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PROPAGATION OF *CANDIDA UTILIS* FOR THE MICROBIAL TRANSFORMATION OF L-TYROSINE TO L-DOPA

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ABSTRACT

L-dopa is an amino acid derivative used for the cure of Parkinson's disease, a degenerative disoreder of nervous system. The present study is concerned with the propagation of *C. utilis* NRRL-Y-1084 for L-dopa production from L-tyrosine as a basal substrate. Yeast cells were cultivated under stationary culture for the biochemical transformation of L-tyrosine to L-dopa. As tyrosinase (Catechol oxidase, *EC 1.10.3.1*) is an intracellular enzyme, pre-grown yeast cells were used as an enzyme source in the reaction mixture. The effect of different cultivation conditions and nutritional requirements such as initial pH, time of incubation, carbon, nitrogen and phosphate sources on the propagation of *C. utilis* was investigated. Optimal production was observed when cells were cultivated at pH 5.0 for 72 h. with the addition of cellobiose at a level of 2.5%, peptone at 1.0% and KH_2PO_4 at 0.2%. A 16 h old inoculum was added at a level of 10% (v/v). During the course of study, the maximum L-dopa production of 1.624 mg/ml was accomplished in the reaction broth with a cumulative tyrosinase activity of 174 U/mg of yeast cells. L-tyrosine consumption was found to be 1.965 mg/ml. All the biochemical reactions were performed aerobically at 50ºC for 1 h on a hotplate with magnetic stirrers.

Key words: Microbial Transformation, Candida Fungi, Inoculum, L-tyrosine, L-dopa

INTRODUCTION

A drug related compound 3, 4-dihydroxy L-phenylalanine (L-dopa,) is a derivative of amino acid, L-tyrosine (Alsina *et al*., 1988; Mercuri *et al*., 1991). It naturally occurs in the human body. In the management of dopa-responsive dystonia and Parkinson's disease, L-dopa is used in clinics (Napolitano *et al.*, 2006). It is converted to dopamine in the brain. It is also found in seedlings, pods and beans of V*icia faba* and in the seeds of *Mucana pruriens* (Buttner *et a l.*, 2005).

Tyrosinase (Catechol oxidase) is a copper containing enzyme. The two copper atoms within the active site of tyrosinase enzymes interact with dioxygen to form a highly reactive chemical intermediate that then oxidizes the substrate (Donald and Gutteridge, 2009). It is also a key enzyme in melanin biosynthesis, involved in determining the colour of mammalian skin and hair. Albinism is a result of mutation in tyrosinase (Sugumaran 1996).

The enzyme tyrosinases have been isolated and studied from a wide variety of plant, animal and fungal sources. *Aspergillus oryzae* has been known as the organism of choice for L-dopa production; however, *Candida utilis* is gaining interest as it is a human friendly yeast species and a normal constituent of the human flora (Chihara *et al*., 1986; Ali and Haq, 2007). L-dopa is produced from L-tyrosine by one-step oxidation reaction by submerged cultivation (Haneda *et al*., 1973; Raju *et al*., 1993).

The present study deals with the propagation of *Candida utilis* NRRL-Y-1084 for the microbial transformation of L-tyrosine to L-dopa. Cultural condition such as initial pH and cultivation period were optimized. Carbon, nitrogen and phosphate sources were also evaluated to determine the nutritional requirements of the culture for optimal growth so that maximum amount of L-dopa could be produced in the reaction mixture.

MATERIALS AND METHODS

The chemicals were of analytical grade and obtained directly from Sigma (USA), BDH (UK), E-Merck (Germany), Acros (Belgium) and Fluka (Switzerland).

Organism and culture maintenance

Candida utilis strain NRRL-Y-1084 was obtained from the available stock culture of the *Institute of Industrial Biotechnology (IIB), GC University Lahore* and was used in the present study. The culture was maintained on GPYE-agar medium containing (g/l): glucose 10, peptone 5, yeast extract 3, agar 20, and pH 5.0.

Reaction procedure and critical phases

The biochemical reaction for L-dopa production from L-tyrosine was carried out in a suspension of intact yeast cells following the method of Haneda *et al.* (1973). The reaction mixture contained (mg/ml): L-tyrosine 2.5, L-

ascorbic acid 5.0, intact dry yeast cells 0.12. It was prepared in acetate buffer (pH 3.5, 50 mM). The reactions were carried out aerobically for 60 min on a hotplate with magnetic stirrers at 50ºC. At the termination of reaction, the sample was withdrawn and centrifuged at 6500×*g* for 20 min (–5ºC). The clear supernatant was kept under dark in an ultra freezer (–20ºC). The data shown in all the figures was maintained under cultivation conditions (Temperature 30ºC, initial pH 5.0), reaction conditions (Temperature 50ºC, pH 3.5, incubation time 1 h), Y-bars show the standard deviation (±sd) among the three parallel replicates, each mean value differ significantly at a level of $p \leq 0.05$.

Propagation of yeast at various cultural and nutritional conditions

Effect of initial pH

The effect of initial pH on the propagation of *C. utilis* NRRL-Y-1084 was carried out for the microbial biotransformation of L-tyrosine to L-dopa. The pH was varied from 4.0 to 6.5 using 500 ml Erlenmeyer flasks under stationary culture conditions.

