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Catalytic Zone, A Novel Screening Approach for the Production of Extracellular Tyrosinase by *Streptomyces vinaceusdrappus* DSV5

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An investigation was carried out to search potential actinobacteria for tyrosinase activity from different environmental samples. Detection of a typical catalytic zone developed on Tyrosine Gelatin Beef extract (TGB) agar indicating the tyrosinase activity by actinobacteria is a unique approach, which was never reported earlier. A total of five isolates of actinobacteria obtained from soils (DSV15 and DPG2) of Gulbarga India and marine sediments (DSV5) from Goa India were proved to be promising during primary and secondary screening for tyrosinase activity, based on the degree of catalytic zone and also intensity of color. Thus, observation of catalytic zone in addition to the intensity of color is a novel approach for the detection of tyrosinase activity. TGB medium has been proved as most suitable (high intensity of color and maximum catalytic zone) for tyrosinase activity, from among eight media, including two conventional and seven modified media, assessed during primary and secondary screening. An isolate of actinobacterium DSV5 has been proved as most efficient with the maximum production of tyrosinase in both Starch Tyrosine mineral (160±0.2U/ml) and TGB (175±0.2U/ml) media. The most potential isolate DSV5 was confirmed as *Streptomyces vinaceusdrappus* based on the molecular characterization by 16S rRNA analysis.

Key words: Streptomyces, Tyrosinase, Screening, Pigmentation, Catalytic zone, Production.

Tyrosinase (EC: 1.14.18.1) is an important copper containing high redox potential enzyme that catalyzes various phenolic substrates. It catalyzes the oxy hydroxylation of monophenols (Monophenolase activity) and the oxidation of oxy diphenols to reactive o-quinones (Diphenolase activity), using molecular oxygen[1]. The o-quinones undergo non enzymatic reactions with various nucleophiles producing an intermediate, which generates dark brown pigments[2]

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Microbial tyrosinases are very prominent and attractive enzymes due to their several biotechnological and industrial applications. Tyrosinase has been employed as biosensor for the detection of various phenolic constituents from polluted soils and water samples[3]. It is also used as probe to sense the level of phenolic constituents in media during large scale/industrial fermentations[4,5]. Immobolization of antibody was achieved via tyrosinase catalyzed reactions[6].Specific release of drug was targeted for melanoma by tyrosinase activity. Tyrosinase was used as an antigen to detect melanoma[7,8,9]. It is used as a biosensor for the detection of herbicides from agricultural soil[10]. It is also used as indicator for the potability of water by analyzing a phenolic compound released from colliforms in

water[11]. Tyrosinases are found in both prokaryotes and eukaryotic microbes, invertebrates, plants and mammals[12,13]. The most extensively investigated tyrosinases are however reported from fungi, actinomycetes and bacteria. The predominant commercial tyrosinase was reported from *Agaricus bisporus*[14,15]. *Neurospora crassa*[16], *Acremonium rutilum*[17], *Agaricus oryzae*[18], *Lentinus edodes*[19], *and Pycnoporus sanguineus*[20], *Streptomyces antibioticus*[21], *Streptomyces glaucescens*[22], *Streptomyces michiganensis* DSM 40015[23], *Streptomyces griseus*[24], *Streptomyces* sp. KY-453[25], *Bacillus thuringiensis*[26] were also reported and known for the synthesis of tyrosinase.

The information on the tyrosinase structure and exact reaction mechanisms is limited. Some reasons for these deficits are difficulties in purification of sufficiently high amounts of tyrosinases from eukaryotic sources due to low enzyme concentrations, contamination with pigments, occurrence of isoenzymes or post translational modifications[27]. Despite of intensive screening investigations for the exploration of microorganisms for the synthesis of tyrosinase, no rapid and reliable methods are in practice. The development of dark brown color in the medium as visual observation, indicating the formation of melanin is the sole conventional method[28], followed for the detection of tyrosinase activity by microorganisms. An investigation was carried out employing various media for the detection of tyrosinase activity by different approach, in addition to the visual observation of dark brown color, aiming at the exploration of actinobacteria for the enhanced production of tyrosinase.

