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Direct Production of an Extracellular Tyrosinase from *Rhizopus oryzae* NRRL-1510 by Solid Substrate Fermentation

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ABSTRACT

Tyrosinase (EC 1.14.18.1) belongs to copper containing metalloproteins essential in melanogenesis pathway. This enzyme finds biotechnological applications in L-dopa production, wound healing in higher organisms, detection and removal of phenolic compounds from waste water. In the present study, we report on the novel production of an extracellular tyrosinase from *Rhizopus oryzae* NRRL-1510 by solid substrate fermentation (SSF). A range of different agricultural by-products including sunflower meal (SFM), soybean meal (SBM), almond meal, canola meal, mustard seed cake and rape seed meal collected from various local markets were evaluated as basal substrate under SSF. Initial moisture content was determined using different diluents including distilled water (pH 7), sodium citrate buffer (pH 8.1), phosphate buffer (pH 7.2) and acetate buffer (pH 3.5). Sodium citrate buffer (100 ml) was used to extract enzyme from the fermented mash culture. A noticeable enhancement in enzyme activity (64.55 U/mg) was observed when the significant process parameters viz. SFM level (15 g), volume of distilled water (20 ml), time of incubation (72 h), temperature (30ºC) and level of sodium citrate buffer at pH 8.1 (100 ml) were determined after Plackett-Burman design. Thin layer chromatography (TLC) of tyrosinase catalysis-products confirmed the presence of an active tyrosinase in the reaction mixture. The value of tyrosinase correlation (0.135*E*+0025) depicted that the model terms are highly significant (*HS*, $p \leq 0.05$) indicating commercial viability of the fungal culture (df = 3, LSD = 0.0385).

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Authors' Contribution
SA conceived and designed the project, analyzed the data and wrote the article. AT executed the experimental work.

Key words
***Rhizopus oryzae*, tyrosinase, batch-culturing, solid state fermentation, extracellular enzyme, 2-factorial design.**

INTRODUCTION

Tyrosinase (EC 1.14.18.1) belongs to a subclass of enzymes known as tyrosinases of class oxidases which catalyze the *o*-hydroxylation of monophenols to *o*-diphenols and subsequently to *o*-quinones (Davis and Blevins, 1979; Mayer, 1987; Matoba and Kumagi, 2006). In the reaction, copper ions react with molecular O_2 to form intermediates which act on monophenols and diphenols. Microbial tyrosinases have been produced more commonly from *Pseudomonas*, *Bacillus*, *Myrothecium*, *Mucor*, *Miriococcum*, *Apergillus*, *Chaetotomastia*, *Ascovaginspora*, *Trametes*, *Pyenoporus*, *Trichoderma* and *Streptomyces* spp. (Wichers *et al*., 1996). There has been a growing interest in exploiting *Neurospora* and *Agaricus* spp. because they can produce high-levels of tyrosinase; however enzyme characterization remained a problem. The production of tyrosinases from *Rhizopus* spp. might have a tremendous scope in fermentation based industry. It is an obligate aerobic filamentous fungus. It has a faster growth rate with fluffy hyphal pattern. Tyrosinases play vital roles to develop organoleptic properties in tea, cocoa, coffee and resins (Martinez and Whitaker, 1995; Inamdar *et al*., 2014).

Solid substrate fermentation (SSF) is considered to be more economical than other methods of tyrosinase production (Aulde, 1992; Jeong *et al*., 2013). It appears to possess several advantages such as improved fermentation productivity, higher end-concentration of products, better product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, lower demand on sterility due to the low water activity and less energy requirements (McMahon *et al*., 2007; Wu *et al.*, 2010). In the present study, a novel fungal culture *Rhizopus oryzae* NRRL-1510 was selected for direct tyrosinase production in flasks under SSF. Two-factorial experimental design was employed to identify significant batch-culture parameters influencing enzyme production.