Time of cultivation period

Time of incubation not only determine the length of exponential phase of yeast growth but also establishes the efficacy of metabolite production (Raju *et al*., 1993). Therefore, the effect of cultivation period on the propagation of *C. utilis* NRRL-Y-1084 for the microbial biotransformation of L-tyrosine to L-dopa was also investigated. Yeast cells were cultured under stationary conditions from 12 to 96 h after inoculation at pH 5.0.

Evaluation of different carbon sources

In the present study, different carbon sources were evaluated for the optimal propagation of *C. utilis* NRRL-Y-1084 for L-dopa production from L-tyrosine. Glucose, maltose, xylose, cellobiose, sucrose and fructose were used as sole carbon source and added individually at a level of 0.5 to 3% (v/v). In all the subsequent studies, the microbial cultivations were carried out at pH 5.0 for 72 h.

Evaluation of different nitrogen sources

Different nitrogen sources were tested for the propagation of *C. utilis* NRRL-Y-1084 for L-dopa production from L-tyrosine. The sole nitrogen sources other than the control (peptone partially replaced by yeast extract and added at a ratio of 5:3) included peptone, yeast extract, tween 80, urea and ammonium nitrite/or nitrate. Their concentrations in the cultivation medium were varied from 0.2 to 1.2% (v/v) for each trial.

Evaluation of different phosphate sources

Different phosphate sources were also evaluated for the propagation of *C. utilis* NRRL-Y-1084 for L-dopa production and L-tyrosine consumption. KH_2PO_4, K_2HPO_4 and Na_2HPO_4 were used as sole nitrogen source, added at a level of 0.05 to 0.3% (v/v) for each trial.

Effect of size and age of inoculum

The effect of size of inoculum on L-dopa production from L-tyrosine by using *C. utilis* NRRL-Y-1084 was investigated under stationary culture conditions. An inoculum level of 2 to 12% (v/v) was used to seed the liquid cultivation media. Yeast cells were grown from 18 to 28 h and their effect on the efficacy of biochemical transformation was studied.

Statistical analysis

Treatment effects were compared by the protected least significant difference method (Spss-10-6, version-4.0, USA) after Snedecor and Cochran (1980). Significant difference among the replicates has been presented as s Duncan´s multiple range in the form of probability (p) value.

RESULTS AND DISCUSSION

Effect of initial pH

The effect of initial pH on the propagation of *C. utilis* NRRL-Y-1084 for the microbial biotransformation of L-tyrosine to L-dopa was carried out. Initially the pH was varied from 4.0 to 6.5 using 500 ml Erlenmeyer flasks under stationary culture conditions. The results are given in Fig 1. At pH 4.0, L-dopa production of 0.216 mg/ml with an L-tyrosine consumption of 0.185 mg/ml was observed in the reaction mixture. The maximal L-dopa production (0.415 mg/ml) was obtained at pH 5.0 with L-tyrosine consumption of 0.342 mg/ml. It was probably due to the best

growth of yeast as all the metabolic pathways were operating normally at this pH (Kandaswami and Vaidyanathan, 1973). The present work is substantiated with the findings of Sih *et al*. (1969) and Haneda *et al*. (1973) who obtained maximal production of L-dopa (0.84 mg/ml) from filamentous fungi, when pH of the cultivation medium was adjusted to 5.0. L-Dopa production declined gradually when pH was further increased beyond the optimal, becoming very low (0.294 mg/ml) at pH 6.5 which was not encouraging ($p \leq 0.05$). However, substrate consumption continued to rise regardless of the decrease in L-dopa formation at higher pH values. It might be due to the insufficient yeast growth and disturbed microbial physiology. Therefore, the best results in term of L-dopa production from L-tyrosine were achieved at pH 5.0 and it was selected for further studies.

Effect of cultivation period

Time of incubation not only determine the length of exponential phase of yeast growth but also establishes the efficacy of metabolite production (Raju *et al*., 1993). In Fig. 2 is depicted the effect of cultivation period on the propagation of *C. utilis* NRRL-Y-1084 for the microbial biotransformation of L-tyrosine to L-dopa. Yeast cells were cultured under stationary culture conditions from 12 to 96 h after inoculation. L-dopa production of 0.072 mg/ml was obtained when 12 h old cells were used as an enzyme source in the reaction mixture. L-tyrosine consumption was noted to be 0.102 mg/ml. When cultivation period was prolonged from 24-60 h, a gradual rise in L-dopa production was observed. However, maximal L-dopa production of 0.532 mg/ml with L-tyrosine consumption of 0.412 mg/ml was accomplished 72 h after incubation. It was possibly due to the increased tyrosinase activity of *C. utilis* cells (150.28 U/mg), as reported by Carvalho *et al*. (2000). Although substrate consumption was markedly rose between 84-96 h, yet L-dopa production fell gradually (0.51-0.468 mg/ml). It might be due to the conversion of L-dopa into other metabolites such as dopamine or dopacrome when cells with late exponential or initial stationary phase were used as a source of enzyme tyrosinase in the reaction mixture So, an incubation period of 72 h was optimized for maximal L-dopa production in the subsequent parameters.