MATERIALS AND METHODS

Collection of samples and isolation of actinobacteria

Soil samples from different ecological habitats such as agricultural fields, barren land, garden, graveyard, limestone quarry and tree bark scraping were collected[29], in sterile polythene bags. Sediment from mangrove near Salim Ali Bird Sanctuary, Goa, India was also collected[30]. All the collected samples were processed aseptically and pretreated[31]. Actinobacteria were isolated from different samples on starch casein agar (SCA)[32], and starch tyrosine mineral agar (STMA) by pour plate culture method[33]. The typical colonies of actinobacteria were identified based on the standard colony and microscopic features[34,35].

Screening of actinobacteria

The identified prominent isolates of actinobacteria were subjected to primary, secondary and tertiary screening for tyrosinase activity. The selected isolates were screened for the synthesis of tyrosinase on STMA, where casein was replaced with tyrosine in SCA and Tyrosine-gelatin-beef extract agar (TGBA)[36], by streak plate culture method[37]. Further, seven modified media including starch-tyrosine-mineral agar (STMA); tyrosine-beef extract agar (TBA); Tyrosine-gelatin agar (TGA); starch-tyrosine agar (STA); starch-tyrosine-beef extract agar (STBA); starch-tyrosine-gelatin agar (STGA) and starch-tyrosine-gelatin-beef extract agar (STGBA) were formulated based on the permutation combination of different ingredients of both conventional media (SCA and TGBA) and examined for the synthesis of tyrosinase. The potential isolates were employed to produce tyrosinase in selected media.

Production and assay of tyrosinase

Production of tyrosinase in a batchwise bioprocess (100ml media in 250ml Erlenmeyer's flask) employing STM and TGB media was carried out as per the procedure prescribed by Konard lerch and Leopold ettlinger (1972)[22]. The quantity of tyrosinase produced was determined by measuring the dopachrome at 475 nm[38], employing molar extinction co-efficient[39]. Standard reaction mixture containing 0.5 ml 4 mM L-dopa, 0.5 ml 0.1 M sodium phosphate buffer (pH 6.8) and enzyme extract in a total volume of 3 ml, mixture brought to 40^0C in BOD incubator for of 10 min. The absorbance at 475 nm was monitored continuously for 3 min in (Systronics) 2201 UV-VIS spectrophotometer. One unit of tyrosinase activity was referred as the amount of enzyme required to catalyze 1 µmol of L-dopa per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome 3600 M^{-1} cm^{-1} [39].

Molecular characterization of the potential isolate

The potential isolate was identified and characterized by the standard colony characters[34], microscopic features and biochemical/ physiological properties[35]. In addition, the 16S rDNA sequence of strain was determined. The strain was cultivated on starch casein agar (SCA)

until sporulation and then the spores were collected. The genomic DNA of *Streptomyces* isolate DSV5 was extracted as prescribed by Rintala et al. (2001)[40] and further purified using Wizard DNA Clean-Up System A7280 (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using 50 µl reaction mixture with 1 µl of template DNA, 25 pmol of primers pA and pH (Oligomer, Helsinki, Finland)[41], 10 nmol of each deoxynucleoside triphosphate (Finnzymes, Espoo, Finland), 5 µl of 10x Phusion HF buffer, 3 % dimethylsulphoxide, and 1.0 U of Phusion DNA Polymerase (Finnzymes, Espoo, Finland). The PCR conditions in DNA Engine DY AD Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) were as follows: heat denaturation at 98 ÚC for 30 s, 30 amplification cycles of denaturation at 98ÚC for 10 s, primer annealing at 61 ÚC for 30 s, and primer extension at 72 ÚC for 45 s. In the end of amplifications 72^{0}C was maintained for 10 min, followed by cooling to 4^{0}C. The 16S rRNA gene was sequenced in duplicate using the universal primers pE (forward and reverse), pB forward and pD forward[41]. The sequencings were done in the DNA Sequencing Laboratory (Institute of Biotechnology, University of Helsinki, Helsinki, Finland) as a purchased service. Sequence analysis was carried out at the Department of Biotechnology, University of Helsinki, Finland and submitted (Genbank ID: JX050266) to NCBI. BLAST search comparison was made against the Genbank databases and the related strains were selected for alignment by CLUSTAL X program [42]. The evolutionary history was inferred using the Neighbor-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method[43]. Phylogenetic analyses were carried out employing software MEGA4.

