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Media Optimization for the Production of Tyrosinase Enzyme from Isolated Bacterial strain *PV24*

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ABSTRACT: Using the Plackett-Burman design, a fermentation medium was created for the current study in order to maximize the synthesis of tyrosinase from the strain that was isolated from the Ahmednagar region. This was done by screening various nutritional and physical characteristics. $CusO_4$, L-tyrosine, D-mannitol, and beef extract were the four fermentation process variables chosen from the One variable at a time (OVAT) method. Plackett Burman statistical analysis was used to assess the chosen variables based on standard effect plot, coefficient value, and significance. Three variables were shown to have a high degree of influence on production: D-mannitol, beef extract, and L-tyrosine. The other variable did not significantly affect production, according to the data. Additionally, the study of the variance value R2 (0.92) demonstrated the significance of the prediction model (p less than 0.05). Plotting the standard effect for every component and its characteristics gave precise information that may be used to choose appropriate variables for additional optimization. Tyrosinase activity was determined to be 49.9U/ml.

Keywords: Tyrosinase, Bacteria, OVAT analysis, Enzyme assay, L- tyrosine, Plackett Burman Analysis, Media optimization, L-DOPA.

INTRODUCTION

Microbial fermentation to produce enzymes have also attracted much concern in the recent past especially to those that deal in biotechnology (Singh *et al*., 2016). Tyrosinase is one of such enzymes classified as a copper containing oxidase being used in synthetic melanin manufacture, biosensors, and biodegradation of phenolic pollutants (Zaidi *et al.,* 2014).

The multipurpose enzyme tyrosinase regulates how animals produce melanin from tyrosine. It facilitates the conversion of monophenols into o-diphenols via hydroxylation, and then oxidizes those diphenols to o-quinones. All domains of life contain the copper-containing enzymes known as tyrosinases (EC 1.14.18.1) (Claus and Decker 2006; Matoba *et al.,* 2006). The sources of tyrosinases are Animals (Sanchez Ferrer *et al.,* 1995; Faccio *et al.,* 2012; Fairhead and Thony-Meyer 2012), Plants (Janovitz-Klapp *et al.,* 1989; Raymond *et al.,* 1993; Friedman 1996; Lee, 1997; Kubo and Kinst-Hori 1998; Rani *et al.,* 2007) and fungi (Strothkamp *et al.,* 1976; Lerch, 1983; Mueller *et al.,* 1996; Kanda *et al.,* 1996; Nakamura *et al.,* 2000; Halaouli *et al.,* 2005; De Feria *et al.,* 2007) are the good source of tyrosinase.

Bacterial tyrosinase isolated from different species such as *Streptomyces,* (Della-Cioppa *et al.,* 1998) *Rhizobium*, *Pseudomonas maltophilia, Marinomonas mediterranea, Thermomicrobium roseum, Bacillus thuringiensis, Ralstonia solanacearum, Verrucomicrobium spinosum* (Claus and Decker 2006) and *Pseudomonas putida* (McMahon *et al.,* 2007).

Tyrosinase has various industrial applications including, production of the L-Dihydroxyphenylalanine (DOPA) (Pialis and Saville 1998; Koyanagi *et al.,* 2005; Ates *et al.*, 2007), *invitro* conjugation of the protein gelatin to the polysaccharide chitosan (Chen *et al.,* 2002), The peptide sericin, which is present in the effluent of the silk textile industry, is converted into conjugates by the enzyme tyrosinase (Anghileri *et al.,* 2007).

The cost and availability of the substrate and producer organism, as well as the efficiency of the upstream and downstream processing, all affect how profitable it is to generate enzymes economically (Becerra and Siso 1996). Prior to any large-scale metabolite production, medium optimization is still one of the phenomena that is most thoroughly studied and comes with a lot of problems. Prior to the 1970s, media optimization was done through the use of antiquated techniques that were costly, time-consuming, and required a lot of tests with questionable accuracy. However, with the development of contemporary mathematical and statistical methods, media optimization has become more dynamic, successful, economical, efficient, and reliable in producing outcomes. The best fermentation conditions (pH, temperature, agitation speed, etc.) and medium components (carbon, nitrogen, etc.) must be determined and optimized in order to design a production medium.