Evaluation of different carbon sources

Carbon is not only the basic element of cells but is also required by the living organisms for the maintenance of their metabolic activities (Conn *et al*., 1987). Different carbon sources were evaluated for the optimal propagation of *C. utilis* NRRL-Y-1084 for L-dopa production from L-tyrosine as a substrate. The sole carbon sources included glucose, maltose, cellobiose, xylose, sucrose and fructose. Their concentration in the cultivation medium was varied from 0.5 to 3% (v/v) for each trial. Among them, maltose, xylose and fructose utilized a minimum quantity of the basal substrate which in turn did not support a better L-dopa production. Cellobiose was found to be the best carbon source. The maximum L-dopa production (0.928 mg/ml) was obtained at a level of 2.5% (v/v). L-tyrosine consumption was recorded to be 1.164 mg/ml. Other carbon sources including glucose and sucrose gave an intermediate level of L-dopa production in the reaction mixture which was competitive with each other. The substrate consumption was also fairly high. Hence, cellobiose at a level of 2.5% (v/v) was found to give the optimal growth of yeast cells and subsequent production formation (Fig. 3a,b).

Evaluation of different nitrogen sources

The nitrogen sources as well as their concentration in the cultivation medium greatly affect the biosynthesis of L-dopa from L-tyrosine using *C. utilis* as an enzyme source (Ho *et al*., 2003). Fig. 4a,b are highlighted the results of evaluation of different nitrogen sources for the propagation of *C. utilis* NRRL-Y-1084 for L-dopa production from L-tyrosine as a substrate. The sole nitrogen sources other than the control included peptone, yeast extract, tween 80, urea and ammonium nitrite. Their concentration in the cultivation medium was varied from 0.2 to 1.2% (w/v) for each trial. Among added nitrogen sources, peptone was found to be the best nitrogen source. The maximum L-dopa production (1.312 mg/ml) was obtained at a level of 1.0% (v/v). Hence, yeast extract was completely replaced by peptone in the cultivation medium. L-tyrosine consumption was recorded to be 1.528 mg/ml. Tween 80 gave an intermediate level of L-dopa production in the reaction mixture which was competitive with the control. The growth of yeast cells reduced with a much longer lag phase resulting in the decreased level of tyrosinase activity in the reaction mixture (85.25 U/mg). The study is substantiated with the findings of Mencher and Hein (1962); Scribhers *et al*. (1973) and Dastager *et al*. (2006). Therefore, peptone added at a level of 1.0% (w/v) was found to give the optimal growth of yeast cells and subsequent production formation.

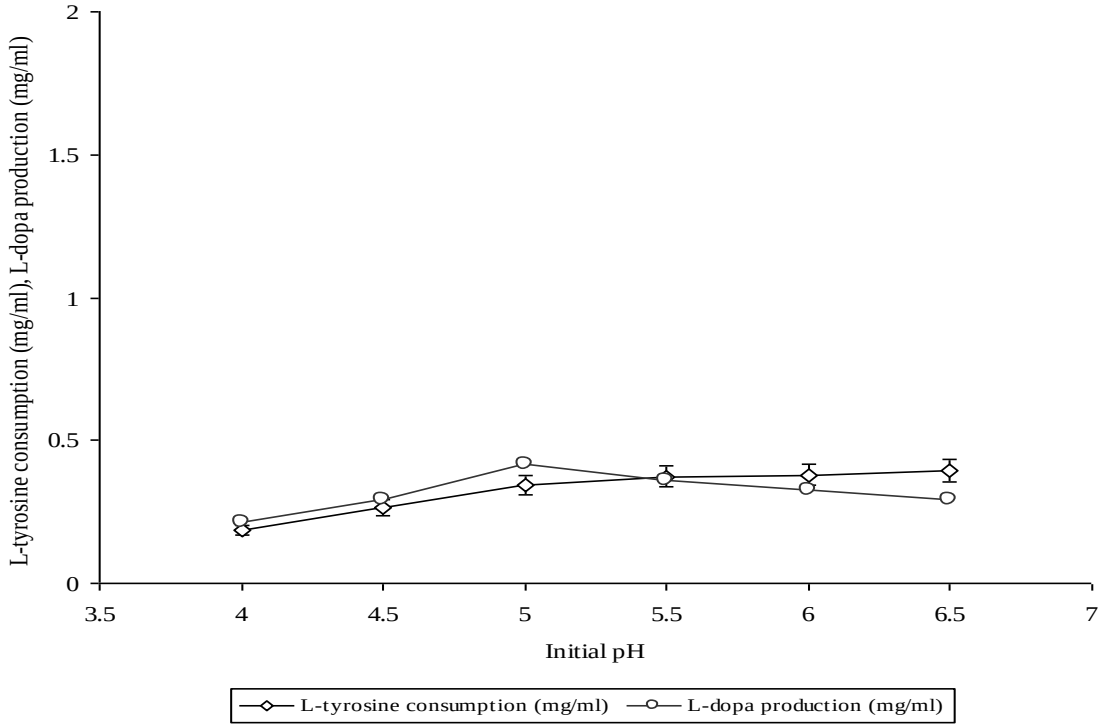

Fig. 1. Effect of initial pH on the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for the microbial biotransformation of L-tyrosine to L-dopa.