RESULTS AND DISCUSSION

Exploration of microorganisms from various ecological habitats constituting unique niche is an ever continuing phenomenon, searching for the potential isolates targeting variety bioactive molecules. Few novel isolates of Streptomyces have been reported[44,45], from the regional soil samples and were explored for different enzymes [46,47]. Though, various stages of isolation and screening of potential microorganisms have been well recorded[48]. Much more rapid and accurate methods are warranted, atleast for certain bioactive molecules. More accurate and different approach for the screening of actinobacteria from various habitats for the detection of tyrosinase activity was investigated. The Streptomyces sp. DSV5 was isolated from Tree bark sample, Chandarki, India.

Primary screening

Two conventional media (SCA and TGBA) and seven modified media (starch-tyrosine-mineral agar (STMA); tyrosine-beef extract agar (TBA); Tyrosine-gelatin agar (TGA); starch-tyrosine agar (STA); starch-tyrosine-beef extract agar (STBA); starch-tyrosine-gelatin agar (STGA) and starch-tyrosine-gelatin-beef extract agar (STGBA) were employed (Table 1) in the primary

Table 1. Composition of different media employed

Composition	Different media								
(g)	SCA	STMA	TGBA	TBA	TGA	STA	STBA	STGA	STGBA
Starch	10.0	10.0	-	-	-	5.0	5.0	5.0	5.0
L-Tyrosine	-	4.0	5.0	5.0	5.0	3.0	5.0	5.0	5.0
Beef extract	-	-	3.0	3.0	-	-	3.0	-	3.0
Gelatin	-	-	5.0	-	5.0	-	-	5.0	5.0
Casein	0.03	-	-	-	-	-	-	-	-
K_2HPO_4	2.0	2.0	-	-	-	-	-	-	-
KNO_3	2.0	-	-	-	-	-	-	-	-
NaCl	1.0	1.0	-	-	-	-	-	-	-
$MgSO_4.7H_2O$	0.05	0.05	-	-	-	-	-	-	-
$CaCO_3$	0.02	0.02	-	-	-	-	-	-	-
$FeSO_4.7H_2O$	10.0	10.0	-	-	-	-	-	-	-

stage for the isolation and screening of actinobacteria aiming at the synthesis of tyrosinase.

Starch casein agar is an exclusive medium prescribed by Kuster and Williams (1964)[32] for the isolation of actinobacteria. Casein was replaced by tyrosine and used for the isolation and screening of actinobacteria for the synthesis of tyrosinase. In addition, TGB agar[36], was also used for the said purpose. Degree of coloration or intensity of color (yellowish, brownish and blackish) was an exclusive conventional method prescribed[28,49], and followed for the detection of microorganisms synthesizing tyrosinase. The actinobacteria isolated and screened from different samples for the synthesis of tyrosinase based on intensity of color are as presented in Table 2. The degree of intensity of color was recorded as poor (+, yellowish), moderate (++, brownish) and higher (+++, blackish) and correlated for the level of synthesis of tyrosinase. TGB agar was never reported to be employed earlier, to the best of our knowledge, for the screening of actinobacteria to detect the tyrosinase activity. However, this

Table 2. Primary screening of soil actinobacteria for the synthesis of tyrosinase in different media based on intensity of color

Soil samples	Tyrosinase activity graded (+/++/+++)* in different media							
	STMA	TGBA	TBA	TGA	STA	STBA	STGA	STGBA
Agricultural field (red)	++	++	+	+	++	++	+	+
Agricultural field (black)	++	++	+	++	+	++	+	+
Barren land	++	++	+	+	+	++	+	+
Fresh water	++	+++	+	++	++	+	+	+
Garden	++	++	+	+	+	+	+	+
Grass land	++	++	+	+	+	+	+	+
Grave yard	++	+++	+	++	++	+	+	++
Limestone quarry	+	+	+	+	+	+	+	+
Mangrove	+++	+++	+	++	++	++	+	++
Rhizosphere	++	++	+	+	+	+	+	+
Tree bark	++	+++	+	++	++	+	+	++
Wood ash	+	++	+	+	+	+	+	+

* +: poor (yellowish); ++: moderate (brownish); +++: higher (blackish)