MATERIALS AND METHODS

The chemicals including L-catechol, L-ascorbic acid, trisodium citrate, sodium acetate, ethylene diamine tetra acetic acid (EDTA) and comassie brilliant blue (G-250) were of analytical grade and procured directly from Fluka (UK) and Sigma (USA). Reagents and other solutions were also of the highest possible purity.

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Organism and culture maintenance

The culture *Rhizopus oryzae* NRRL-1510 (initially procured from Northern Regional Research Labs, Peoria, USA) was obtained from the available stock culture of *IIB*. It was maintained on malt extract agar slopes containing 20 g/l malt extract, 5 g/l peptone, 20 g/l dextrose, 15 g/l agar, pH 4.8. A small amount of spores from the master slant culture was inoculated to the freshly prepared slants of the same medium. For growth, the cultures were incubated at 30°C for 3-5 days until maximal sporulation. Sub-culturing was carried out every 2 weeks. The slant cultures were stored at 4ºC in a cold-cabinet (1410V, Sanyo, Tokyo, Japan).

Inoculum preparation and cell count

Ten millilitre of sterilized 0.05% (w/v) diacetyl ester of sodium sulpho succinic acid (monoxal O.T.) was added to a 3-5 day old slant culture of *R. oryzae* NRRL-1510. The clumps of spores were broken by an inoculum wire loop. The tube was gently shaken till a homogenous suspension was obtained. A haemocytometer was used for spore count and found to be 1.25×10^6 CFU/ml at A_{546nm}~1.

Mycelial propagation and critical phases

Ten grams of sunflower meal as a solid substrate was taken in a 500 ml capacity Erlenmeyer flask. It was moistened by 15 ml of distilled water. The medium was autoclaved (KT-40L, ALP Co, Ltd 3-3-10, Midorigaoka, Hamara-shi Tokyo, Japan) at 15 lbs/in^2 pressure (121°C) for 15 min. After cooling at room temperature, it was seeded with 1 ml of spore suspension as an inoculum. The incubation was carried out in a fungal growth chamber (MIR-153, Sanyo, Japan) at 30°C for a period of 48 h. All the cultivation experiments were performed in a set of three parallel replicates, under aseptic conditions.

Microbiological reactions

After optimal incubation time period, 0.5% of L-ascorbic acid prepared in 100 ml of acetate buffer (pH 3.5, 50 mM) was added to the flask. The reactions were performed aerobically at 30°C for 60 min in a shaking water bath (SV-1422 Moemmert GmbH-Co KG, Germany) at 160 rpm and a digital hot plate with magnetic stirrers (1224mi, Technico Scientific Supply, Lahore, Pakistan). Fungal biomass (2.5 mg/ml) was used as a source of enzyme. The samples were withdrawn, filtered and stored at −20°C under dark.

Analytical techniques

Extracellular tyrosinase activity (as polyphenol oxidase) was determined following the method of Kandaswami and Vaidyanathan (1973). One unit of tyrosinase activity is equal to a ΔA_{360nm} of 0.01 per min at pH 6.5 (25ºC) in 1 ml of reaction mixture containing L-catechol and L-ascorbic acid. For enzyme assay, 2.6 ml of potassium phosphate buffer, pH 6.5 (50 mM), 0.1 ml of 50 mM L-catechol solution, 0.1 ml of 2.1 mM L-ascorbic acid solution alongwith 0.1 ml of 0.065 mM ethylene diamine tetra acetic acid (EDTA) were transferred to a glass cuvette. The filtrate (0.1 ml of test solution) was also added to the reaction cuvette. The mixture was vortex twice. A_{360nm} was observed on a spectrophotometer (D-21496, Irmeco GmbH, Heidelberg, Germany) every 1 min for a period of 5 min, consecutively. A control was also run parallel replacing the filtrate with 0.1 ml of distilled water while keeping all other conditions as constant. The decrease in absorbance was observed in comparison to the control. Enzyme activity was determined using the below mentioned formula and expressed in U/mg.