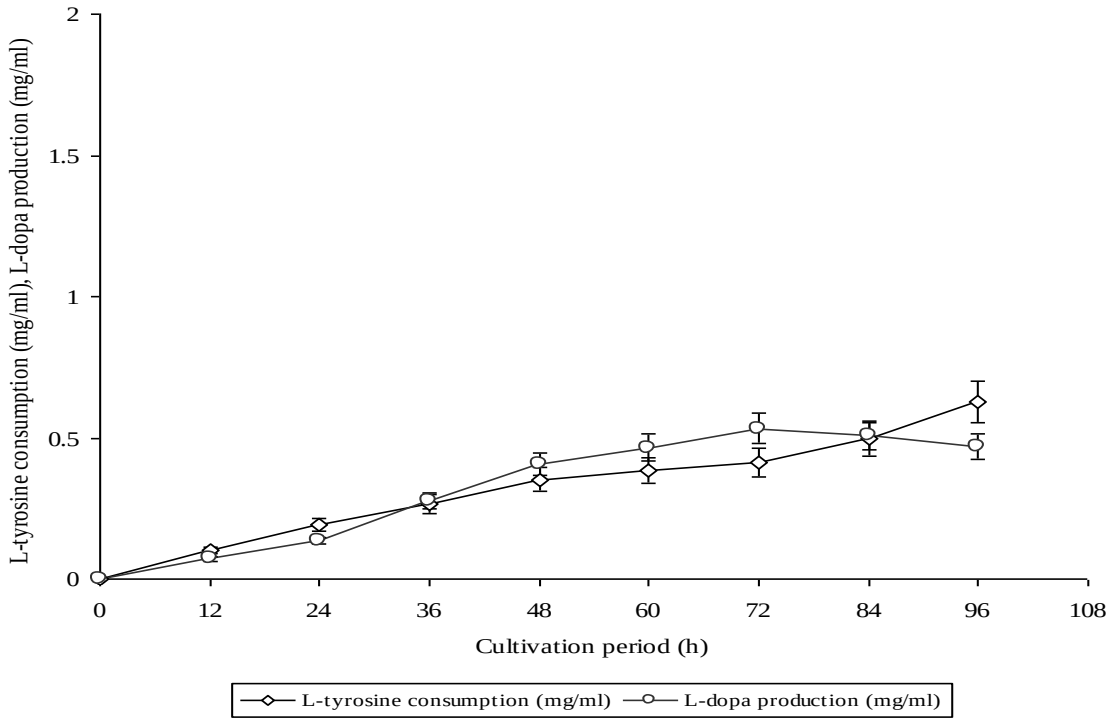

Fig. 2. Effect of cultivation period on the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for the microbial biotransformation of L-tyrosine to L-dopa.

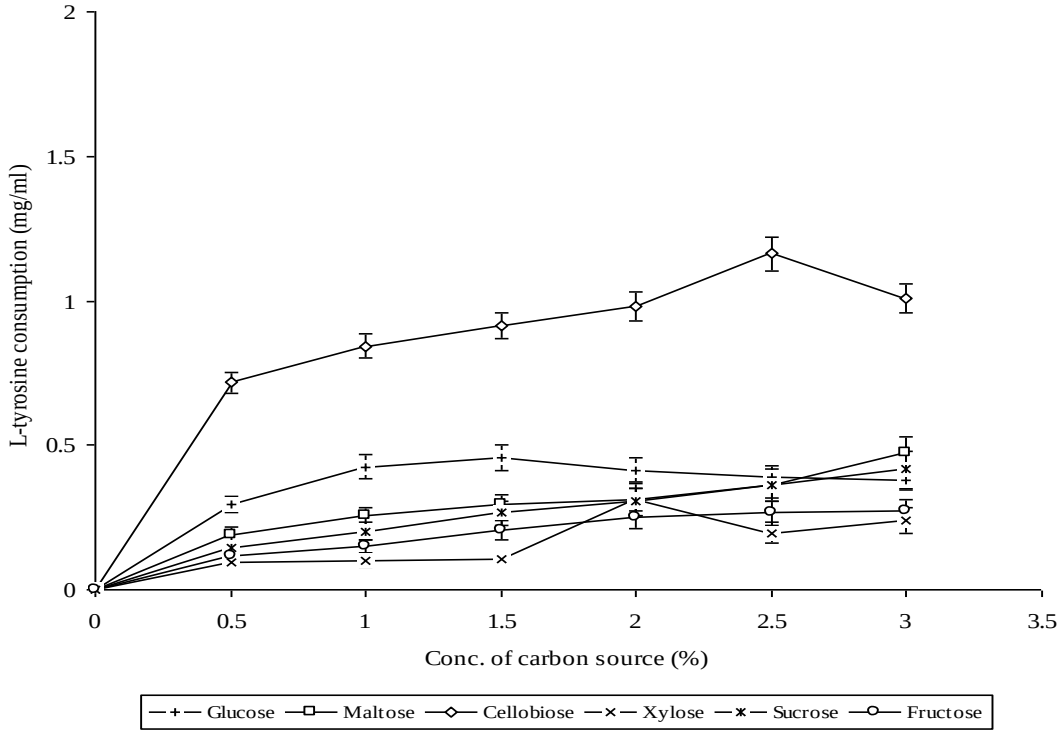

Fig. 3a. Evaluation of different carbon sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-tyrosine consumption.