Additionally, maximal product concentration may be attained by fine-tuning the previously mentioned parameters (Gupte and Kulkarni 2003; Franco-Lara *et al.,* 2006; Wang *et al.,* 2006).

Plackett-Burman design (PBD) is a traditional method for screening large variables. The quantity of the data is significantly reduced by the Plackett Burman design, which helps to prioritize the crucial elements and their optimal values (Plackett and Burman 1946). Plackett Burman design was used for theoptimization of enzymes like chitinae from *Brevundimonas diminuta*, lactase from *Bacillus* (Karlapudi *et al.,* 2018), lipase from *Bacillus* (Vasiee *et al.,* 2018).

Present research shows this to be of value for a variety of enzymes of microorganisms, including the specified tyrosinase. The current article seeks to utilise the Plackett-Burman design to improve the media for increasing the production of tyrosinase using the isolated bacterial strain PV24 for possible industrialisation. Thus, in methodologically developing media optimization as a means of enhancing the cost-efficacy and mass-scale yield-prolificacy of microbial enzymes, this study is moderately to highly relevant to the current state of the art in the boosting of microbial enzymes.

MATERIAL AND METHODS

Chemicals and Microorganism. All the chemicals were purchased from Himedia (India) and the tyrosinase producing bacterial strain *PV 24* was isolated from the Kapurwadi lake (Latitude: 19.114736, Longitude: 74.780445°) mud sample, District Ahmednagar (M.S.), India. The sample was serially diluted and streaked on the Sterile Tyrosinase agar (supplemented with 0.1% L-Tyrosine).

Growth and enzyme production. The production of tyrosinase was carried out in a 250 mL Erlenmeyer flask that held 50 mL of the baseline medium, which included 0.15% Yeast Extract, 0.15% Peptone, 0.5% NaCl, and 0.1% Tyrosine. Optical Density of the organism was measured at 660 nm by using the Visible spectrometer (Cystronics type 105) Enzyme assay was performed by Dopachrome method. 3 ml of 0.02M L-dopa, 0.1 millilitres of 0.1 M potassium phosphate buffer [pH 6.8] for a blank, and 0.1 millilitres of cell-free extract (the cell-free supernatant was produced by centrifuging at 8000 rpm for ten minutes) were included in the standard reaction mixture. For the purpose of producing dopachrome, the absorbance was measured at 475 nm in a visible spectrophotometer for five minutes at 30°C. The amount of enzyme required to oxidize one mol of L-DOPA per minute under the given conditions is known as one unit of tyrosinase activity. This amount was calculated using the molar extinction coefficient [3600 M^{-1} cm^{-1}] of dopachrome (Vachtenheim *et al*., 1985). Protein concentration was determined by the Folin-Lowry method (Lowry *et al.,* 1951).

Optimization of carbon source. D-glucose, starch, D-mannitol, lactose, and D-glycerol, were added to the basal medium in order to determine which carbon source was optimal for the production of enzymes. Apart from the carbon source, the basal medium composed of yeast extract as nitrogen source and L-tyrosine acted as substarte (Valipour *et al.,* 2015; 2016).

Optimization of nitrogen source. Peptone, beef extract, tryptone, casein, tri-ammonium citrate, L-asparagine, L-glutamine, and L-cysteine were supplemented to the media to identify the optimal source of the nitrogen. The organism was inoculated for five days, the enzyme's activity was assessed spectroscopically every 24 hours for five days. (Valipour *et al.,* 2015; 2016).

Optimization of substrate concentration. The medium was supplemented with L-tyrosine at concentrations ranging from 0.1 gm/L to 1 gm/L. Organism was inoculated and production was carried out for 5 days. Enzyme activity was determined to assess the production. (Valipour *et al.,* 2015; 2016).

Optimization of the $CuSO_4$ concentration. As tyrosinase is a Copper containing enzyme it requires the Copper Sulphate at a trace amount. To determine the optimum concentration the enzyme production was tested with concentrations of $CuSO_4$ 0.1mM, 0.25mM and 0.5mM (Valipour *et al.,* 2015; 2016)

Environment parameter. To determine the optimum environmental parameters enzyme production was also tested to determine the optimum pH and temperature for the maximum production of the enzyme.