Table 3. Primary screening of soil actinobacteria for the synthesis of tyrosinase in different media based on catalytic zone

Soil samples	Tyrosinase activity measured (mm) in different media (Average ± SD)							
	STMA	TGBA	TBA	TGA	STA	STBA	STGA	STGBA
Agricultural field (red)	0	8 ± 1.0	1 ± 0.2	3 ± 0.1	5 ± 2.0	4 ± 1.0	1 ± 0	2 ± 0.1
Agricultural field (black)	0	9 ± 0.4	2 ± 0.2	5 ± 0.2	5 ± 0.5	6 ± 0.1	4 ± 0.2	4 ± 0.2
Barren land	0	8 ± 1.0	2 ± 1.0	2 ± 0.1	3 ± 0.4	3 ± 0.1	1 ± 0.4	4 ± 1.0
Fresh water	0	9 ± 0.3	1 ± 0	4 ± 0.4	7 ± 0.2	5 ± 0.2	2 ± 0	1 ± 0.1
Garden	0	8 ± 1.1	2 ± 0	2 ± 0.4	2 ± 0.2	1 ± 0.4	2 ± 0.4	1 ± 0.2
Grass land	0	7 ± 0.6	1 ± 0.4	4 ± 0.1	3 ± 0.2	3 ± 0.2	3 ± 0.1	4 ± 0.4
Grave yard	0	15 ± 0.3	2 ± 0.2	2 ± 0.4	7 ± 1.0	5 ± 0.1	0 ± 0	1 ± 0.2
Limestone quarry	0	3 ± 1.0	1 ± 0.3	1 ± 0.2	1 ± 0	1 ± 2.0	1 ± 0	1 ± 0.1
Mangrove	0	25 ± 0.5	3 ± 0.2	7 ± 0.2	5 ± 0	4 ± 1.0	1 ± 0.1	3 ± 0.6
Rhizosphere	0	7 ± 0.1	1 ± 0.3	1 ± 0.4	3 ± 0.1	5 ± 2.0	2 ± 0	3 ± 0
Tree bark	0	20 ± 0.4	1 ± 0.2	4 ± 1.2	10 ± 0.1	5 ± 0.2	2 ± 0	7 ± 0.5
Wood ash	0	5 ± 1.0	2 ± 0.4	3 ± 0.4	2 ± 2.0	2 ± 0.4	1 ± 0.1	3 ± 0.1

medium[36] was recorded, to be used for the differentiation of *Streptomycete* isolates, but never reported the catalytic zone for the tyrosinase activity. Surprisingly, this medium exhibited catalytic zone (a clear zone around the colony catalyzing the tyrosine), indicating the synthesis of tyrosinase. Table 3 exhibit the degree of catalytic zone on different media (TGBA and modified media) by the isolates of actinobacteria obtained from various soil samples. The catalytic zone developed was measured (mm) and considered as novel criteria for the selection of potential isolate based on the degree of catalytic zone. Seven different modified media were also examined for the synthesis of tyrosinase by actinobacteria obtained from different soil samples. Tyrosinase is an induced enzyme[50], and tyrosine is an amino acid being the exclusive protein source, significantly revealing the tyrosinase activity. The typical colonies of actinobacteria isolated from different samples showing the maximum tyrosinase activity based on the intensity of color and catalytic zone were selected and examined further for the confirmation of tyrosinase synthesis.

Secondary screening

Three selected test isolates of actinobacteria (DSV5, DSV15 and DPG2) were subjected for secondary screening to confirm the synthesis of tyrosinase on STM agar based on intensity of color and catalytic zone on TGB agar (Table 4). It is interesting to note that, the graded intensity of color on STM agar can be matched

Table 4. Secondary screening of Streptomyces for the synthesis of tyrosinase on STM agar based on intensity of color and on TGB agar based on catalytic zone

