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Isolation and characterization of tyrosinase-producing bacteria from industrially contaminated soil

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Abstract

Parkinson's disease is a progressive neurological disorder that affects the nerve cells in the brain that produce dopamine. L-3, 4-dihydroxyphenylalanine (L-DOPA) is a natural precursor for the production of dopamine by the central nervous system required for normal movement of the body. Microbial tyrosinase by a series of reactions produces L- DOPA as an intermediate. The present work emphasizes on the isolation and characterization of potential bacteria with the ability of L-tyrosine to L-DOPA biotransformation from industrially contaminated soil. The contaminated soils from different industries of the Mandsaur and Neemuch district of Madhya Pradesh and the Chittorgarh district of Rajasthan were exploited for the present study. Out of the 48 isolates, seven were found with prominent tyrosinase production activity. Among these 7 strains, MUTY-5 is found to be the most potent tyrosinase-producing strain with tyrosinase production indexing 3.07±0.18. Microbial strain MUTY-5 is further characterized by biochemical and molecular methods. Based on 16S rRNA gene sequencing, we have identified tyrosinase-producing strain as *Bacillus cereus.* The study indicates the potential of *Bacillus cereus* MUTY-5 isolated from industrially contaminated soil for extracellular tyrosinase enzyme production which further can be explored for the transformation of L-tyrosine into L-DOPA under stress conditions.

Keywords: Parkinson's disease, L-DOPA, Tyrosinase, Biotransformation, Industrially contaminated soil.

Introduction

Modern lifestyles, characterized by sedentary behaviour, poor diet, high-stress levels, and insufficient sleep, contribute to the risk of developing different disorders. Neurological disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and stroke have

become increasingly prevalent in contemporary society, and present lifestyle factors significantly influence their incidence and progression. Parkinson's disease is one of the most common neurological disorders in elderly people which generally onset at an average age of 55. In this disease, there is impairment in motor and non-motor system (van Rooden et al., 2009). It is a common disease of unknown etiology and characterized by bradykinesis, tremor, rigidity, and impaired postural reflexes. Its relentless progression results in severe disability.

While the exact cause of Parkinson's disease remains unclear, it is believed to involve complex mechanisms such as environmental toxins acting on susceptibility genes, oxidative stress from mitochondrial dysfunction, and disruptions in the ubiquitin-proteasome system leading to the buildup of misfolded proteins and endoplasmic reticulum stress. Additionally, the activation of microglia in response to inflammation may contribute to these changes, ultimately resulting in programmed cell death of dopaminergic neurons. Dopamine is synthesized in these neurons from L-3,4-dihydroxyphenylalanine (L-DOPA), which is produced from tyrosine by the enzyme tyrosine 3-monooxygenase (Nagatsu & Sawada, 2009).

L-DOPA (levodopa) is used for the treatment of Parkinson's disease, which is believed to be caused due to low concentration of dopamine in some parts of the brain. For the treatment of Parkinson's disease, levodopa is taken orally, which converts into dopamine and crosses the blood-brain barrier. Thus, the increase in dopamine concentration improves nerve conduction and resolves movement disorders in Parkinson's disease. Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase that catalyzes the o-hydroxylation of monophenols to the catechols and then the oxidation of monophenols to the corresponding o-quinones. It plays a role in the biosynthesis of melanin. It catalyzes the ortho hydroxylation of tyrosine a monophenol to 3,4-dihydroxyphenylalanine (DOPA) an orthodiphenol that further oxidized to dopaquinone an orthoquinone. This o-quinone is then transformed into melanin pigments through a series of reactions. L-DOPA is an intermediate of the series (Peng et al., 2022). The total market volume of L-DOPA is $101 billion per year worldwide (Xun & Lee, 1998*)* and thus alternative production methods are being explored for the production of this drug (Ates et al., 2007). The global market for L-DOPA is increasing as a sign of rise of 250 tons per year (Koyanagi et al., 2005). In the past few decades, the enzymatic production of L-DOPA has been industrialized using microbial sources like *Erwinia herbicola* (Enei et al., 1972), *Pseudomonas putida* (Koyanagi et al., 2012)*, Aspergillus oryzae* (Haq & Ali, 2006), *Yarrowia lipolytica* (Ali & Shultz, 2007), *Acremonium rutilum* (Krishnaveni et al., 2009) and *Bacillus* sp. JPJ (Surwase &

Jadhav, 2011). As per the commercial requirement of L-DOPA, with increasing cases of Parkinson's disease, new sources need to be identified.