$$\text{Enzyme activity (U/mg)} = \frac{\Delta A_{560nm}/\text{min Test} - \Delta A_{560nm}/\text{min Control}}{0.01 \times \text{mg Enzyme / Reaction mixture}}$$

Significant production conditions

Various agricultural by-products such as sunflower meal, mustard seed cake, almond meal, canola meal, soybean meal and rape seed meal were evaluated as potential solid substrates for the production of an extracellular tyrosinase from *Rhizopus oryzae* NRRL-1510. The level of sunflower meal was varied from 2.5-25g. Different moistening agents including phosphate buffer (pH 7.2), acetate buffer (pH 3.5), potassium phosphate buffer (pH 6.5), citrate buffer (pH 4.8) and distilled water were used at a level of 1:1. The level of phosphate buffer (found optimal) was varied from 5-30 ml for each batch culture. The incubation temperature was ranged from 25 to 55°C while microbial cultivations were carried out from 12 to 96 h (McMahon *et al*., 2007; Leona *et al*., 2008). Citrate buffer was used for enzyme extraction.

Thin layer chromatography (TLC)

Tyrosinase catalyzed reaction mixture was analyzed by TLC using silica-56 coated glass plates (E-Merck). Each fraction was run in hexane-ethyl acetate (4:1) and later monitored through hexane-ethyl acetate (9:1) following the procedure of Saewan *et al.* (2011). The plates were subsequently flashed with dichloromethane-ethylacetate-acetone (18:1:1) and further developed by Sephadex LH-20 with ethanol.

Statistical analysis and application of Plackett-Burman experimental design

Duncan's multiple range tests (Spss-21, version 14)

were applied under one-way analysis of variance (ANOVA-I) and the treatment effects were compared after Snedecor and Cochran (1980). The significant batch culture conditions affecting improved tyrosinase productivity were identified using a 2-factorial system *i.e.*, Plackett-Burman experimental design (Ahuja *et al.*, 2004). The variables were denoted at two widely spaced intervals and the effect of individual parameters on enzyme production was calculated by the following equations,

$$E_o = (\Sigma M_+ - \Sigma M_-) / N \qquad I$$

$$E = \beta_1 + \Sigma \beta_2 + \Sigma \beta_3 + \beta_{123} \qquad II$$

In Eq. I, E_o is the effect of first parameter under study while M+ and M– are responses of enzyme production by the fungal strain. N is the total number of optimizations. In Eq. II, E is the significant parameter, β_1 is the linear coefficient, β_2 the quadratic coefficient while β_3 is the interaction coefficient among significant process parameters.

RESULTS AND DISCUSSION

The direct production of tyrosinase from *Rhizopus oryzae* NRRL-1510 was accomplished under solid substrate fermentation (SSF) in laboratory scale Erlenmeyer flasks. Different agricultural by-products including mustard seed cake, almond meal, sunflower meal (SFM), soybean meal (SBM), canola meal and rape seed meal were evaluated as a basal substrate for enzyme production. The results are given in Figure 1A. SBM gave 26.84 U/mg of enzyme activity. However, the maximal enzyme production (30.42 U/mg) was achieved when SFM was used as a potential substrate. It was possibly due to the optimal fungal growth in SFM which contained appropriate amounts of carbon, nitrogen and potassium elements along with other major essential nutrients such as glucose. In contrast to present study, Marques *et al.* (2002) studied the production of tyrosinase (14.55 U/mg) by a strain of *Pleurotus pulmonarius* under SSF using wheat bran as a substrate. In some other useful investigations, Ogel *et al.* (2006) produced extracellular tyrosinase (19.12 U/mg) from *Scytalidium thermophilum* by growing it on a glucose-containing medium while Nikitina *et al.* (2010) maximized tyrosinase activity (16.60 U/mg) in bacteria of the genus *Azospirillum* using submerged fermentation technique (SmF). Conversely, the enzyme production from all other substrates was not encouraging in the present study. Among these, canola meal and rape seed meal gave an enzyme production of 14.72 and 8.16 U/mg, respectively, which were significantly less ($p \leq 0.05$) compared to the optimal. Mustard seed cake and almond meal exhibited the least amount of enzyme activity that was recorded to be 6.58 and 2.06 U/mg, respectively. In another set of experiments, different levels of SFM and SBM were partially replaced with each other (8:02, 6:04, 4:06, and 2:08 g). Maximum enzyme activity (25.28 U/mg) was observed at 8:02. A gradual decrease in enzyme activity (21.52-6.64 U/mg) was observed from 6:04 to 2:08 ratio. As the maximum enzyme production by partially replacing SBM with SFM at optimal ratio remained less compared to the addition of