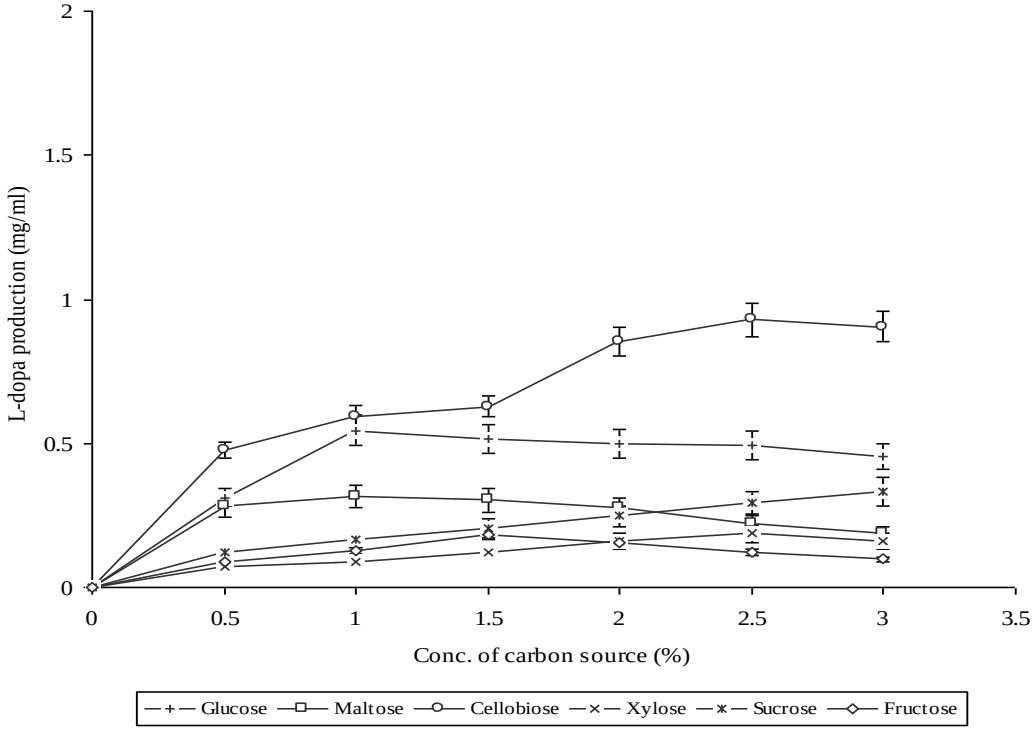

Fig. 3b. Evaluation of different carbon sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-dopa production.

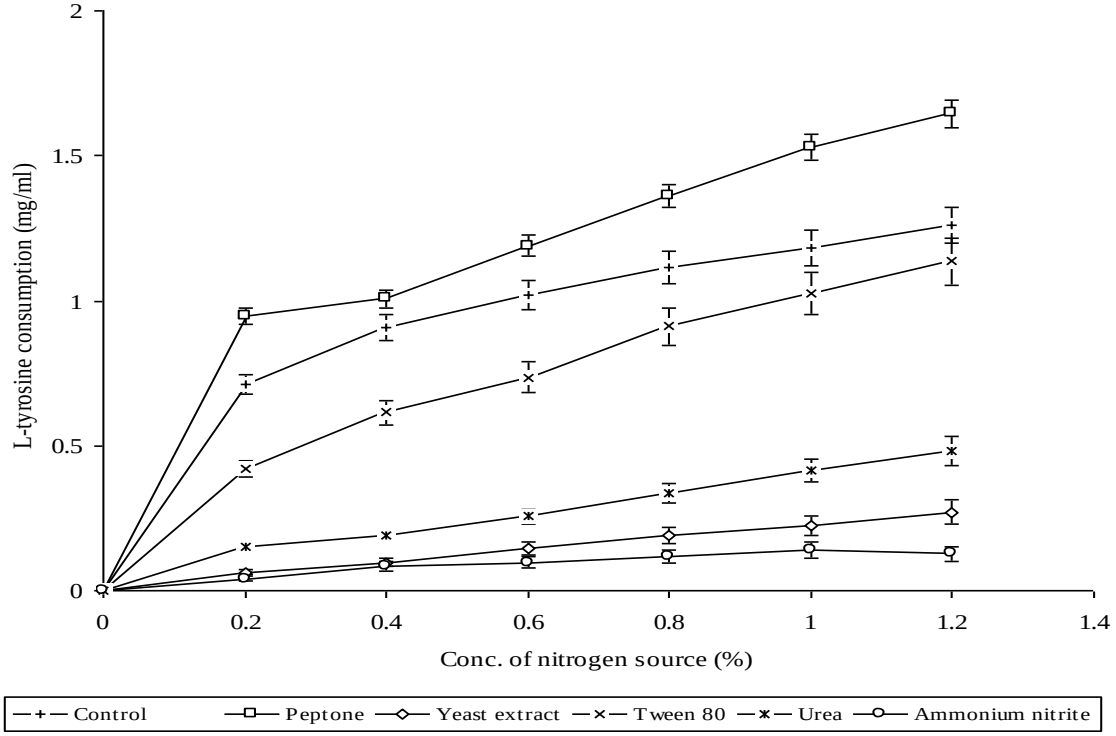

Fig. 4a. Evaluation of different nitrogen sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-tyrosine consumption.