Plackett and Burman design. The elements of the media that had a substantial impact on tyrosinase production were screened using the Plackett- Burman design. Different medium components and culture parameters have been assessed for screening purposes. Every factor was analyzed at two different levels, (−1) for a low level and (+1) for a high level, using the Plackett-Burman factorial design. To create the Plackett-Burman design Minitab version 22 (free trial) was used. (Plackett and Burman 1946; Karlapudi *et al.,* 2018; Shehata *et al.,* 2014). The parameters were selected by one variable at one time and their lower and a higher values were determined (Table 1). The design of matrix is represented in Table 2.

The first-order polynomial equation serves as the foundation for the Plackett-Burman experimental design.

$$Y = \beta 0 + (\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots\ldots\ldots + \beta_n x_n)$$

Table 1: Media components and their lower and higher values used to construct the Placket Burman design.

Variables	Codes to the variables	Lower value	Higher value
Mannitol	A	1gm/L	5gm/L
Beef Extract	B	1gm/L	5gm/L
$CuSO_4$	C	0.1mM	0.5mM
L-Tyrosine	D	0.5gm/L	1gm/L

By using the design, the twelve runs were made. Enzyme activity was determined and by using the Minitab software version 22 (free trial) the statistical analysis was carried and results were interpreted.

Statistical Analysis. All the statistical analysis was done with the help of the MS Excel 2019 and the

Minitab version 22 (free trial). All the experiments were carried out in the multiple of the three and the values are interpreted at S.D. ± 0.5.

RESULT

Growth curve v/s Enzyme Activity. Upon inoculating the organism into the media, growth and enzyme activity were analyzed. The highest tyrosinase activity, reaching 43.3 U/ml, was observed on the third day, in the stationary. This clearly indicates that enzymes, as secondary metabolites, are produced at their peak during the organism's stationary phase (Fig. 1A).

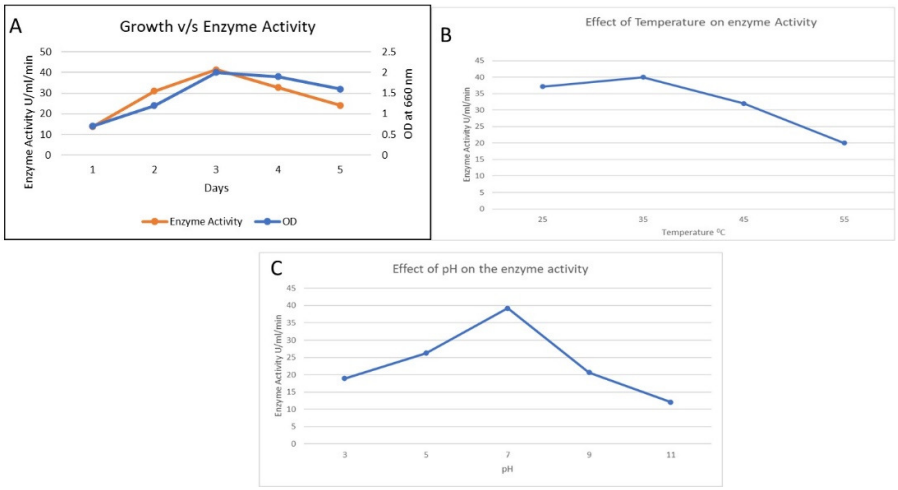

Fig. 1. Impact of the A: days of incubation, growth, B: Temperature, and C: pH on the enzyme production.

Carbon source. D-mannitol exhibited the highest enzyme activity among the tested carbon sources, with 39.61 U/ml observed on the third day. This highlights mannitol as a crucial carbon source for maximizing tyrosinase production (Valipour *et al.,* 2015; 2016) (Fig. 2A).

Nitrogen source. When bacteria were grown on different nitrogen source, maximum activity and production was observed with the beef extract, that is 43.05U/ml. The peptone 39.61 U/ml served as second best nitrogen source with enzyme activity and production of tyrosinase (Valipour *et al.,* 2015; 2016) (Fig. 2B).