As the tyrosinase enzyme shows stress oxidative property, industrially contaminated soil can be considered as a possible source of tyrosinase-producing bacteria. Industrial contamination often leads to soils with high levels of heavy metals, hydrocarbons, and other toxic substances, creating a selective pressure that favors microorganisms with specialized metabolic capabilities and stress resistance. Therefore, the present study was carried out with the motive of isolation and characterization of potential bacteria from industrially contaminated soil for extracellular tyrosinase enzyme production which further can be explored for the transformation of L-tyrosine into L-DOPA under stress condition.

Material and Methods

Chemicals and reagents

All chemicals/reagents used throughout the work were of high-quality analytical grade and were purchased from Himedia (India).

Soil sample collection

The sampling sites chosen for the present study were the Mandsaur and Neemuch districts of Madhya Pradesh and the Chittorgarh district of Rajasthan states of India. The soil samples were collected from the surrounding area (contaminated soil) of different industries located in the aforementioned districts by wearing pre-sterilized gloves and placed individually in pre-sterilized polybag (Himedia, India) with proper labeling. Following collection, the samples were brought to the Faculty of Life Sciences, Mandsaur University, M.P., India laboratory for further processing and bacterial isolation.

Isolation of bacteria

Preliminary isolation of bacteria from the contaminated soil was done on Nutrient Agar (NA) medium. One gram of soil sample was weighed and added to 9 ml of sterile saline solution. Serial dilutions were prepared in a saline solution up to 10^{-6} dilutions and 500 µl of different diluted samples were spread on nutrient agar (NA) plate. Plates were then incubated at 28 ± 2 °C in BOD incubator for 24 hours and observed for bacterial growth after completion of the incubation. Based on the morphological characteristics, single colony from different plates have been picked and streaked on NA plates for culture purification. Pure cultures were maintained through periodic subculturing on NA plates.

Screening of tyrosinase-producing bacteria

For the screening of tyrosinase-producing potential bacterial strains, different bacteria isolated from the contaminated soil on NA plate were inoculated on Tyrosine agar medium (TAM) containing L-tyrosine 1.0 g/l, casein hydrosylate 10.0 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 0.25 g/l and agar 1.5 g/l, with pH adjusted to 5.5. Plates were incubated at 37 °C for 48-72 hours (Dalfard et al., 2006). Following the incubation, the plates were observed for brown-colored colonies with clear zone formation as a positive indicator for tyrosinase production activity.

Tyrosinase production efficiency grading of potential bacterial isolates

Tyrosinase production efficiency grading of potential bacterial isolates were performed by using tyrosinase production indexing to find out the most prominent one among the positive isolates. Tyrosinase production indexing was performed by using the spot inoculation method. All 7 positive bacterial isolates were spot inoculated from freshly grown culture (OD^{600} adjusted to 0.2; 10^8 CFU $ml^{-1)}$ at the centre of petriplates of Tyrosine agar medium (TAM). All experiments were carried out in triplicate. Plates were incubated at 37 °C for 48-72 hours followed by the measurement of colony and clearing zone. The tyrosinase production index was calculated by the following formula-

$$\text{Tyrosinase Production Index (TPI)} = \frac{\text{Clearing zone diameter}}{\text{Colony diameter}}$$

Based on TPI, most prominent bacterium MUTY-5 was chosen for the further study.

Morphological and Biochemical characterization of isolate MUTY-5

The promising strain MUTY-5 was examined for morphological and biochemical characteristics following the standard procedures with reference to Bergey's Manual of Systematic Bacteriology (Parry et al., 1983). Gram reaction, endospore staining, capsule staining, and Methyl Red-Voges Prauskauer (MR-VP) test were performed with standard kit (Himedia, India). Catalase test was performed by adding 100 µl overnight grown bacterial culture in 1 ml of H_2O_2 and observed for gas bubble formation. Motility test, mannitol fermentation test, urease activity, and amylase test were performed by inculcating bacteria on 0.35 % NA medium, phenol red mannitol broth medium (pH 7.3), urea broth, and starch minimal medium respectively. Other biochemical tests like Indole, Citrate utilization, Nitrate reduction, Gelatin hydrolysis, Glucose fermentation, Protease test, Oxidase, and Urease test were performed by amendment of the growth medium with the respective enzyme substrate.