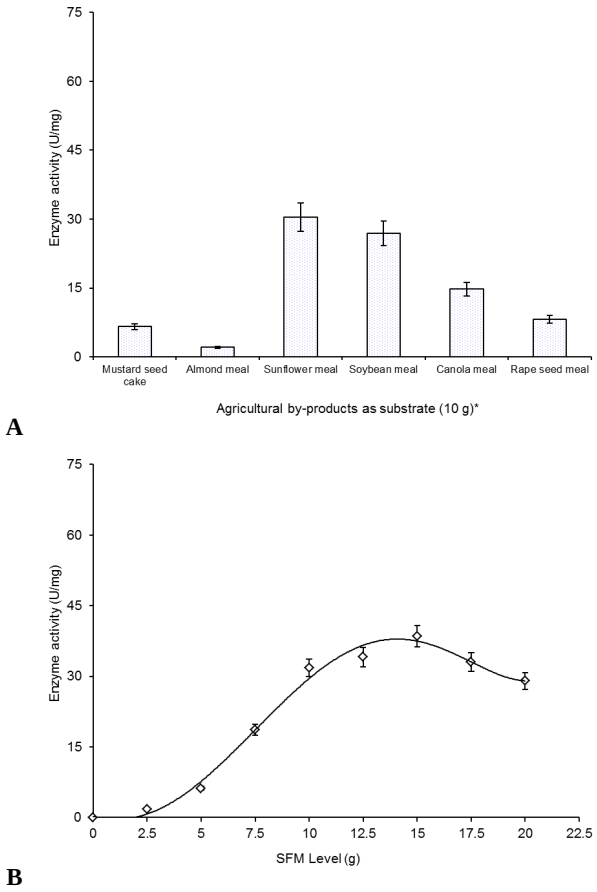

Fig. 1. Effect of different by-products as substrate for tyrosinase production from *R. oryzae* NRRL-1510 (a. Agricultural by products, b. Different levels of SFM). Incubation was carried out at 30°C for 72 h using distilled water at pH 7 as a moistening agent and 100 ml acetate buffer as enzyme extractant. Ascorbic acid (0.5%) was added into the assay medium as an enzyme inducer. Y-bars show the standard deviation (±sd) among the three parallel replicates.

sole substrate, therefore SFM as a substrate was optimized for enzyme production. The effect of different levels of SFM as a substrate (2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5 and 25 g) was also investigated on enzyme production (Fig. 1B). An enzyme production of 1.38 U/mg was obtained when 2.5 g sunflower was used. A gradual increase in enzyme production was observed (4.61 to 33.94 U/mg) as the level of substrate was further increased from 5-12.5 g/500 ml flask. Maximal enzyme production was however, achieved at 15 g of substrate, which declined to 26.52 U/mg at 20 g, 18.85 U/mg at 22.5 g and 9.05 U/mg at 25 g of the substrate. In the present study, the optimal enzyme production (36.15 U/mg) was achieved at 15 g of substrate which was due to the provision of optimal level of growth nutrients. At higher substrate level, the lower enzyme production was due to the carbon catabolite repression as reported by Marques *et al.* (2002).