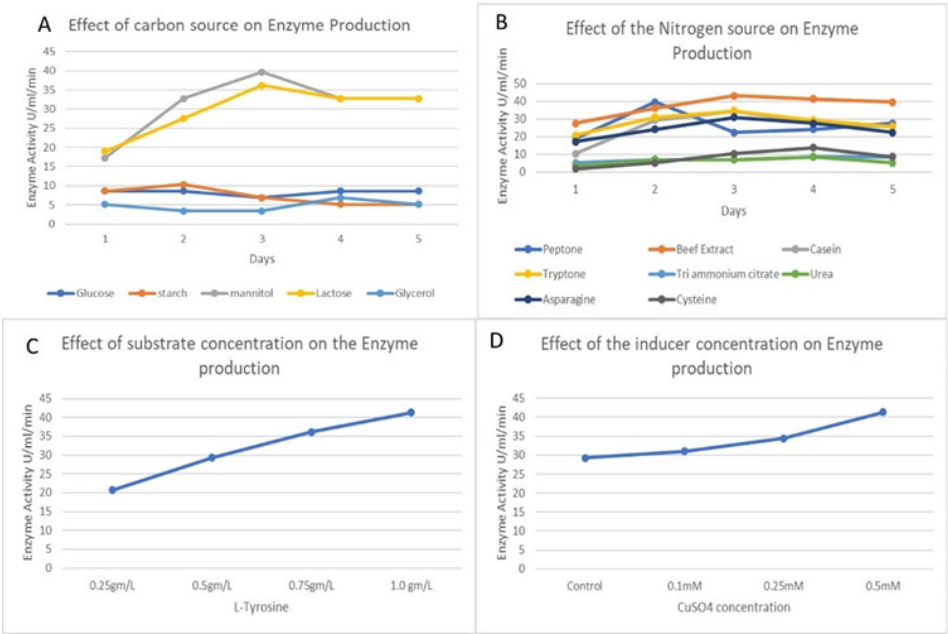

Fig. 2. Impact of the various media components on the enzyme production. A: Carbon source, B: Nitrogen source, C: Substrate Concentration, D: Concentration of the inducer. Enzyme production was determined by measuring the activity at 475 nm of the cell free extract.

Substrate concentration. L-Tyrosine serves as the substrate for the enzyme tyrosinase. When different concentrations of the L-tyrosine were supplemented in the medium and the enzyme activity was tested against it, the optimum concentration of L-tyrosine was 1gm/L.

The maximum activity at optimum concentration was 41.33 U/ml (Valipour *et al.,* 2015; 2016) (Fig. 2C).

Inducer. Tyrosinase enzyme belongs to the class oxidoreductase, it requires the Cu for the oxidation and reduction of its substrate. So $CuSO_4$ concentration also plays a vital role in the production of the enzyme. Organism showed the maximum production and activity at the concentration of the 0.5mM $CuSO_4$ with the activity 46.5 U/ml. Whereas the control had 29.4 U/ml activity (Valipour *et al.,* 2015; 2016) (Fig. 3D).

Temperature and pH. Each organism requires an optimum pH and temperature to synthesize the enzyme. Change in pH and temperature can affect the yield. The isolated strain showed the optimum growth and enzyme production at 35°C with the activity 40 U/ml and pH 7 with 39.234 U/ml activity (Valipour *et al.,* 2015; 2016) (Fig. 1B and C).

Plackett Burman design. When examining how process variables affect yield, the Plackett-Burman design is a crucial tool since it can drastically cut down on the number of repeated trials needed for a follow-up optimization study that use a response surface methodology. By carrying out the 12 trials (runs) that the model suggested, the impact of the factors on tyrosinase was ascertained (Table 2). The Plackett-Burman design was used to generate the regression equation, which predicted the response-affecting components.

Table 2: Plackett Burman design with the Response (Enzyme Activity).

Run	Mannitol (A)	Beef Extract (B)	$CuSO_4$ (C)	L-Tyrosine (D)	Enz. Activity (U/ml/min)
1	5gm/L	5gm/L	0.1mM	1gm/L	49.94444444
2	1gm/L	1gm/L	0.5mM	1gm/L	31
3	1gm/L	5gm/L	0.1mM	0.5gm/L	36.16666667
4	1gm/L	5gm/L	0.5mM	1gm/L	34.44444444
5	1gm/L	1gm/L	0.1mM	1gm/L	31
6	5gm/L	5gm/L	0.1mM	1gm/L	49.94444444
7	5gm/L	5gm/L	0.5mM	0.5gm/L	43.05555556
8	5gm/L	1gm/L	0.5mM	1gm/L	36.16666667
9	5gm/L	1gm/L	0.5mM	0.5gm/L	36.16666667
10	5gm/L	1gm/L	0.1mM	0.5gm/L	32.72222222
11	1gm/L	1gm/L	0.1mM	0.5gm/L	29.27777778
12	1gm/L	5gm/L	0.5mM	0.5gm/L	32.72222222

Table 3: Coded coefficients of Plackett Burman Analysis.