Molecular characterization of tyrosinase producing bacteria

Isolation, extraction and purification of genomic DNA were done using the protocol of Sambrook and Rusell with slight modification (Sambrook & Russell, 2006). The purity of the isolated genomic DNA was assessed by the A^{260}/A^{280} ratio. For the amplification of the 16S rDNA gene, universal PCR forward primer (5'-AAGGAGGTGATCCAGCCGCA-3') and reverse primer (5'-GAGAGTTTGATCCTGGCTGGCTCAG-3') were used (Cheng et al., 2010). The PCR was accomplished in a total volume of 25 µl using the thermal cycler (SureCycler 8800 Agilent Technologies) equipment. The reaction mixture consisted of 7.5 µl of nuclease-free water, 1 µl of each forward and reverse primer, 3 µl of template DNA (50 ng/ µl) and 12.5 µl of Gene Dire X's PCR master mix. A thermal cycler was programmed under the following conditions: denaturation at 94 °C for 5 minutes followed by a run of 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1.5 minutes, and the final extension at 72 °C for 10 minutes.

For the assessment of the gene, the PCR product was loaded in the 1 % agarose gel (wt/vol) containing EtBr and run for 45 min at 85 Volts in 1X TAE buffer. Observation for the DNA band was done by visualizing gel for the DNA band in the Syngene gel doc system. The amplified PCR products were purified using EXOSAP clean-up according to the instructions provided by the manufacturers. The sequencing of the final PCR products has been done through outsourcing at Eurofins Pvt Ltd, Bangalore.

16S rRNA gene sequence homology and phylogeny analysis

Molecular identification of the bacterium isolate MUTY-5 was done by homology analysis of 16S rRNA gene sequence obtained through sequencing using the Basic Local Alignment Search Tool (BLASTn) program of the National Centre or Biotechnology Information (NCBI) available at the site https://blast.ncbi.nlm.nih.gov/Blast.cgi. Based on the maximum identity score, relevant sequences were selected for further analysis. Some of the non-similar sequences were also selected as out-group organisms. Selected sequences were subjected to multiple sequence alignment using Clustal W and checked for the gap.

The evolutionary history of MUTY-5 was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the MUTY-5. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the

branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1570 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2004, 2021).

Results and Discussion

Isolation and Screening of tyrosinase-producing bacteria

Soil is a natural habitat of millions of microorganisms. The collected industrially contaminated soil samples were examined for tyrosinase-producing positive strains. From the industrially contaminated soil of 6 different regions, a total of 48 bacterial isolates were isolated and designated as MUTY-1 to MUTY-48.

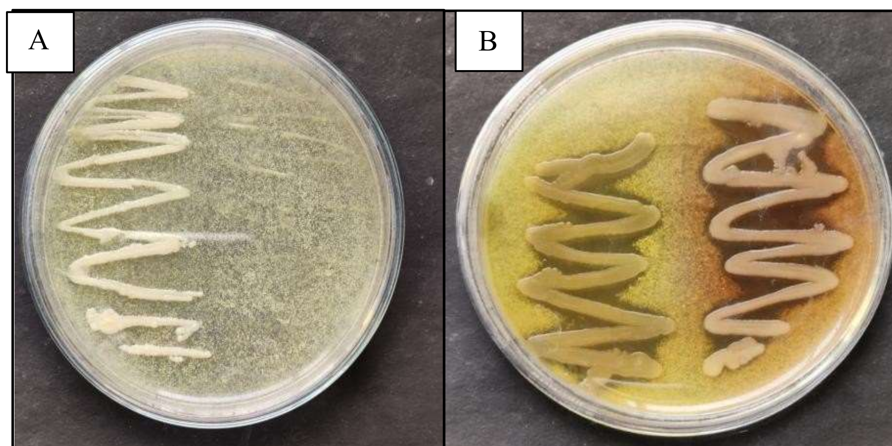

Fig 1: Screening of bacteria on Tyrosine Agar **A.** Tyrosinase negative **B.** Tyrosinase positive

Tyrosinase-producing activity of the isolated bacteria from the industrially contaminated soil was evaluated on the growth medium containing L-tyrosine as a substrate. The tyrosinase activity was expressed as the production of brown-colored colonies with clear zone formation. Out of the 48 isolates, 7 isolates, namely MUTY-2, MUTY-5, MUTY-18, MUTY-29, MUTY-35, MUTY-42 and MUTY-47 were found with prominent Tyrosinase production activity as they exhibited clear zone along with brown pigmented colonies on the TAM (Fig 1). Further, the tyrosinase production index was calculated for the most prominent isolate screening.