Incubation Period (hrs)	Secondary Screening					
	Tyrosinase activity (intensity of colour) by selected isolates graded (+/++/+++)* on STM Agar			Tyrosinase activity (zone of catalysis) by selected isolates measured (mm) on TGB Agar(Average ± SD)		
	DSV5	DSV15	DPG2	DSV5	DSV15	DPG2
24	+	+	+	9±0.57	7±0.09	6±0.2
48	++	++	+	15±0.02	12±0.5	7±1.19
72	+++	++	+	20±0.5	18±1.0	12±0.25
96	+++	++	++	25±0.7	22±1.0	15±0.1
120	+++	+++	+++	30±0.18	24±0.57	18±1.52
144	+++	+++	+++	32±0.31	26±1.0	20±1.89

* Yellowish = +; brownish = ++; blackish = +++

Table 5. Tertiary screening of the selected isolates of streptomyces for the production of tyrosinase in STM and TGB media

Incubation Period (hrs)	Tertiary Screening					
	Tyrosinase activity(U) in STM medium (Average ± SD)			Tyrosinase activity(U) in TGB medium (Average ± SD)		
	DSV5	DSV15	DPG2	DSV5	DSV15	DPG2
24	28±1.0	23±0.5	25±0.2	25±1.0	21±0.4	24±0
48	62±0.2	47±0.6	54±0.4	53±0.6	40±0.6	58±0
72	101±0.1	93±0.4	78±0.4	105±0.4	98±0.2	102±0.2
96	138±0.4	124±0.4	98±0.2	140±0.6	122±0.2	125±0.5
120	160±0.2	155±0.2	140±0	175±0.2	165±0.4	160±0.2
144	152±0.2	140±0.1	121±0.2	172±0.4	153±0.4	158±0.2

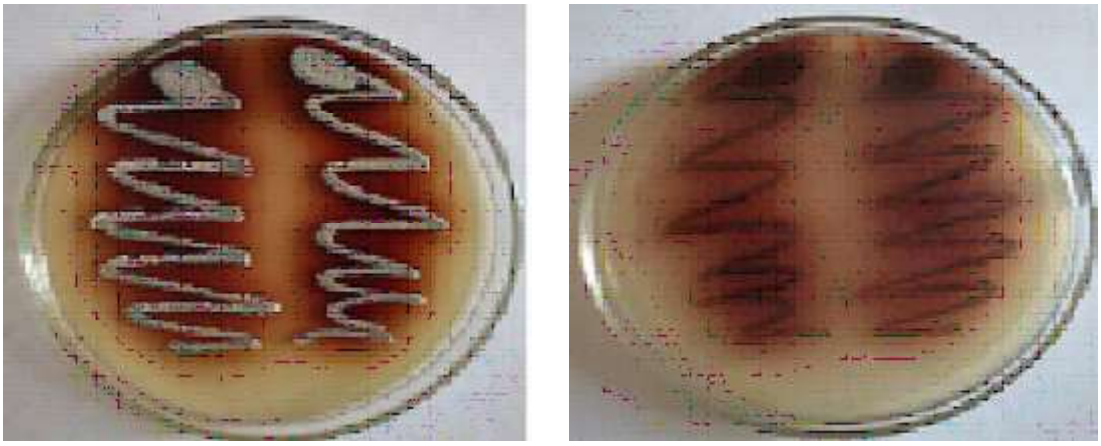

Fig.1. Synthesis of tyrosinase by the potential isolate of Streptomyces DSV5 on STM agar based on the intensity of color.

Fig. 2. Synthesis of tyrosinase by the potential isolate of Streptomyces DSV5 on TGB agar based on the intensity of color and catalytic zone.

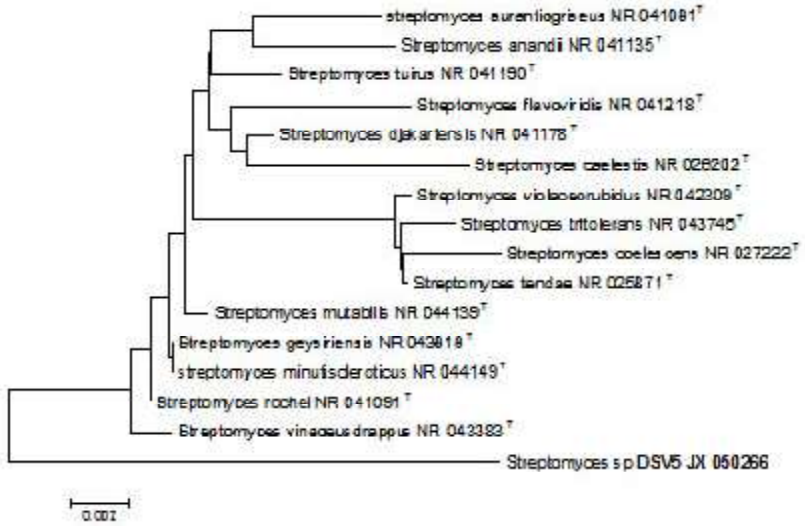

Fig.3. Phylogenetic tree showing the systematic position of the potential isolate of Streptomyces - DSV5.