In another series of batch culture studies, the effect of different initial moisture content on tyrosinase production from *R. oryzae* NRRL-1510 under SSF in 500 ml Erlenmeyer flask was undertaken. The diluents including distilled water (pH 7), phosphate buffer (pH 7.2), acetate buffer (pH 3.5) and sodium citrate buffer (pH 8.1) were added at a level of 15 ml/15 g substrate i.e., 1:1 ratio. Phosphate buffer as the moistening agent was not found effective for enzyme production (17.25 U/mg). Similarly, enzyme production by acetate buffer (23.46 U/mg) and sodium citrate buffer (32.14 U/mg) was also not found encouraging ($p \leq 0.05$). However, optimal enzyme production was obtained with distilled water (38.32 U/mg). The enhancement in enzyme production by replacing sodium citrate and acetate buffers with distilled water was due to the incorporation of correct proportion of additional nutrients supplemented into the solid substrate. The low activity with other moistening agents was due to the inability of organism to acclimatize with the imbalanced nutritional level (Christopher *et al.*, 1997). Figure 2 shows a comparison for the effect of different levels of distilled water and sodium citrate buffer as initial moisture content (5, 10, 15, 20, 25 and 30 ml) on tyrosinase production. The production was less effective from 5-15 ml of distilled water. A marked increase in the production was noticed (42.68 U/mg) when 20 ml distilled water was used to moisten 15 g of SFM. Beyond the optimal level, enzyme production declined (31.55 U/mg at 30 ml of diluent). At low levels of distilled water enzyme activity remained low probably due to lower solubility and less availability of nutrients to organism as reported by Antonio *et al.* (1997). High moisture content is known to affect O_2 diffusion in the substrate as well as causing water logging of the substrate. Enzyme production declined (17.25 U/mg) at 20-25 ml level of diluent. Similar kinds of findings have also been reported by Holker *et al.* (2004).

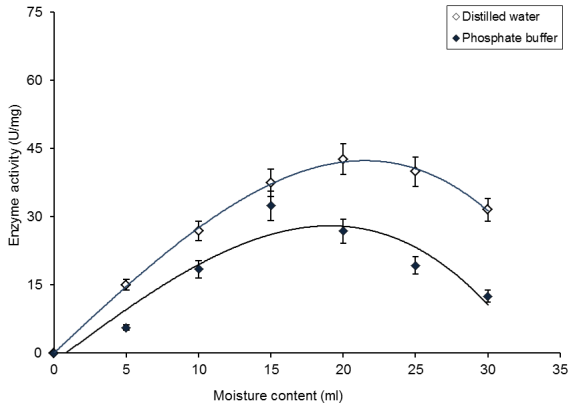

Fig. 2. Effect of distilled water and sodium citrate buffer as moistening agents on tyrosinase production from *R. oryzae* NRRL-1510. Incubation was carried out at 30°C for 72 h. using 15 g SFM as substrate and 100 ml acetate buffer as enzyme extractant. Ascorbic acid (0.5%) was added into the assay medium as an enzyme inducer. Y-bars show the standard deviation (±sd) among the three parallel replicates.

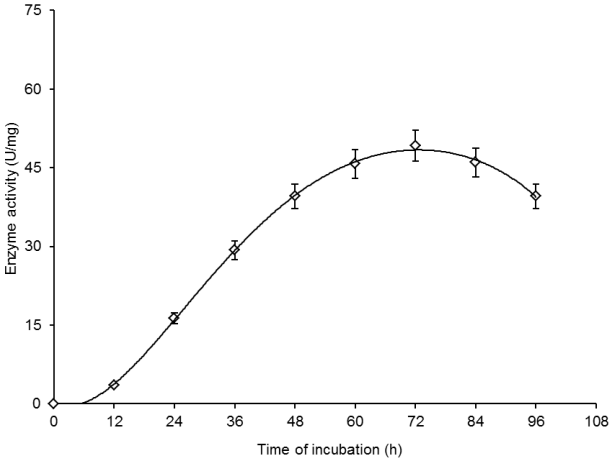

Fig. 3. Time of incubation for tyrosinase production from *R. oryzae* NRRL-1510. Incubation was carried out at 30°C using 20 ml distilled water as moistening agent and 15 g SFM as a substrate alongwith 100 ml acetate buffer as enzyme extractant. Ascorbic acid (0.5%) was added into the assay medium as an enzyme inducer. Y-bars show the standard deviation (±sd) among the three parallel replicates. Each mean value differ significantly at a level of $p \leq 0.05$.