Tyrosinase production efficiency grading of potential bacterial isolates

The tyrosinase production indexing was calculated for the Tyrosinase production efficiency grading of potential bacterial isolates. Among the positive isolates, MUTY-5 was found with the

highest TPI=3.07±0.18 followed by MUTY-42 while MUTY-18 was found with the lowest TPI=1.26±0.11. Based on the TPI of all positive isolates, MUTY-5 found with 58 % more potential than MUTY-18 in tyrosinase production. On the other hand, MUTY-5 found with 28 % more potential than the nearest one MUTY-42 in tyrosinase production. Other positive isolates, MUTY-2, MUTY-29, MUTY-35 and MUTY-47 were found 46 %, 39 %, 41% and 32% respectively less significant than MUTY-5 (Fig 2). Based on the interpretation, the most prominent bacterium isolate MUTY-5 was chosen for further study

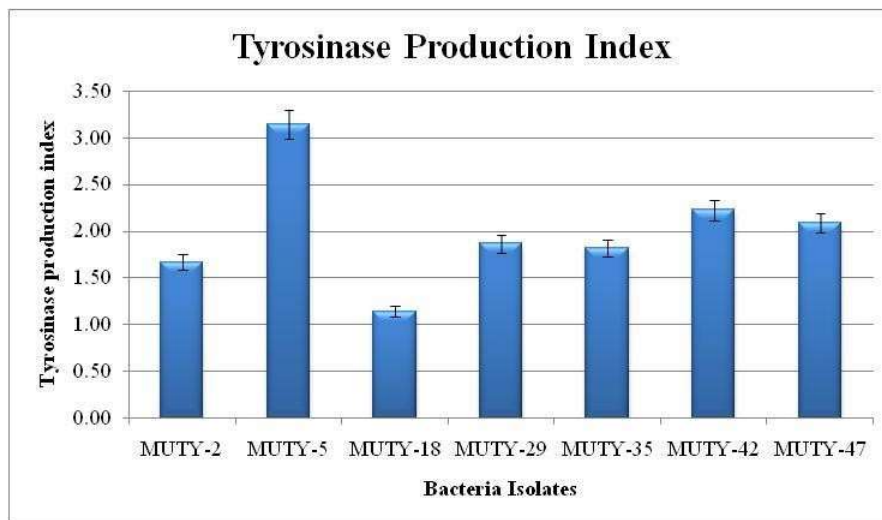

Fig 2: Comparative analysis of tyrosinase production capacity of bacterial isolates

Morphological and Biochemical characterization of the isolate MUTY-5

Morphological and biochemical characterization was done for preliminary identification of bacterial isolate MUTY-5. Based on morphological properties, it was found rod shaped, gram positive, endospore and capsule forming bacterium with off-white, round, opaque, flat white colour colonies on NA medium. Upon various biochemical test, MUTY-5 found positive for the Voges Prauskauer, Citrate utilization, Catalase, Nitrate reduction, Gelatin hydrolysis, Fermentation of glucose and Protease test. On the other hand, it was found negative for the Indole, Methyl Red, oxidase and urease test. Hence, based on the morphological and biochemical results stain was identified as *Bacillus* sp. and further confirmation was done by molecular characterization.

16S rRNA sequence analysis and phylogeny analysis of isolate MUTY-5

Nowadays 16S rRNA sequencing is considered as the most powerful tool for the identification of prokaryotic phylogeny (Bavykin et al., 2006). Molecular characterization of tyrosinase producing bacterium MUTY-5 was done by amplifying 16S rRNA gene from genomic DNA by

using universal 16S rRNA primers. On the agarose gel a sharp band around 1.5 kb was observed. Upon interpretation of 16S rRNA gene sequence obtained from Sanger sequencing through BLASTn similarity tool, MUTY -5 found highest similarities with *Bacillus cereus*. During taxonomy lineage interpretation of BLAST result, 82 hits out of 100 were found with the *Bacillus cereus* group wherein around 40% hits found with *Bacillus cereus* only. MUTY-5 exhibited maximum identity with *Bacillus cereus* strain 2YT241 as evident from Fig. 3 while found distantly correlated with other species of *Bacillus* genes and out group organism.