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		36.884	0.701	52.62	0.000	
Mannitol	8.898	4.449	0.701	6.35	0.000	1.00
beef Extract	8.324	4.162	0.701	5.94	0.001	1.00
$CusO_4$	-2.583	-1.292	0.701	-1.84	0.108	1.00
L-Tyrosine	3.731	1.866	0.701	2.66	0.032	1.00

From the Table 3 and 5, the coefficient of R^2, which was 0.92, was used to express the equation for the tyrosinase. The model equation for enzyme yield can therefore be expressed as follows once the less significant components (p higher than 0.05) have been eliminated:

Enzyme Activity U/ml = 36.884 + 4.449 Mannitol(A) + 4.162 beef Extract(B) - 1.292 $CusO_4$(C) + 1.866 L-Tyrosine (D). The design was significant in predicting the effects of the variables on strain PV24 's production of tyrosinase, as indicated by the coefficient R2 value of 0.92. The model terms were significant if the p-value was less than 0.05. By comparing the p values of the 4 variables, Mannitol (A), Beef extract (B) and L-tyrosine(D) were most significant parameters for the high level of tyrosinase production. Whereas the parameter C, $CuSO_4$ was insignificant in this instance (Table 3 and 4).

Table 4: Analysis of Variance.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	507.193	126.798	21.50	0.000
Linear	4	507.193	126.798	21.50	0.000
Mannitol	1	237.531	237.531	40.28	0.000
beef Extract	1	207.870	207.870	35.25	0.001
CusO4	1	20.021	20.021	3.40	0.108
L-Tyrosine	1	41.772	41.772	7.08	0.032
Error	7	41.277	5.897		
Lack-of-Fit	6	41.277	6.880	6.96556E+09	0.000
Pure Error	1	0.000	0.000		
Total	11	548.471			

Table 5: Model Summary.

S	R^2	R^2(adj)	R^2(pred)
2.42833	92.47%	88.17%	77.88%

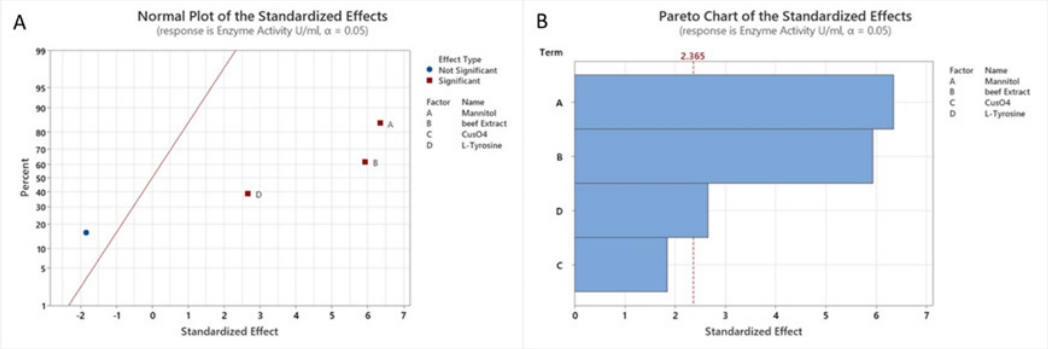

Fig. 3. Main Effect of variables on Enzyme production. A: Normal plot of the standardization effect. B: Pareto chart of the standardization effects.

The graph (Fig. 3) confirmed that mannitol, as a carbon source, significantly enhanced tyrosinase enzyme production by supporting bacterial growth. Beef extract was the most important nitrogen source for growth and enzyme production, while L-tyrosine, acting as a substrate, induced tyrosinase production. In contrast, CuSO4 concentration had an insignificant effect, as indicated by the Plackett-Burman design. Runs 1 and 6 showed the highest tyrosinase activity (49.94 U/ml), while the basal medium reached 43 U/ml.