The rate of tyrosinase production from *R. oryzae* NRRL-1510 in Erlenmeyer flasks under SSF was assessed and depicted in Figure 3. The time of incubation was varied from 12-96 h after inoculation. Initially at 12 h of incubation, 3.52 U/mg of enzyme activity was achieved. Enzyme production in the fermented mash increased gradually with the increase in incubation period from 24-60 h. However, the best results (49.16 U/mg) in terms of enzyme productivity were achieved at an incubation period of 72 h and are substantiated with the findings of Krishnaveni *et al*. (2009) who reported optimal tyrosinase activity (41.95 U/mg) at an incubation time of 72 h and temperature 25°C. Afterwards, the enzyme production declined gradually (84-96 h), becoming very low 39.56 U/mg at 96 h of incubation. This sharp decline in enzyme production after 72 h was due to the exhaustion of nutrients and cessation of fungal growth in the fermented mash culture which resulted in the inactivation of enzyme. The low enzyme activity was attributed to the inactivation of enzyme by toxic metabolites or inhibitors produced at later stages of batch culture fermentation (Marusek *et al*., 2006).

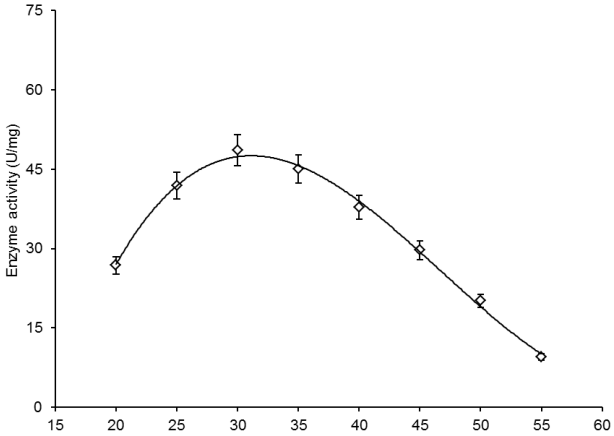

Fig. 4. Effect of incubation temperature on tyrosinase production from *R. oryzae* NRRL-1510. Incubation was carried for 72 h using 20 ml distilled water as moistening agent and 15 g sunflower meal as substrate and 100 ml acetate buffer as enzyme extractant. Ascorbic acid (0.5%) was added into the assay medium as an enzyme inducer. Y-bars show the standard deviation (±sd) among the three parallel replicates. Each mean value differ significantly at a level of $p \leq 0.05$.

The incubation was carried out at various temperatures (20, 25, 30, 35, 40, 45, 50 and 55ºC), keeping all other conditions at their optimal levels. Results are shown in Figure 4. At 20ºC, enzyme activity was 26.82 U/mg. It was increased as temperature was further increased and became maximum (48.62 U/mg) at 30ºC possibly due to the optimal growth of fungal mycelia in the cultivation medium. Any variation in temperature above or below the optimal lead to a decline in enzyme production due to the mesophilic nature of the fungus as reported by Halaouli *et al*. (2005). Toshiteru *et al*. (1991) carried out enzyme production and its extraction from mycelia of *Aspergillus oryzae* at a temperature ranging from 30-40°C. As the temperature was increased up to 45ºC, a slight decrease in enzyme production (29.68 U/mg) was observed. Afterwards it was gradually declined to 20.12 U/mg and then sharply reduced to 9.48 U/mg at a higher temperature such as 55ºC, which became economically insignificant ($p \leq 0.05$). It was due to the denaturation of enzyme with loss of active binding sites which eventually lead to a disturbed mycelial growth and subsequently decreased the extent of enzyme activity (Katsuji and Isao, 1974; Faccio *et al*., 2013).