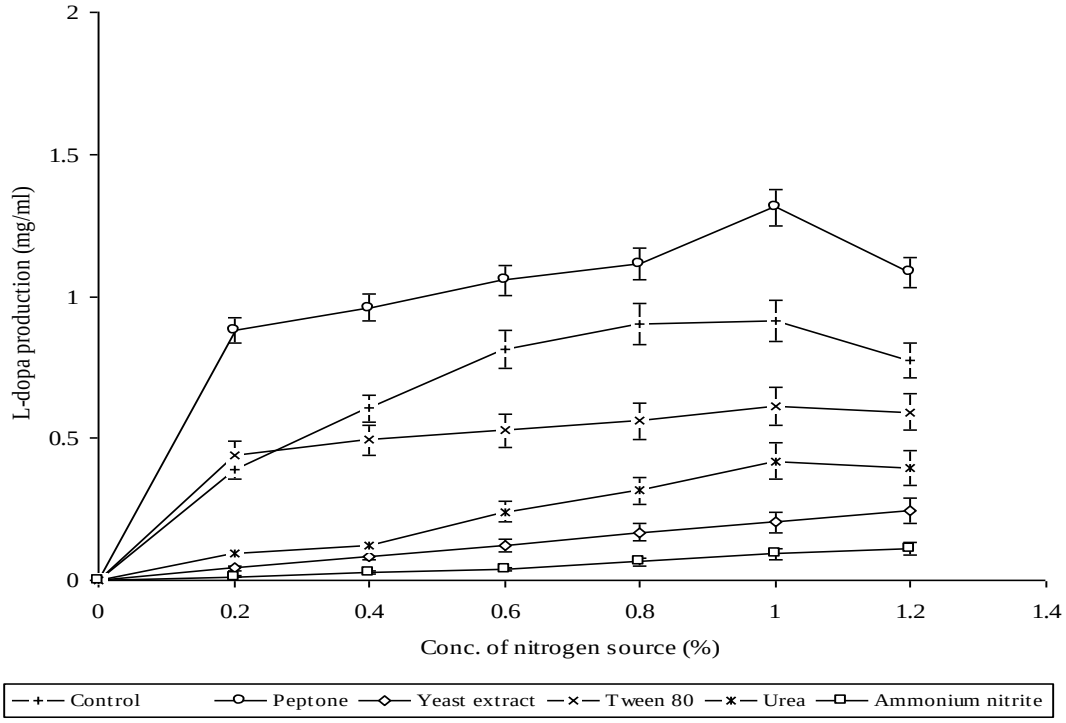

Fig. 4b. Evaluation of different nitrogen sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-dopa production.

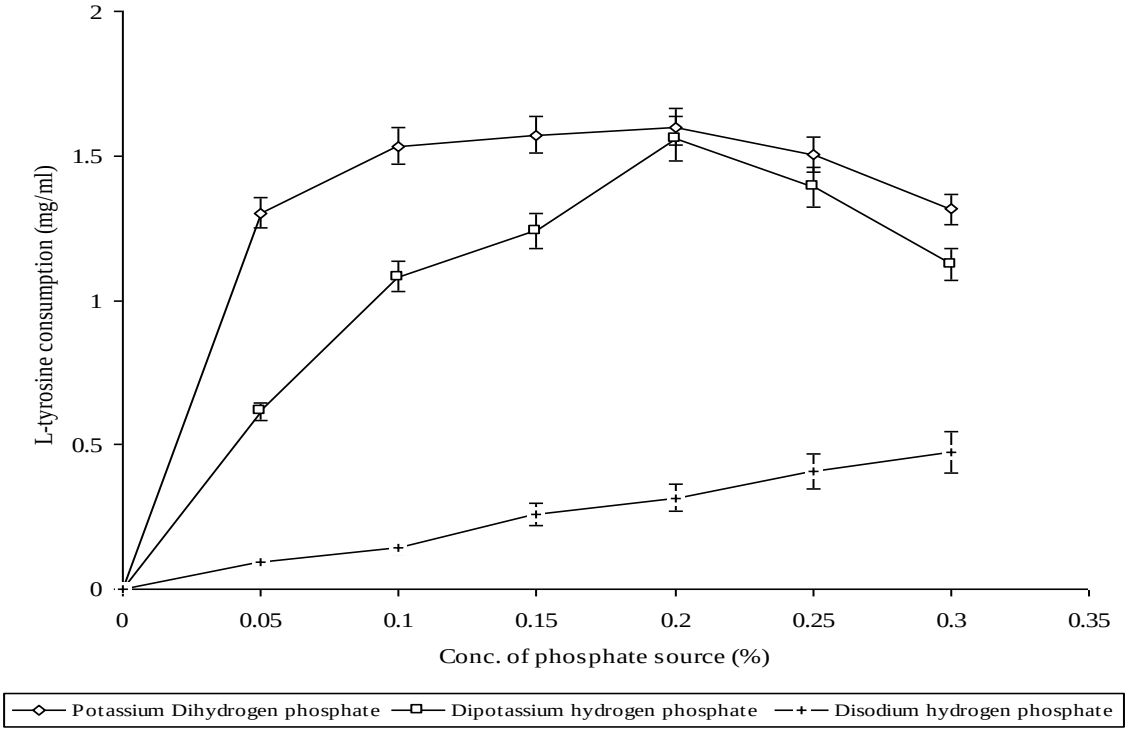

Fig. 5a. Evaluation of phosphate sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-tyrosine consumption.

