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Optimization of medium composition for increased production of tyrosinase enzyme in recombinant *Bacillus megaterium.*

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ABSTRACT

Tyrosinase is a copper-containing enzyme which catalyzes the conversion of L-tyrosine to L-DOPA and melanin. L-DOPA is a preferred drug for treatment of Parkinson's disease and melanin has many pharmaceutically and therapeutically uses. In this study a native *Bacillus megaterium*producing tyrosinase enzyme was isolated from soil sample and the effect of various amounts of L-tyrosine and trace element were studied and the optimum amount of them was opted. Enzyme production by the native *Bacillus megaterium* was analyzed at different inoculums ages and inoculumssize for the enzyme production was selected. Also the effect of oxygen was tested using different volume and rpm. By testing the enzyme activity for different inoculum ages at 48 hours intervals, the optimum incubation age was recorded. According to the findings of this article, 100was the optimum volume and 150 as optimum rpmwerekept as optimum for the enzyme production. The addition of tries element also effect on enzyme production, which addition (1.1mM) L-tyrosine to the production medium was highly effective on enzyme production. Consequently (0. 5ml) inoculum was optimum inoculum size, after optimization, 1070IU tyrosinase enzyme per milliliter of medium culture was obtained.

Keyword: inoculum size, L-tyrosine,inoculum age, Tyrosinase, optimization

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INTRODUCTION

Tyrosinases are the enzymes that are majorly involved in the formation of the naturally occurring pigment Melanin. Tyrosinases being involved in the melanin formation have inherent properties like absorption of UV radiation, metals, sound and also have anti-oxidant and semi-conductor properties (Krset al2002). Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of diphenols to *o*-quinones followed by aseries of nonenzymatic steps resulting in the formation of melanin (Oode et al 2016).

The enzyme has an important rolein biosynthesis and medical applications such as theproduction of L-DOPA, the preferred drug in thetreatment of Parkinson's disease (FairheadandThöny 2010), production of hydroxyl tyrosol as a food additive (Guptaet al 2002), production ofestrogenic compounds (Hernándezet al 2005), production of melanin fortherapeutic uses (Zhanget al 2007), treatment of neurological diseasesand production of antibiotic lincomycin(Essam et al 2012),cosmetic application as a self-tanning agent andthe production of dye (Raval et al 2012). Another use of the enzymeis in food manufacturing, such as the production of theaflavins, a major group of polyphenol compounds660 in black tea with strong antioxidant, anticancer andother bioactive properties (Haudecoeur et al2014).

Tyrosinase is a type 3 copper-containing enzymes thathas been found widely distributed in microorganisms, plants and animals (Surwase et al 2012). Tyrosinase plays an importantrole in wound healing and the primary immune response of plants, sponges and many invertebrates (Jhadav et al 2009). In fungi, this enzyme is of crucial importance in survival (Xuet al2012) and virulence, reproductive organ differentiation, spore formation and tissue protection after injury (Surwase et al2011). In bacteria, tyrosinase enzyme is the key enzyme in initiating the melanin biosynthesis pathway and plays an important protective and survival role.

Optimization of the culture conditions (nutritionaland physical parameters) for enzyme productionis of crucial importance. Production efficiencycan be increased and the production process can beeconomized by this approach. Classical optimizationwas carried out using a one-factor-at-a time method.The best production yield reported till date for tyrosinase was about 1g/l, which was produced by cultivating the *Trichoderma reesei*a filamentous fungus for 6days(Reni et al 201). Today, researchers tend to use static methods, suchas response surface methodology (RSM), for enzymeproduction, which provides important practical information(Shuster and Fishman 2009).

The objective of this study was to increasethe production of tyrosinase*using Bacillus megaterium*by optimize the best conditions for production.

MATERIALS AND METHODS

Isolation and Identification of tyrosinase Bacteria

Among 22 different bacterial strains isolated from soil, a potent strain which gave a high yield of tyrosinase was chosen for further study. The isolated strain was fully identified using morphological, biochemical and molecular biology technique as *Bacillus megaterium*. This strain was routinely grown on(NA) agar medium at 35 °C for 72 hours. After thisstage, the grown cells were collected in glycerolsolution (50%) and stored in Cryogen vial at -80°C asmaster/working cells bank.