The extraction of tyrosinase was carried out by adding 100 ml of different extracting buffers (phosphate buffer, pH 7.2; acetate buffer, pH 3.5 and sodium citrate buffer, pH 8.1) into each fermented mash culture individually. Figure 5a highlights the results. The enzyme extraction was not encouraging with acetate (49.36 U/mg) or phosphate buffer (36.64 U/mg). However, maximal extraction achieved (63.92 U/mg) using sodium citrate buffer as extracting agent was due to the fact that it allowed better interaction between both the liquid and solid phases which promoted the optimal extraction of tyrosinase under the specified conditions. Similar kinds of studies have also been reported by Herter *et al*. (2011). In disparity, Zhou *et al.* (2010) used phosphate buffer as an extractant, while Haq *et al*. (2002) emphasized on the use of distilled water as an enzyme extractant. In another study, Raju *et al*. (1993) exploited acetate buffer for L-dopa production from L-tyrosine under liquid culture. In Fig. 5b is depicted the effect of level of sodium citrate buffer as an optimal extracting agent for tyrosinase production. The level was varied from 25-200 ml for separate flasks. At 25 ml, enzyme activity was 12.26 U/mg. It was increased as volume of sodium citrate buffer was further increased and became maximal (64.55 U/mg) at 100 ml volume. The enzyme activity was gradually reduced to 52.94 U/mg at 150 ml and then sharply declined to 25.02 U/mg at higher volumes such as 200 ml. Thus, 100 ml was selected as the best level of sodium citrate buffer.

The potential products of tyrosinase catalysis of L-tyrosine were run on a TLC plate. The standard mixture was run with three purified samples (levodopa, dopamine, o-dopaquinone) as depicted in lanes 1 to 3.

Relative front (R_f) values were compared after the resulting products were developed by ethanol. The spot of o-dopaquinone were appeared just below the solvent front and confirmed the presence of an active tyrosinase in the reaction mixture.

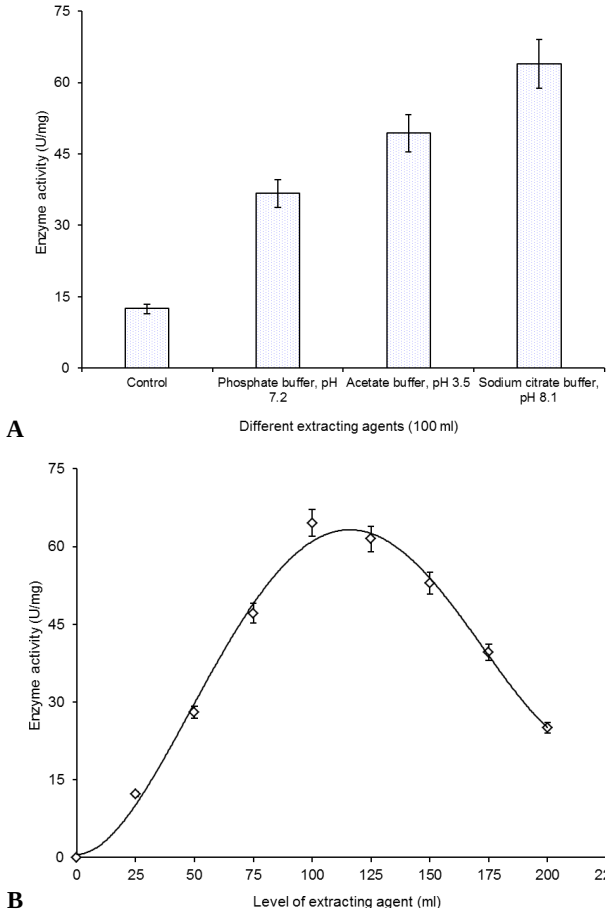

Fig. 5. Effect of different extracting buffers on tyrosinase production from *R. oryzae* NRRL-1510 (a. Buffers, b. Varying levels of sodium citrate buffer). Incubation was carried at 30°C for 72 h using 20 ml distilled water as moistening agent and 15 g sunflower meal as substrate. Ascorbic acid (0.5%) was added into the assay medium as an enzyme inducer. Y-bars show the standard deviation (±sd) among the three parallel replicates.