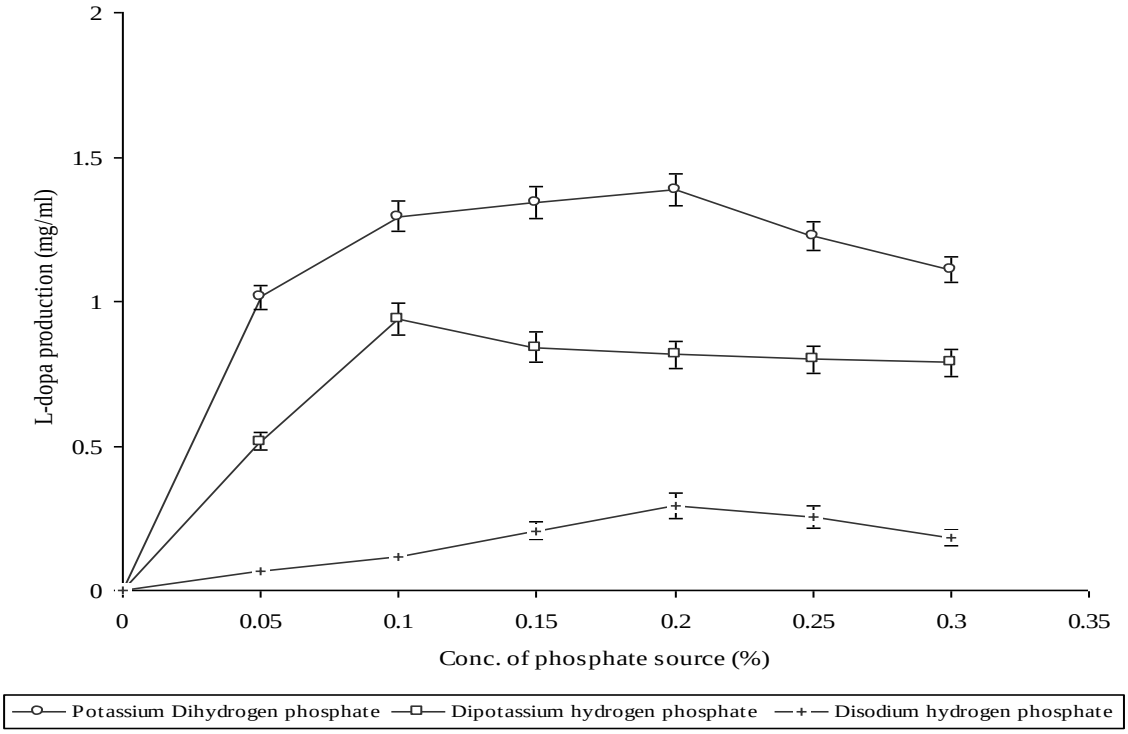

Fig. 5b. Evaluation of phosphate sources for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-dopa production.

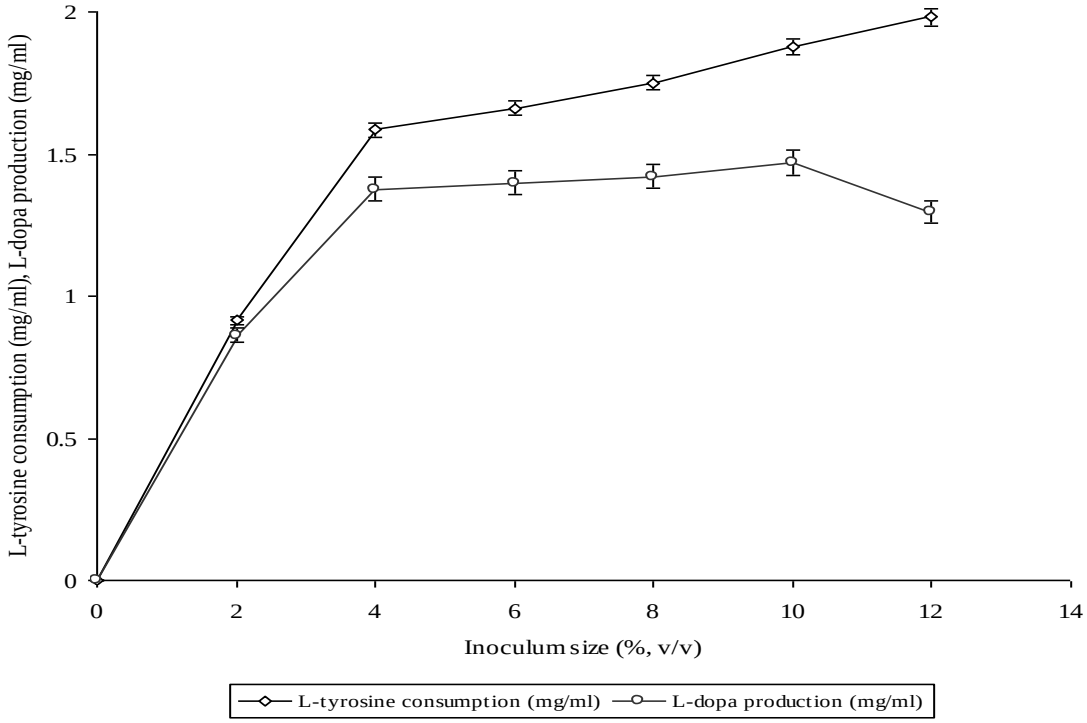

Fig. 6a. Effect of size of inoculum on for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-tyrosine consumption and L-dopa production.