DISCUSSION

Due to the vast array of applications of Tyrosinase enzyme, it is very important to find out the process of media optimization to get the maximum yield from an isolated strain of bacteria from Kapurwadi Lake, Ahmednagar region. There are many substrates which can enhance the enzyme production during laboratory processes. Here we have used and tried different factors which can give a maximum yield. Carbon source which is very important for the process, here we used 5 different carbon sources which show simple to complex media- as simple as D-glucose and as complex as starch. In the same way we used different nitrogen sources like beef extract, tryptone, casein, tri-ammonium citrate etc. Here we think that it can start production earlier and get maximum yield of enzyme.

Substrate concentration is also one of the crucial factors for the production of enzymes. Hence testing was done with different concentrations. In this case, 0.1 gm/L is the best concentration as above this concentration the process cannot be initiated. Optimization of $CuSO_4$ was also studied for different concentrations. Environmental parameters like temperature and pH also studied during study.

The Plackett Burman design was selected for the process optimization because it is the best, accurate and easy to perform method for the optimization process. The most important advantage of the design is that it reduces the efforts of multiple runs and decreases the complexity of the experiment. This method gives a regression equation for the optimization of the process. Hence it seems to be the most accurate method and hence it was adopted for the experiments.

Total 12 runs were done with 4 factors with their maximum and minimum concentrations. Time factor versus enzyme activity was tested which shown maximum activity on third day of inoculation of an isolated organism in the media. Most probably this is because the secondary metabolites and their production is maximum when the organism is in stationary phase.

Mannitol was found to be the best carbon source for enzyme production in this experiment, which gave the highest activity. Some reports suggested that the D-glucose served the best carbon source. (Valipour *et al.,* 2015). Beef extract emerged as the best nitrogen source as it is concentrated protein media. Even tryptone (Suravase *et al.,* 2012), Casein (Valipour *et al.,* 2015) was also reported to enhance the production of the tyrosinase. In case of substrate concentration, the maximum the concentration of L-tyrosine, the maximum was the enzyme activity, which is quite normal.

$CuSO_4$ was the inducer used in the experiment, which gives very less and late growth in enzyme activity. Results obtained from Plackett Burman formulation, $CuSO_4$ has negative impact on production of enzyme. Therefore, concentration of $CuSO_4$ is insignificant in the media optimization. (Suravase *et al*., 2012)

Environmental factors affect much on the production of enzyme. As we can see steep declines in the activity with the change in pH, means the production decreases in both acidic and alkaline condition. Also, it gave maximum yield at 35°C (Valipour *et al.,* 2015).

Plackett Burman method showed the process optimization with the maximum yield of enzyme activity in 49.94 U/ml in Run 1 and Run 6which has the carbon source- mannitol, nitrogen source- beef extract, substrate- L-tyrosine with 1 gm/L concentration and minimum concentration of inducer $CuSO_4$.

The highly significant factor was mannitol and nonsignificant factor was $CuSO_4$. The model equation gave the value of 46.07U/ml by elimination of non-significant factor. Run 1 and Run 6, gave maximum production of enzyme with maximum activity enzyme quantity of 49.94U/ml, which is 14.59% more than the predicted value 46.07U/ml.

ANOVA test was used to observe the significance within group. The mannitol was found to be the best factor with f value 40.28, $p< 0.0001$.Hence, this experiment gives the optimized process for the

production of tyrosinase enzyme (with Plackett Burman method) for given bacterial strain.

CONCLUSIONS

The optimization of process parameters for the newly identified bacterial strain PV24, known for its robust tyrosinase activity, revealed significant findings. Mannitol and beef extract emerged as critical components, leading to an impressive peak tyrosinase activity of 49.9 U/ml. These results highlight the potential of this isolated bacterium as a valuable and reliable industrial source of tyrosinase.

FUTURE SCOPE

Further studies regarding the improved media for the production of the production of the tyrosinase from bacterial strain PV 24 may involve the studies on the industrial scale production, gene manipulation to increase the enzyme production and on possibility on its application in the cosmatic, pharmaceutical and environmental fields which includes skin pigmentation treatments, biosensors and bioremediation of the phenol containing effluents.

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Conflict of Interest. None.

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