The 2-factorial experimental system *i.e.*, Plackett-Burman design was applied to determine the significant process parameters involved in tyrosinase production from *R. oryzae* NRRL-1510 (Table II). The validation of the model was investigated under the conditions predicted against the responses obtained for improved enzyme production. A slightly differential correlation was noted between the observed and predicted values as reported by Burkert *et al.* (2006). The optimal levels of the parameters for improved enzyme production under SSF were SFM level (15 g), volume of distilled water (20 ml), time of incubation (72 h), temperature (30ºC) and level of sodium citrate buffer at pH 8.1 (100 ml). The statistical analyses of the responses for tyrosinase production were also performed (Table I). The correlation (0.135*E*+0025) of A, B, C and D for E values depicted that the model was highly significant ($p \leq 0.05$). Correspondingly, the lower probability values indicated that the model terms are valid. The analysis of linear, quadratic and interaction coefficients were performed on the batch culture results which highlighted that enzyme production was a function of the independent parameters (Ahuja *et al*., 2004). The addition of distilled water as a diluent (degree of freedom 3) was found necessary for maintaining the possible spatial conformation of enzyme and thus have an important physiological role in the enzyme activity. According to these results, the fungal strain of *R. oryzae* NRRL-1510 could be considered as an organism of choice for tyrosinase productivity.

Table I.- Comparison of statistical analysis of significance level and probability values for tyrosinase production from *R. oryzae* NRRL-1510*

Significant process parameters	Sum mean values	F-value	Degree of freedom (df)	Proba-bility < p >
A	12.58	1.12	1	0.0741
B	25.33	2.58	1	0.0622
C	39.75	8.54	2	0.0553
D	64.96	17.36	3	0.0385
E	50.42	12.05	1	0.0564
Correlation	0.135*E*+0025			

*The letters represent significant process parameters (SFM level, volume of distilled water, time of incubation period, temperature and level of sodium citrate buffer, pH 8.1) for tyrosinase production. CM – 12.39; R^2 – 0.285.

CONCLUSIONS

In the present study, extracellular tyrosinase activity by *Rhizopus oryzae* NRRL-1510 was improved to a maximum of 64.55 U/mg by optimizing the cultural conditions such as temperature, time of incubation, initial moisture content and enzyme inducer. An overall improvement of more than 55% in terms of enzyme activity was accomplished when the significant process parameters were determined after Plackett-Burman design, and confirmed by TLC analysis of tyrosinase-catalysis products. The value of tyrosinase correlation

Table II.- Application of 2-factorial design at various process parameters for tyrosinase production from *R. oryzae* NRRL-1510*

Process parameters at 2-factorial design					Tyrosinase production (U/mg)	
SFM (g)[A]	**Distilled water (ml)[B]**	**Time of incubation (h)[C]**	**Temp (°C)[D]**	**Sodium citrate buffer (ml)[E]**	**Observed**	**Predicted**
10	na	48	20	50	11.84	19.15
12.5	10	48	25	50	24.02	32.70
12.5	15	60	25	75	39.66	53.45
15	20	72	30	100	64.55	71.16
17.5	25	84	40	125	53.26	67.38

*The different letters represent significant process parameters for tyrosinase production. Statistical analysis of the model was based on 2-factorial experimental design. 'na' means that nitrogen source was not added.

(0.135*E*+0025) depicted that the model terms are highly significant (*HS*, $p \leq 0.05$) indicating commercial utility of the fungal culture (df = 3, LSD = 0.0385). This is perhaps the first ever report on tyrosinase production from *R. oryzae* under SSF. However, biochemical characterization of the enzyme is prerequisite for scale up studies.

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Conflict of interest declaration

The authors declare that they have no competing interests.

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