Culture Conditions and Sample Preparation

Tyrosinase production was carried out in 250 ml flask contain 50 ml/flask of basal medium of Sharma et al.(2006) containing (g/l): casein,10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25; tyrosine, 10; (pH 6.5), then sterilized by autoclaving at 121°C and 1 atm Pressure. The inoculum was prepared by inoculating the flasks with a loop of the potent isolate and the flasks were then incubated on a rotary shaker at 200 rpm and 37°C.

For the cultivation processes, the flasks were inoculated with 5% (v/v) of the previously prepared vegetative cells. The effect of various inoculum ages (24-120 hr) was investigated. Various concentrations of Tyrosine (0.1-1.5 M)were addedto the basal medium as a supplemented in order to study their effect oftyrosineconcentrations on tyrosinaseproduction,which the controlwas carried out without tyrosine. By

addition of various trace element solutions at 0.2% w/v concentration contained ($FeSO_4$ $7H_2O$, $ZnSO_4$, Li, Ni_2O_3, $BaSO_4$, $CaCl_2$ $4H_2O$, Se, $PbCl_2$, NaCl, $CaCl_2$ $2H_2O$, and $CuSO_2$. $5H_2O$)was studies.

Concerning the experiment of testing oxygen influenceon growth and tyrosinase production, the broth medium was prepared in different volume and rpm in 250 mL Erlenmeyer flasks. *Bacillus megaterium* was inoculated (0.5-5) ml in tyrosine broth medium to study the effect of inoculum size.All the experiments were conducted in triplicate.At the end of each experiment, final pH, bacterial growth, total protein content, and tyrosinase activity were determined.The results are average of three independent experiments

Determination of cell dry weight

Cell dry weight was determined spectrophotometrically at 600 nm. During cultivation, samples of cell growth were taken periodically and the O.D of the bacterial growth was measured. Afterwards, the sample was filtered using Whatmann No.1 filter paper, twice washed, dried overnight at 65^{o}C, and then weighed to get the cell dry weight. A standard curve was drawn between the cell dry weight and the corresponding optical density, and was used to derive the linear cell dry weight equation (Renet al 2013).

Determination of total protein content

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.The enzyme produced is measured spectrophotometrically and the concentration was estimated on the standard graph.

Determination of tyrosinase activity

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1M sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA , and the formation of dopachrome is monitored by measuring the absorbance at 475 nm. Dopachrom “coloured intermediate” is an intermediate of melanin biosynthesis that is made from o-quinones by nonenzymatic oxidation. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 µmol of L-tyrosine to dopachrom per minute under the above conditions (Rao et al 2013).

RESULTS AND DISCUSSION

Optimization of cultivation conditions

The optimization of different cultivation conditions was achieved by cultivating the *Bacillus megaterium*in the basal cultivation medium, followed by evaluating tyrosinase production, final pH, bacterial growth and total protein content.

Effect of different inoculum ageson cell growth and tyrosinase production

Figure 1 shown that five different inoculum age of *Bacillus megaterium* (age of colony), while the best inoculum age for production of tyrosinase enzyme by *Bacillus megaterium* was 48 h. growing in fermentation medium (flask) capacity 250 ml^{-1}. Thisresult was agreementwithRenwho suggested that the best inoculum age for *Escherichia coli* was 48as a pre culture (Renet al 2013)

