77 IU/ml by strain S10 and a maximum of 68.61IU/ml by strain S13; and catecholases production in the range of 20-61 IU/ml with the minimum of 20.24 IU/ml by strain S10 and a maximum of 61.59 by strain S6, respectively (Table 1). The selected strains results expressed a significant amount of enzyme activity was further identified using the molecular technique.

Molecular identification of organisms

The genomic DNA from the selected strains (S2, S4, S13, S17 and S20) was extracted and quantified using UV spectrophotometer at 260nm, 50ng/µl of DNA used to amplified the 1500bp of 16SrRNA gene and sequenced (Fig: 2a, 2b) . The sequence was assembled and confirmed with Nucleotide sequence BLAST to identify the 1400bp that the isolated Bacillus species matched and deposited in the GenBank database. The following were the genbank IDs: *Bacillus cereus* S022-VK1 (ON564688), *Bacillus albus* S035-VK2 (ON564690), *Bacillus subtilis* S207-VK3 (ON564692), *Bacillus thuringensis* S281-VK4 (ON586859), *Bacillus tropicus* S317-VK5 (ON586861) (Wang and Ash, 2015; Xiong *et al*., 2022). Using a joining tree made of 10 different species, Phylogenetic neighbourhood, *Bacillus* organisms were determined to be closely related to species-specific (Fig 2c).

Polyphenol oxidases have garnered much attention recently because of their remarkable ability to oxidise aromatic molecules. Due to this characteristic, PPOs, tyrosinase and catecholase are used in biotechnological applications in food, pulp and paper, textiles, medicine, and environmental technology (Padró *et al*., 2021). Soil is a solid substrate that holds many industrially important microbial abundances shaped by biotic and abiotic influences. In congruence with our study, a report by Dalfard *et al*. (Dalfard *et al*., 2006) documented the isolation of 93 different soil bacteria to be screened for PPOs. It successfully came up with 4 potent bacteria for PPO's activity. Several investigations stated the microbial structures and functions associated with wetlands are important and interconnected with various ecological services, including biogeochemical cycling, restoration, survival, biodegradation and specific transformations such as nitrification, mineralization, absorption, humification, as well as the sustainability of wetlands (Chandra *et al*., 2020; Singh *et al*., 2020; Urakawa and Bernhard, 2017; Zhu *et al*., 2022). In general, the microbial communities of the wetland are well preferred due to their higher adaptability and ability to produce many useful enzymes and metabolites (Panis and Rompel, 2022).

Phenolic chemicals are the primary substrates for PPO. A substrate is usually preferred by PPO, like many other enzymes. PPOs are highly active when exposed to substrates with a strong affinity or predilection (Wu *et al*., 2019). The present study used Syringadazine as a substrate in screening out the bacteria for PPOs production (Fig. 1). Twenty-five different bacterial isolates showed promise in producing the PPOs, which were evaluated for their potential in producing tyrosinases and catecholases (Table 1). A wide variety of sources of PPO have been reported in the scientific literature, including fungi, bacteria, shellfish, mushrooms, and plants (Adamian *et al*., 2021). The PPO in shrimp and lobster is responsible for the darkening of crustacean segments after preservation (Faccio *et al*., 2012). PPO has been purified and characterised from a range of plants, such as the sapodilla plum, apple, avocado, marula, potato, Chinese cabbage, banana pulp, cauliflower, and truffles (Nikolaivits *et al*., 2021). PPO may also be produced by other plant components, including