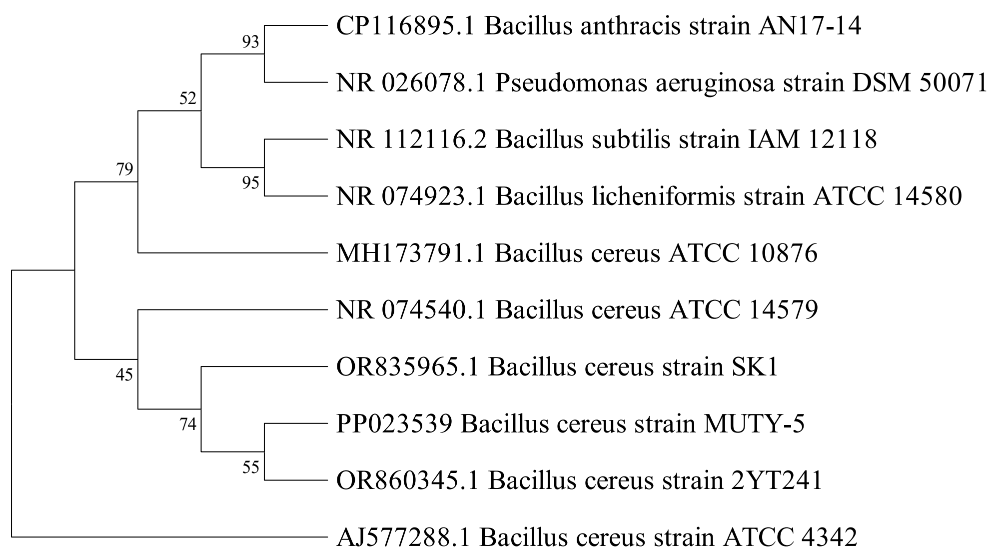

Fig 3: Phylogenetic tree based on the 16S rRNA sequence of the MUTY-5 and that of closely related *Bacillus* sp. following the multiple sequence alignment (neighbour joining tree).

According to the obtained results, the isolate was identified as *Bacillus cereus* strain MUTY-5. The size range of 16s rRNA regions of *B. cereus* MUTY-5 was about 817 bp and the partial sequence of the same was deposited in the GenBank under the accession number PP023539. This is the first report on the production of tyrosinase by any bacteria isolated from industrially contaminated soil.

Discussion

Tyrosinases have been exploited for a variety of biotechnological applications as it plays an important role in the undesirable browning of fruits and vegetables, antibiotic resistance, pigmentation of skin, melanogenesis (Mermer & Demirci, 2023; Halaouli et al., 2006). The production of L-DOPA (L-3,4-dihydroxyphenylalanine), a critical drug for the treatment of

Parkinson's disease, by bacterial systems has garnered significant attention in recent years due to its potential for cost-effective and sustainable biosynthesis. In the present study, a bacterial strain MUTY-5, capable of producing L-DOPA was isolated and identified as *Bacillus cereus* following the 16S rDNA gene sequencing and phylogenetic analysis.

Most of the studies concerning the production of L-DOPA was observed to the fungal species such as *A. oryzae* (Ali & Saleem, 2016), *A. rutilum* (Krishnaveni et al., 2009), *A. niger* (Ali & Haq, 2010), *Penicillium copticola* (Salah Maamoun et al., 2021) isolated from different sources. Different studies on the role of actinomycetes (Rudrappa et al., 2022; Shivaveerakumar & Hiremath, 2019) and bacteria (Muniraj et al., 2021; Shuster & Fishman, 2009) as tyrosinase producers have been also reported from different sources. The present study focuses on isolation and screening of tyrosinase-producing bacteria from industrially contaminated soil. We have exploited industrially contaminated soil samples from three different regions Neemuch and Mandsaur districts of Madhya Pradesh and Chittorgarh district of Rajasthan, and a total of 48 bacterial isolates were isolated from these regions. This appears to be the first report on the exploration of tyrosinase-producing bacteria from industrially contaminated soil.

The isolation of tyrosinase-producing microorganisms is crucial for biotechnological applications, including the biosynthesis of L-DOPA and melanin. This process involves several key steps, beginning with the collection of environmental samples from diverse habitats such as soil, decaying organic matter, and marine environments, known for harboring a variety of microorganisms with unique metabolic capabilities. For instance, Seo et al. (2003) successfully isolated tyrosinase-producing strains from soil and compost samples by using selective enrichment techniques that promote the growth of tyrosinase producers. Shivaveerakumar & Hiremath (2019) isolated *Streptomyces* sp. from Davanagere University soil for the extracellular production of tyrosinase enzyme. They performed various biochemical tests for the identification of promising strains of tyrosinase producers. At the same time, Salah Maamoun et al. (2021) isolated nineteen different fungal isolates as a potential tyrosinase producer on Czapek's-Dox media, among this isolates *A. niger* and *P. copticola* were reported as the most potent tyrosinase producers. These positive isolates were confirmed by the molecular identification based on their internally transcribed spacer (ITS) sequences. In 2021, Muniraj et al. isolated tyrosinase and laccase-producing *Bacillus aryabhattai* bacteria from soil sample of the forest ecosystem. They studied the role of tyrosinase in the polymerization of phenols for the treatment of phenol-rich wastewater. Rudrappa et al. (2022) screened out a total of 40 actinomycetes from the soil sample.