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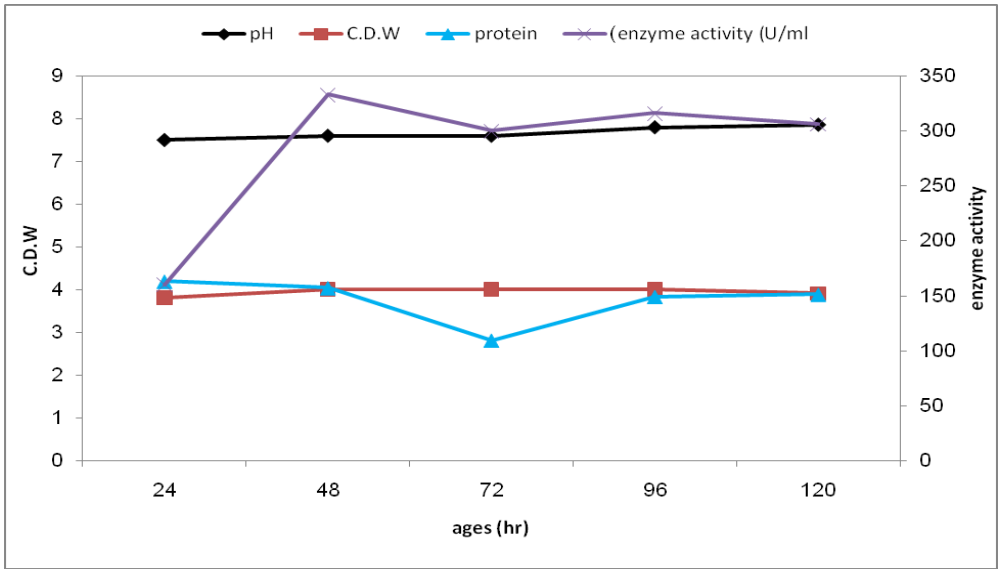

Figure (1) Effect of different inoculum ageson cell growth and tyrosinase production

Effect of different L-tyrosine concentration on cell growth and tyrosinase production

Due to the some previous reports, supplementation of culture medium by L-tyrosine enhances the production of tyrosinase enzyme.Figure 2 showed the effect of different concentrations from L-tyrosine were used. The maximum tyrosinase productivity was attained in the presence of L-tyrosine concentration of 1.1% (w/v). However, similar results were pointed at 0.4 mg/ml of l-tyrosine as optimum concentration for enzyme productionbyBacillus sp.,this result was in accordance with others findings (Surwase et al 2012).

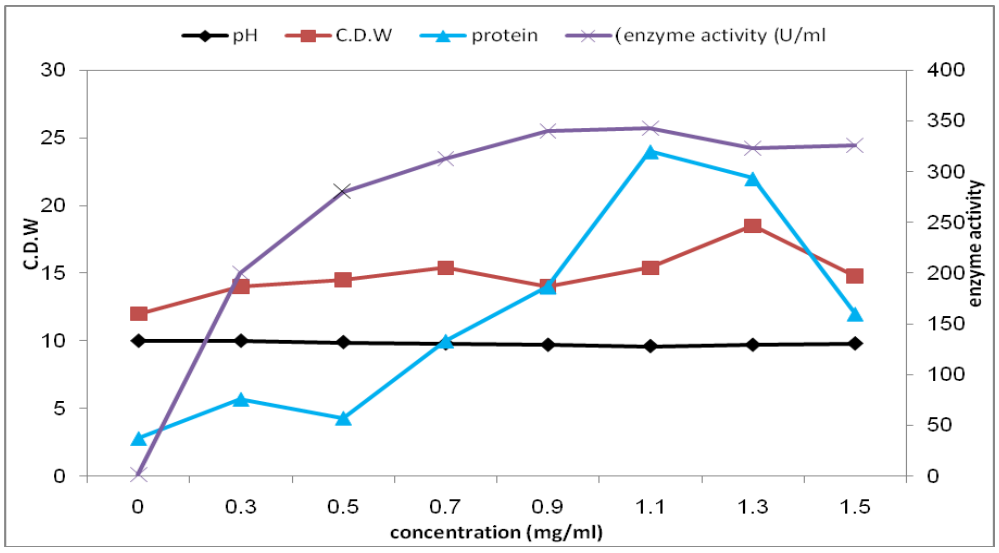

Figure (2)Effect of different L-tyrosine concentration on cell growth and tyrosinase production

Effect of trace element on cell growth and tyrosinase production

Supplemented medium with 0.1% trace element positively affected the enzyme production was shown in table (1).A stronger inhibitory effect was observed in the presence of Sodium Chloridewhich loss20%oftotal enzyme activity. However Esp´ın and Wichers(1999) reported thatSodium chloride and EDTA did not cause severeinhibition to any of the tyrosinase. Whilethe activity of tyrosinase increased in the presence of Copper Sulphate 3.5 fold. Ithas been reported that SDS participates in activation of sometyrosinase. The mechanism is likely related to conformationalchanges, which open the active site and permit substrate's access(Moore and Flurkey, 1990).