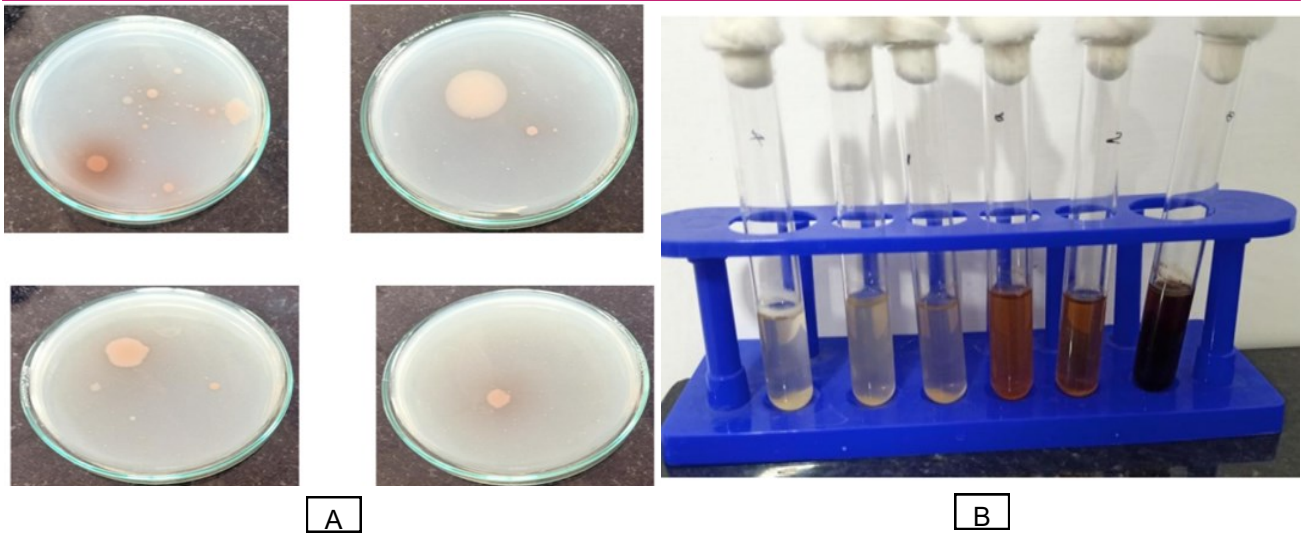
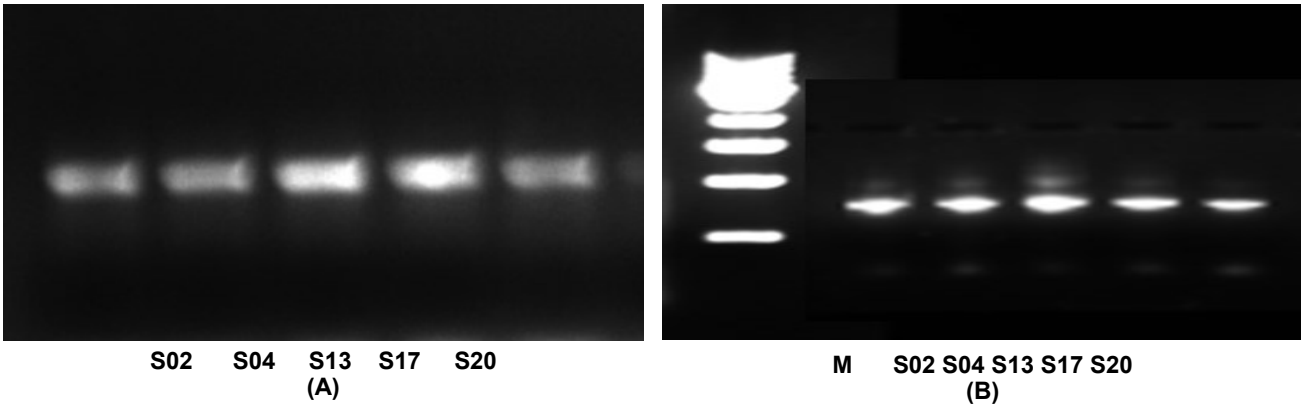
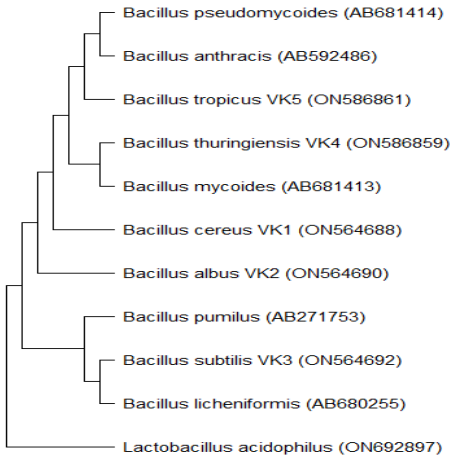


Fig 1. *Isolation & screening of soil microorganisms A) Positive reaction as indicated by culture producing brown pigments ; B)Brown coloration indicating the production of polyphenol oxidase and related copper containing enzymes*





(C)

Fig. 2. *Molecular identification of soil microorganisms, A) Genomic DNA from selected strain; B) PCR amplification of 16S rRNA gene; C) Phylogenetic Neighbour Joining tree for selected Bacillus organisms*

the pulp of fruits and vegetables, including bananas, cauliflower, and truffles (Nikolaivits *et al*., 2021).