with the catalytic zone on TGB agar. However, incidentally TGB agar exhibits not only the catalytic zone but also the intensity of color similar to the STM agar. This ensures the accurate matching of the intensity of color and catalytic zone. Fig. 1 and 2 exhibiting the high intensity of color on STM agar and catalytic zone along with intensity of color on TGB agar by DSV5 significantly reveals it as the most potential isolate for the synthesis of tyrosinase.

Tertiary screening

The qualitative attribute for the synthesis of tyrosinase shown during the primary and secondary screening was assessed and confirmed, by subjecting the selected isolates of actinobacteria to tertiary screening. Tyrosinase activity is generally measured by either determining the consumption of oxygen during the reaction or spectrophotometrically by following the increase of absorbance at 475 nm due to dopachrome formation[51]. The quantitative production of tyrosinase in STM and TGB media by all the three test isolates of actinobacteria are as shown in Table 5. In tertiary screening also, isolate DSV 5 has been proved as most potential isolate in both STM and TGB media. However TGB medium has resulted in more production (175±0.2U) of tyrosinase than STM (160±0.2U) medium. Thus, the intensity of color (a conventional approach) and the catalytic zone can be perfectly correlated for the synthesis of tyrosinase. This significantly reveals that, the catalytic zone can be an accurate and novel approach to screen actinobacteria for the synthesis of tyrosinase, which may be applied in general for screening of any microorganisms for the synthesis of tyrosinase. Recent available literature reveals 141 U/ml intracellular tyrosinase by *Acremonium rutilum*[17], 94 U/ml extracellular tyrosinase by *Bacillus thuringiensis*[26], and 72 U/ml extracellular tyrosinase by actinomycetes[52]. Highly varied range of tyrosinase activities from Streptomyces were reported by several researchers. Konard lerch and Leopold ettlinger (1972)[22], reported 24400 units by *Streptomyces glaucescens,* following oxygen consumption method using oxygen electrode and Stephan Phillip *et al*. (1991)[23] reported 22700 nkat units by *Streptomyces michiganensis*, employing YSI Biological Oxygen Monitor model 5300. Various methods with different principles, type of substrates and their concentrations employed in the process of enzyme assay would result such higher values. Molar extinction coefficient of Dopachrome is not an uncommon practice being followed by majority of researchers to determine tyrosinase activity. The potential isolate DSV5 detected in the present investigation was proved to be highly efficient with production of 160±0.2 U/ml (STM media) and 175±0.2 U/ml (TGB medium) extracellular tyrosinase.

Actinobacteria were being analyzed[53], at various levels to gain information suitable for constructing databases and effecting identification. The highest level is the genome and its direct expression as RNA. Sequence analysis of various genes provides stable classification and accurate identification, which has become the cornerstone of modern phylogenetic taxonomy. The potential isolate in the present investigation was assigned to the genus *Streptomyces* based on colony characters and microscopic features. Further, the isolate was characterized by 16S rRNA analysis and has been confirmed (Fig. 3.) as *Streptomyces vinaceusdrappus* NR 043383^T based on the phylogenetic illustration.

CONCLUSIONS

Search for newer organisms with higher level production of tyrosinase is ever continuous phenomenon and essential for commercial viability. Screening of potential isolates from natural habitats is utmost important. Detection of catalytic zone, a novel approach can be a more reliable, accurate and simple method for rapid screening of microorganisms from natural sources for tyrosinase activity. The most potential isolate DSV5 *Streptomyces vinaceusdrappus* can be explored further for the large scale industrial production of extracellular tyrosinase with a greater commercial value. TGB medium may be developed further for the much enhanced production of extracellular tyrosinase.

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