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Table (1) Effect of trace element on cell growth and tyrosinase production

elements	pH	enzyme activity(U/ml)	CDW (g/L)	Protei(mg/L)
Sodium Chloride	8.2	120	4.3	720
control	8.3	150	0.37	694
Lied hydroxide	8.3	163.3	4.09	609
Ferrous Sulphate	7.9	166	4.24	816
Zinc Sulphate	8.2	166	3.93	595
Calcium Chloride	8.2	196	3.83	576
Lead Chloride	8.3	200	4.57	691
Selenium	8.3	243	4.03	609
Barium Sulphate	8.3	253	4.28	604
Nickel Oxide	7.8	266	2.44	691
Cadmium Chloride	7.3	280	3.55	787
Copper Sulphate	8.3	426	4.77	792

Effect of different Volume of medium on cell growth and tyrosinase production

The data of Figure 3 shows the effect of different volumes of the basal medium on the production of tyrosinase by *Bacillus megaterium*, respectively. The volume of the fermentation medium varied from 25-150 of the total volume on 250 mL capacity Erlenmeyer flasks and production of tyrosinase 923U/ml was achieved at a basal volume of 100mland rpm 150. The data also shows that as the volume of fermentation medium was increased above 100 ml, the production of the enzyme started to decrease gradually. The growth of the organism and production of enzyme at lower volumes of basal medium (below 100ml) were also insignificant. The dissolved oxygen content was also effect of increasing volume of fermentation medium on the oxygen supply to the microorganism. It might be due to the improper agitation and inadequate aeration (oxygen supply) resulting from higher volumes. Similar kinds of findings have also been reported by Viitanen et al (2003) and Ekwealor&Obeta(2005) who have optimized 20 and 25 % volume of the medium for the production of lysine and single cell proteins by *Bacillus megaterium* and *E. coli* respectively.

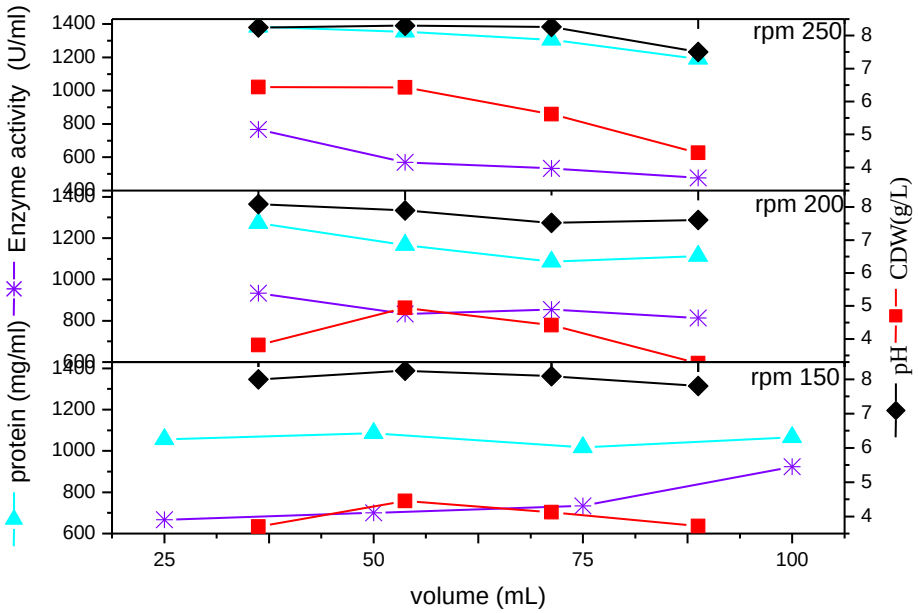

Figure (3): Effect of different volume on cell growth and tyrosinase production

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Effect of different inoculums size on cell growth and tyrosinase production

Size of inoculum is an important biological factor in the production of the enzyme. Maximum enzyme production (Figure 4) was obtained when medium was inoculated with 0.5 ml of inoculum. At lower inoculums levels, the yield was very low. The decrease seen with large inoculums size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture (Hesseltine et al., 1976) and reduced sugar and oxygen uptake rate and also enzyme release. From the survey of literature it can be seen that the 2% of inoculum size gave maximum production reported byRenet al 2013.