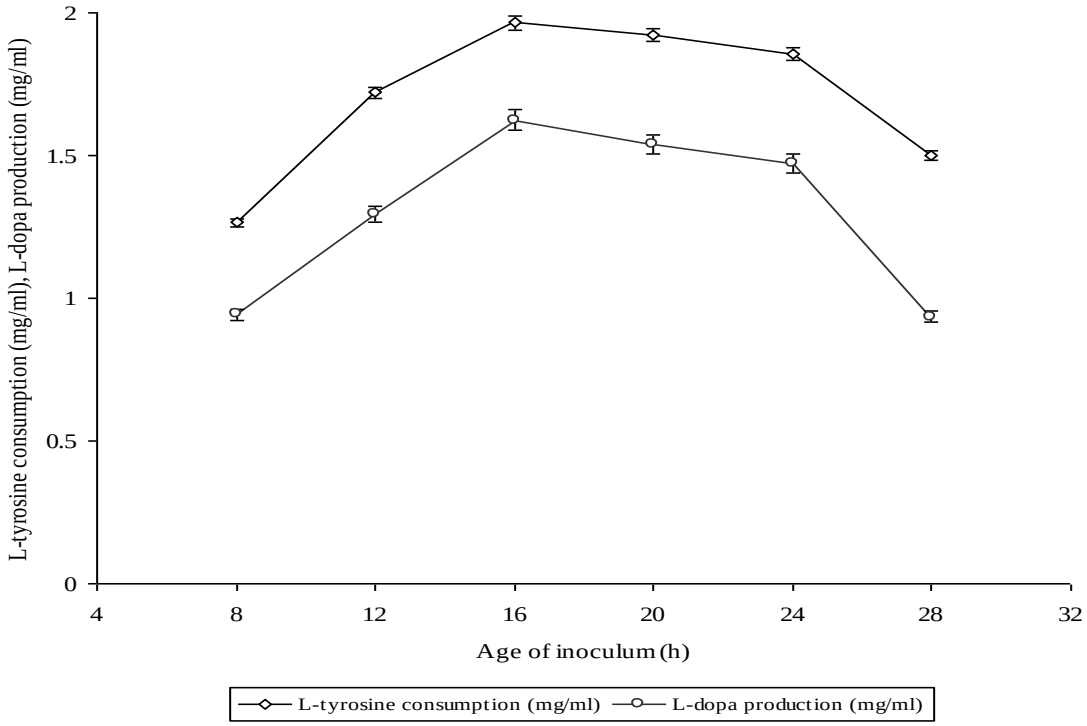

Fig. 6b. Effect of age of inoculum on for the propagation of *C. utilis* NRRL-Y-1084 under stationary culture for L-tyrosine consumption and L-dopa production.

Evaluation of different phosphate sources

Different phosphate sources were also evaluated for the propagation of *C. utilis* NRRL-Y-1084 for L-dopa production and L-tyrosine consumption. The sole phosphate sources KH_2PO_4, K_2HPO_4 and Na_2HPO_4 were used under stationary culture conditions. Their concentration in the cultivation medium was varied from 0.05 to 0.3% (v/v) for each trial. The results are shown in Fig. 5a,b. K_2HPO_4 and Na_2HPO_4 supported lower L-dopa production at all the concentrations tested. The consumption of L-tyrosine was also not encouraging ($p \leq 0.05$). However, the maximum L-dopa production was observed when 0.2% (w/v) KH_2PO_4 was added as a sole phosphate source in the cultivation medium. The maximum L-dopa production was 1.386 mg/ml with the consumption of 1.6 mg/ml L-tyrosine as a basal substrate. So, KH_2PO_4 at a level of 0.2% (v/v) was found to be the best for L-dopa production from L-tyrosine in the reaction mixture.

Effect of size and age of inoculum

The effect of size of inoculum on L-dopa production from L-tyrosine by using *C. utilis* NRRL-Y-1084 was investigated under stationary culture conditions. An inoculum level of 2 to 12% (v/v) was used to seed the liquid fermentation media. The results are shown in Fig 6a. Maximal L-dopa production (1.468 mg/ml) and L-tyrosine consumption (1.876 mg/ml) was obtained when the inoculum size was 10% based on the working volume of solid substrate. Further increase in inoculum size from 10 to 12% decreased the production of L-dopa. It might be due to the overgrowth of yeast cells in the reaction broth (Soto *et al*., 2006; Donald and Gutteridge, 2009). So, an inoculum size of 10% was optimized for L-dopa production. In Fig 6b showed the effect of age of inoculum on L-dopa production from L-tyrosine. Yeast cells were grown from 18 to 28 h. Maximal L-dopa production (1.624 mg/ml) was obtained at 16 h. Further increase in inoculum age gradually decreased both the L-tyrosine consumption and L-dopa production. At 28 h old inoculum, L-dopa production was not found encouraging (0.936 mg/ml) and remained significantly below ($p \leq 0.05$) than the average. It was possibly due to the fact that the activity of enzyme was decreased due to catabolic repression of enzyme. The finding of current study is similar as reported by Conn *et al*. (1987) and Odin *et al*. (2008). Therefore, 16 h old inoculum added at a level of 10% was optimized for L-dopa production from L-tyrosine.

Conclusions

In the present study, a novel strain of *Candida utilis* NRRL-Y-1084 was used for the propagation of yeast cells for L-dopa production from L-tyrosine as a substrate. The organism was capable of releasing enzyme tyrosinase aerobically in an acidic reaction mixture and converting tyrosine derivatives into L-dopa with other corresponding products. The strain was grown in a medium containing carbon, nitrogen and phosphate sources with the essential nutrients. To obtain optimal yield of L-dopa, it is imperative to add L-ascorbic acid to the reaction broth to prevent melanin formation. Since tyrosinase appeared to be as inducible enzyme, its activity should be further enhanced prior to the optimizations of biochemical reaction, for the maximum transformation of substrate to a stable product.

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