However, bacteria are regarded as the primary microbial sources reported for significant PPOs production. The production capacities of isolates varied significantly with enzyme concentrations.. A study by Mohammad *et al*. (Mohammad and Alireza, 2007) also reported the efficiency of 16 aerobic endospore-forming *Bacillus sp.* from fermented tea to produce polyphenol oxidase activities, out of which the *Bacillus sp.* TB3 produced a

Table 1. Polyphenol oxidase-associated enzyme expression of selected Gram-positive *Bacillus Sp* strains

Strain No.	PPO Assay IU/ml	Protein Con. ug/ml	Specific Enzyme Activity IU/ml	Tyrosinase Assay IU/ml	Protein Conc. ug/ml	Specific Enzyme Activity IU/ml	Catecholase IU/ml	Protein Conc. ug/ml	Specific Enzyme Activity IU/ml
S1	55.6± 1.82	1.75± 0.047	31.77	25.93± 1.82	1.17± 0.015	22.23	65.93±1.82	1.67± 0.11	39.56
S2	166.8± 4.23	1.93± 0.040	86.42	65.83± 4.23	1.16± 0.031	56.59	100.87± 7.35	1.86± 0.12	54.13
S3	62.4± 2.70	1.62± 0.036	38.52	35.80± 3.74	1.04± 0.025	34.31	75.80± 3.74	1.54± 0.11	49.11
S4	178.2± 3.77	1.89± 0.031	94.29	78.63± 3.77	1.25± 0.040	62.91	78.63± 2.81	1.78± 0.17	44.09
S5	59.8± 2.41	1.32± 0.036	45.30	35.83± 4.34	0.94± 0.025	37.99	69.17± 2.41	1.38± 0.13	50.24
S6	55± 3.65	1.47± 0.020	37.41	34.90± 3.65	1.17± 0.020	29.83	96.70± 1.40	1.57± 0.17	61.59
S7	63.8± 2.52	1.41± 0.026	45.25	43.10± 2.52	0.84± 0.030	51.31	73.10± 2.52	1.33± 0.11	54.83
S8	62.2± 2.65	1.22± 0.026	50.98	41.37± 2.65	0.99± 0.031	41.93	74.70± 3.27	1.24± 0.07	60.08
S9	39.2± 1.96	1.35± 0.017	29.04	26.03± 4.10	1.14± 0.017	22.84	52.70± 5.96	1.27± 0.11	41.39
S10	48.6± 2.59	1.78± 0.021	27.30	25.77± 3.47	1.27± 0.021	20.24	65.77± 3.47	1.71± 0.11	38.54
S11	55.8± 1.21	1.53± 0.026	36.47	35.60± 1.21	1.14± 0.026	31.23	65.60± 1.21	1.57± 0.06	41.69
S12	69.6± 3.28	1.48± 0.040	47.03	45.77± 3.75	1.14± 0.036	40.15	65.77± 3.75	1.44± 0.07	45.67
S13	195± 4.29	1.87± 0.020	104.2	94.00± 4.29	1.37± 0.020	68.61	94.00± 4.29	1.77± 0.17	53.11
S14	66.2± 0.89	1.29± 0.025	51.32	46.50± 0.89	1.06± 0.030	43.87	76.50± 0.89	1.32± 0.07	57.95
S15	49.8± 3.29	1.31± 0.031	38.02	28.93± 3.29	1.04± 0.031	27.91	55.60± 4.06	1.30± 0.03	42.66
S16	83.4± 2.31	1.48± 0.025	56.35	43.20± 2.31	1.15± 0.035	37.67	73.20± 2.31	1.48± 0.03	49.57
S17	188.2± 3.64	1.85± 0.031	101.7	88.80± 3.64	1.34± 0.031	66.10	65.47± 2.75	1.74± 0.17	37.55
S18	79.6± 2.25	1.42± 0.021	56.06	56.10± 4.09	1.15± 0.038	48.92	66.10± 4.09	1.31± 0.17	50.33
S19	69.4± 3.56	1.59± 0.031	43.65	35.70± 3.52	1.25± 0.040	28.56	75.70± 3.52	1.48± 0.17	51.03
S20	193.6± 2.77	1.91± 0.042	101.3	92.87± 2.77	1.43± 0.020	64.94	46.20± 3.22	1.73± 0.28	26.71
S21	82.4± 2.75	1.35± 0.026	61.04	65.83± 3.73	1.33± 0.071	49.62	75.83± 3.73	1.33± 0.07	57.16
S22	64.4± 1.82	1.48± 0.030	43.51	44.07± 1.82	1.38± 0.070	31.93	54.07± 1.82	1.41± 0.12	38.25
S23	58.8± 2.82	1.22± 0.026	48.20	37.53± 2.82	0.94± 0.032	39.79	64.20± 4.65	1.28± 0.13	50.29
S24	77.2± 2.15	1.27± 0.025	60.79	47.03± 2.15	0.91± 0.036	51.68	67.03± 2.15	1.30± 0.07	51.56
S25	71± 1.60	1.53± 0.031	46.41	34.40± 4.50	1.14± 0.031	30.26	64.40± 4.50	1.44± 0.18	44.83