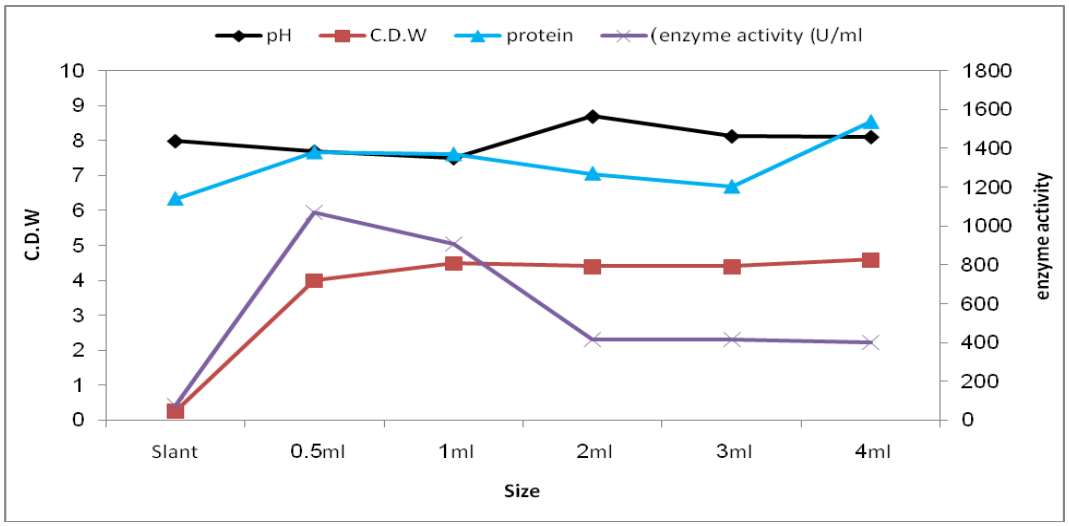

Figure (4) Effect of different inoculums sizeon cell growth and tyrosinase production

To our knowledge, there is poorly published information, about the relation of inoculum size, inoculum age and protease production from bacteria. So, this is considering the first published information in this relation.

Increase in inoculum size was seen to adversely affect the enzyme production. During fermentation medium are changes as a result of evaporation and metabolic activities(Baysol and Aytekin 2003).

CONCLUSION

The present study revealed that it was possible to determinethe optimum medium conditions for tyrosinase production. Studies on tyrosinase production were significantly affected by the interaction of *Bacillus megaterium* cells were grown in basal medium. The addition of trace elementtothe production medium was highly effective on enzyme production. Consequently, Changing of volume medium improved the growth and synthesis of tyrosinase. The optimum values of the tested variables were obtained using both inoculum ages 48 h, inoculum size 0.5%, and 1.1% of tyrosine concentration i.e. at these optimized conditions the model predicted 1070 U/ml of tyrosinase activity.

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REFERENCES

[1] Baysol Z., Uyar F. and Aytekin C. (2003) Solid state fermentation for production of alpha amylase by a thermotolerant*Bacillus subtilis* from hot spring water. Process Biochem 38: 1665-68.

[2] Ekwealor, I.A. and ObetaJ.A.N.(2005) Studies on lysine production by *Bacillus megaterium*. African J. Biotechnol., 4(7): 633-638.

[3] Esp´ın, J.C., Wichers, H.J., (1999) Activation of a latent mushroom (Agaricusbisporus) tyrosinase isoform by sodium dodecyl sulfate (SDS). Kinetic properties of the SDS-activated isoform. J. Agric. Food Chem. 47, 3518–3525,

[4] Essam F. A1 Juamily and Bushra H.(2012) Optimization conditions of production fibrinolytic enzyme from *Bacillus lichniformis* B4 local isolate. British Journal of Pharmacology and Toxicology. 3(6):289-95.

[5] Fairhead M., Thöny-Meyer L. (2010) Cross-linking and immobilisation of different proteins with recombinant Verrucomicrobium spinosum tyrosinase.Journal of biotechnology. 150(4):546-51.

[6] Gupta R., Beg Q. and Lorenz P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology. 59(1):15-32.