In the present study wherein 48 bacteria were isolated from industrially contaminated soil, 7 isolates, namely MUTY-2, MUTY-5, MUTY-18, MUTY-29, MUTY-35, MUTY-42, and MUTY-47 were found with prominent tyrosinase production activity as they exhibited clear zone along with brown pigmented colonies on the TAM.

Owing to its oxidative stress properties, tyrosinase is utilized in biotechnological applications involving small phenolic compounds and protein-associated phenolic groups. One significant application is the bioremediation of polluted soil and wastewater contaminated with phenolic compounds (Marino et al., 2011). The isolation of tyrosinase-producing microorganisms from industrially contaminated soil presents unique challenges and opportunities due to the stressed conditions in such environments. These conditions can significantly influence the microbial community structure and the functional potential of these ecosystems. Several studies have been reported on exploring the soil samples from industries to isolate different important enzyme-producing microbes. Sridevi et al. (2018) isolated heavy metal-resistant bacteria from soil samples of two industries that are Delta Paper Mill and Tanuku Sugar Factory located in the west Godavari district of Andhra Pradesh. The study was extended next to the *in-vitro* application of the isolated bacterial cultures in the bioremediation of metal-polluted soil. In 2013, Sagar et al. isolated lipolytic bacteria from the waste dumping site of Tezpur University, Assam. This study suggests that waste-contaminated soil can serve as an excellent breeding ground for the isolation of lipid-degrading bacteria of industrial importance. An industrially important enzyme is lipase which catalyzes the hydrolysis of triglycerides to fatty acid and glycerol. Habibollahi and Salehzadeh (2018) isolated a lipase-producing bacteria and identified it using morphological, physiological, and biochemical characteristics as well as molecular identification based on 16S rRNA sequence analysis. The bacteria identified using this polyphasic approach was *Pseudomonas* sp. The tannery industry is one of the primary leather processing units in the entire leather industry. Megharaj et al. (2003) isolated a bacterium *Arthrobacter* sp. having high efficiency in reducing chromium. This great potential of this bacteria can be used for the detoxification of Cr (VI) contaminated soil and water. Massaccesi et al. (2002) isolated filamentous fungi *A. terreus, Cladosporium cladosporioides, Fusarium oxysporum, Gliocladium roseum, Penicillium* sp., *Talaromyces helices,* and *Trichoderma koningii* from industrially polluted sediments in La Plata Argentina having cadmium removal capacities. Okoye et al. (2020) isolated hexadecane degrading bacteria from oil-polluted soil in Gio Community, Niger Delta, Nigeria. They isolated about 22 bacteria, among them *Pseudomonas* sp., *Bacillus* sp.,

Achromobacter sp., *Proteus* sp., and *Serratia* sp. demonstrated higher biodegradation potential for hexadecane. From this study, they found that extant autochthonous bacteria can effectively degrade hexadecane and could be a good candidate for bioremediation of the polluted site. As far as there is no report on the isolation of tyrosinase-producing bacteria isolation from the industrially contaminated soil. We have reported *B. cereus* MUTY-5 isolated from the industrially contaminated soil as a potent producer of tyrosinase with the Tyrosinase Producing Index 3.07±0.18. The *B. cereus* MUTY-5 can be explored further for the transformation of L-tyrosine into L-DOPA under stress conditions.

Conclusion

The industrially contaminated soil is a potent habitat for numerous microorganisms with the potential to survive in stressful conditions. From the literature studied so far it is found that various microorganisms with different applications in various fields isolated till now. Bacterial strain *B. cereus* MUTY-5 exploited in the present study is the first time notably from the industrially contaminated soil with the tyrosinase production potential, an enzyme of clinical and biomedical significance. It plays a major role in the production of a pharmaceutically active compound L-DOPA which is considered one of the important drugs for the treatment of Parkinson's disease. Further, elucidation of an insight into optimization, purification, and characterization of the tyrosinase enzyme of *B. cereus* MUTY-5 may enable in development of a simpler and industrially feasible bacterial system for achieving higher yields and cost-effective production of L-DOPA.

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