maximum of 5.48 units mL^{-1}. In general, these enzymes are produced by soil-dwelling microbes for various reasons, including defense mechanisms, ontogeny and for nutrient acquisition (Sinsabaugh, 2010). The use of PPO will even make protein engineering a more important field of research (Kamali *et al*., 2023). Most of the allelochemicals by the plant systems also influence the production of PPOs by soil microbes. A report said that the levels of different enzymes, including PPOs are significantly altered due to flavone by allelopathic rice plants (Gu *et al*., 2009). Their application in different fields mainly relies on their ability to oxidise phenolic groups from both small molecules, such as tyrosine, to polymeric substrates, such as proteins (Lugani *et al*., 2019). Another study reported a new group of PPOs from the soil bacterium *Azotobacter chroococcum* during melanogenesis and encystment stage in the fixation (Herter *et al*., 2011). Till date, there have been many studies focused on bacterial tyrosinases production, however, the characterization was very limited and only to a certain extend. A study reported that from a soil bacterium *Pseudomonas putida* F6, tyronases and laccases were isolated using its cell extracts (McMahon *et al*., 2007).

The enzyme catecholase belongs to the class of oxidoreductases mainly involved in catechol degradation into benzoquinone and oxygen (Mukherjee *et al*., 2013). It was reportedly produced by many of the microorganisms from different soil substrates. Like present study, the isolation of novel marine bacterium reported by Solano *et al.* (1997) produced all types of PPOs, including Tyrosinases and catecholases. Another report stated that a new bacterium *Pseudomonas* sp. BSC-6 isolated from industrial soils (paper, pulp, textile, pharma and dye) produced a maximum of 52.32 IU/L catecholase after scaling up (Lugani *et al*., 2019). The present report showed a higher catecholase production, with a maximum of 62IU/ml (Table. 1). There is a growing concern about the use of PPOs in various industries. The pigmented and polyphenol substances can be cleaned, bleached, and oxidized with enzymes that cause enzymatic oxidation. Studying laccase as an alternative for industrial applications has revealed new laccase varieties (Jiang and Penner, 2022). Numerous medications can prevent the PPO enzyme from oxidising the phenolic compounds found in certain food components (Bayrak *et al*., 2020). Strong enzyme inhibitors will transfer compounds that enhance the product's shelf life (Soliman *et al*., 2022). The morphological and biochemical methods provide clues for classifying the microorganisms; however, nucleic acid extraction is the critical step in accommodating their genetic grouping. The conserved 16srRNA amplification and PCR sequencing notably contributed to molecular identification and sequence-based microbial ecology taxonomy (Macrae, 2000).

The PPO enzyme has numerous applications in the skin, hair, and eye pigmentation (Mubashshir *et al*., 2023). The bacterial PPOs have higher oxidative potentials for a wide range of substrates and significantly evinced their applicability in various industries that opt phenolic biotransformation including food processing (Jukanti and Jukanti, 2017), cosmetics (Alamelumangai *et al*., 2013), textile industries (Antunes *et al*., 2018), crop management (Babu *et al*., 2015) and even in effluent treatment (Mukherjee *et al*., 2013) and waste management (Eid *et al*., 2023). Microbial identification is the basic part of any microbial-based work. A higher yield of such enzymes can also be tailored with the advancement in molecular biology techniques, including genome editing and these enzymes can be earmarked for their benefits. The present preliminary research thus provides the insights into isolating the potent PPOs-producing bacteria from the wetland of Tirupur. Further exploitation of these reported soil *Bacillus* may highlight their benefits for utilization in various industrial processes, even to stabilize the pigment in the hair.

Conclusion

The present study successfully isolated, screened, and molecularly characterized five *Bacillus species* from four sites of Kulathupalayam wetland ecosystems of Tirupur, elucidating their significant capability to produce polyphenol oxidase (PPO) and associated enzymes, namely tyrosinase and catecholase. The identified strains *B. cereus* S022-VK1*, B. albus* S035-VK2*, B. subtilis* S207-VK3*, B. thuringiensis* S281-VK4*, and B. tropicus* S317-VK5 have demonstrated substantial enzymatic activities, showing their possible applications in various industrial contexts. Besides exploring substrate specificity, the significant activity of these enzyme productions and further purification and optimization may exemplify the potential microbial resources of such enzymes in industrial applications, advocating lesser investment and broader benefits.

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Supplementary Information

The supplementary information is being provided as Fig. S1 and Table S1. The author(s) is responsible for the content or functionality of any supplementary information. Any queries regarding the same should be directed to the corre-sponding author. The supplemen-

tary information is downloadable from the article's webpage and will not be printed in the print copy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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