[7] HaudecoeurR.,Gouron A., Dubois C.,Jamet H.,Lightbody M.,Hardré R.,Milet A.,BergantinoE.,Bubacco L., BelleC.,et al. (2014) Investigation of binding-site homology between mushroom and bacterial tyrosinases by using aurones as effectors. Chem. Biol. Chem. 15, 1325–1333.

[8] Hernández-Romero D., Solano F. and Sanchez-Amat A. (2005) Polyphenol oxidase activity expression in *Ralstoniasolanacearum*.Applied and environmental microbiology. 71(11):6808-15.

[9] Hesseltine, C. W., Swain, E. W., and Wang, H. L. (1976) Production of fungal spores as inocula for oriental fermented foods. Dev IndMicrobiol 17 (55),101-115

[10] JhadavA.,VamsiK.K.,KhairnarY.,BorasteA.,GuptaN.,TrivediS.,PatilP.,Gupta, G., GuptaM.,MujaparaA.K.,JoshiB. and MishraD. (2009)Optimizationof production and partial purification of laccase by PhanerochaeteChrysosporium using sub merged fermentation. Int. J. Microbiol. Res.1(2),09–12.

[11] Krs S.R. andMahalaxmi Y. (2012) Laccase-and peroxidase-free tyrosinase production by isolated microbial strain.Journal of microbiology and biotechnology. 22(2):207-14.

[12] LowryO.H.,RosebroughN.J.,FarrA.L.,RandalR.J.(1951) Proteinmeasurement with theFolinphenolreagent.J.Biol.Chem.193,265–275.

[13] Moore B.M. andFlurkey W.H., (1990) Sodium dodecyl sulfate activity of plant polyphenoloxidase. J. Biol. Chem. 265, 482–488.

[14] Oode C., Shimada, W., Yokota M., Yamada Y. and Nihei K. (2016) Dihydroresveratrolcellobioside and xylobioside as effective melanogenesis activators. Carbohyd. Res. 436, 45–49

[15] Rani M.H.S., Ramesh T., Subramanian J., Kalaiselvam M. (2013) Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaeawerneckii*. International Journal of Pharma Research & Review. 2(8):9-17.

[16] Rao K., Tripathy N.K., Rao D.S., Prakasham R.S. (2013) Production, Characterization, Catalytic and Inhibitory activities of Tyrosinase. Res J Biotechnol. 8(1):83-95.

[17] Raval K.M., Vaswani P.S., Majumder D. (2012) Biotransformation of a single amino acid L tyrosine into a bioactive molecule L-DOPA. Int J Sci Res., 2:2250-3153.

[18] Ren Q, Henes B, Fairhead M and Thöny-Meyer L. (2013) High level production of tyrosinase in recombinant *Escherichia coli*. BMC Biotechnology, 13 (18):1-10

[19] Shraddha, Shekher R., Sehgal, S., Kamthania, M., Kumar, A. (2011) Laccase: microbial sources, production, purification, and potential biotechnological applications.Enzy Res., http://dx.doi.org/10.4061/2011/217861.

[20] Shuster V., Fishman A. (2009) Isolation, Cloning and Characterization of a Tyrosinase with Improved Activity in Organic Solvents from *Bacillus megaterium*. Journal of Molecular Microbiology and Biotechnology, 17(4):188-200.

[21] Surwase S.N. andJadhav J.P. (2011) Bioconversion of L-tyrosine to L-DOPA by a novel bacterium *Bacillus* sp. JPJ.Amino Acids. 41(2):495-506.

[22] Surwase S.N., Patil S.A., Jadhav S.B., Jadhav J.P. (2012) Optimization of l-DOPA production by Brevundimonas sp. SGJ using response surface methodology. Microbial biotechnology, 5(6):731-7.

[23] Viitanen, M.I., VasalaA., NeubauerP. and AlatossavaT. (2003) Cheese whey-induced high-celldensity production of recombinant proteins in *Escherichia coli*. Microbial Cell Factories, 2: 2-7.

[24] Xu DY, Chen JY, Yang Z. (2012) Use of cross-linked tyrosinase aggregates as catalyst for synthesis of L-DOPA. BiochemEng J., 63:88-94.

[25] Zhang J, Cai J, Deng Y, Chen Y, Ren G. (2007) Characterization of melanin produced by a wild-type strain of *Bacillus cereus*. Front Biol China, 2(1):26-9.