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Research Article

COMPARATIVE STUDY OF LACCASE PRODUCTION BY *STREPTOMYCES CHARTREUSIS* IN SOLID STATE AND SUBMERGED FERMENTATION

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ABSTRACT
A novel *Streptomyces chartreusis* strain NBRC 12753 (GenBank accession number JQ086575) was isolated and evaluated for Laccase production by Solid state Fermentation (SsF) and Submerged Fermentation (SmF). Various process parameters like substrate screening, incubation temperature, pH of the medium, initial moisture content, particle size, inoculum size, carbon and nitrogen supplements, time course study, effect of inducers on Laccase production were investigated in both types of the fermentation process. Additional parameters like effect of agitation, rate of aeration and oxygen solubility using alumina were studied for submerged fermentation. The maximum Laccase production in the SsF system was as high as 72 U/gm. Laccase productions in SsF were found to be twofold of that in SmF. One of the main reasons for higher Laccase production in SsF compared to SmF was possibly due to the presence of superior proteolytic activity in SmF. Strong proteolytic activity in SmF apparently caused consequent Laccase degradation, which lesser the vital laccase production in Submerged fermentation.

Key Words: *Substrate Screening, Solid State Fermentation, Submerged Fermentation, Laccase*

Introduction

Laccases are multicopper containing enzymes, which condense molecular oxygen to water and concurrently execute one electron oxidation of a variety of aromatic substrates (Nyanhongo *et al.*, 2002). The electron transfer steps in these redox reactions are coordinated in two copper centers that usually contain four copper atoms. Despite its putative role in various processes in nature, their low substrate specificity allows them to oxidize a wide range of compounds without releasing toxic peroxide intermediates (Claus, 2004; Nyanhongo, 2002). This enzyme, which is believed to possess infinite biotechnological potential and a high market value, is widely circulated among Basidiomycetes, particularly those associated with wood decay or terminal stages of decomposition (Wood *et al.*, 1980). Laccase production has also been reported among higher plants, insects, bacteria and fungi (Cavallazzi *et al.*, 2005). This makes them suitable for biotechnological and environmental applications (Alimin and Annuar, 2009; Annuar *et al.*, 2010; Cavallazzi *et al.*, 2005). Extracellular lignin peroxidase has been demonstrated in different *Streptomyces* strains such as *S. viridosporus*, *S. chromofuscus* and *S. psammoticus* (Ramachandra *et al.*, 1987; Pasti *et al.*, 1990). Apart from LiP, the *Streptomyces* strains remain as a good source for laccase (Arias *et al.*, 2003, Suzuki *et al.*, 2003). Solid state and submerged fermentation techniques are common and conventional biotechnology processes in view of production of value-added products such as enzymes, biopharmaceuticals, organic acids, biosurfactants, vitamins, flavoring compounds, biofuels, biopesticides etc. SsF is described as the fermentation in absence or near absence of free water. Submerged fermentation (SmF), more strongly developed from the 1940s onwards because of the necessity to produce antibiotics on a large scale has been characterized as fermentation in the presence of excess water. A direct comparison between SsF and SmF cultivation techniques is difficult to make because the two processes are quite different. Studies on filamentous bacterial enzyme production in SsF have shown that SsF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with a higher

temperature or pH stability. Solid state fermentation (SSF) is environmental friendly as it resolves the problem of solid wastes disposal. Production of biocatalysts using agro-biotech substrates under solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc. Furthermore, most of these agro wastes contain lignin or/and cellulose and hemicelluloses, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more reasonable.

MATERIALS AND METHODS

Organism

Screening for laccase-producing microbes on Bennet's agar plates containing coloured indicators resulted in isolation of 20 *Streptomyces* strains. Bacterial isolates showing positive Bavendamm's reaction were maintained on Bennets agar at 30°C and stored at 4°C. The best Laccase producing isolates was identified by comparing the partial 16S ribosomal RNA gene partial sequence deposited in GenBank data base and identified as *Streptomyces chartreusis* strain NBRC 12753 (Accession number JQ086575). This isolates is use here for comparative study.

Chemicals

2, 2-Azino-bis (3ethylbenzthiozoline-6-sulphonic acid) (ABTS) and syringaldazine was purchased from Sigma (St. Louis M.O., U.S.A.). Yeast Malt agar, Casein enzyme hydrolysate, Yeast extract powder, Sodium chloride, Dextrose and Guaiacol were procured from Hi-Media (Mumbai, India). Ortho-anisidine and para-anisidine were procured from CDH (Mumbai, India). Pyrogallol, vanillic acid, cupric sulphate and ferulic acid were procured from S.D.Fine chem (Mumbai, India). All other chemicals were of analytical grade procured from Qualigens (Mumbai, India). Wheat bran, rice bran, wheat straw, sugarcane baggase and banana waste were collected locally and used as lignocellulosic substrates. Guaiacol was added to the media before autoclaving, ortho and para anisidine after autoclaving as sterile-filtered 50 % acetone solutions. Cupric sulphate and Vanillic acid were dissolved in sterile water.

Substrate screening for SsF & SmF

The utilization of cheap and easily available agro waste for the production of value added product is one of the suggested advantages of solid-state fermentation. Different agro wastes were screened to identify the suitable substrate for laccase production in solid-state and submerged fermentation. The substrates used were wheat bran, rice bran, wheat straw, banana pulp and sugarcane bagasse (Winquist *et al*, 2008; Singhania *et al*, 2009; Couto and Sanroman, 2005).

Substrate preparation for SsF

5 grams of substrate were added to a 250 ml Erlenmeyer flask and was moistened with a salt solution containing (g/L): yeast extract - l.0, $(NH_4)_2SO_4$ - 0.2, $MgS0_4$ 0.2, $CaCO_3$ - 0.04, $CuSO_4$ - 0.002. Thirteen mL of the moistening solution was added to the substrate and the initial moisture level in the substrate was adjusted to 50 % by adding an adequate quantity of distilled water. After sterilization by autoclaving at 121 °C for 30 min, the medium was cooled to room temperature and inoculated with l.4 x 10^7 CFU of inoculum and incubated at 30 °C for 96 hours (Gómez *et al*., 2005).

Optimization of substrate weight for SsF & SmF

Each substrate in three separate sets of flasks containing 3, 4 and 5 g were incubated in order to find out the optimum substrate concentration for maximum laccase yield. After carrying out the fermentation for optimum time, the contents of the flasks were extracted for laccase and its activity was measured (Chhaya and Gupte *et al*., 2009)

Optimization of particle size and initial moisture content for SsF

Process parameters such as particle size of the substrate and initial moisture content were diverse at different levels and the properties were studied. The particle size was varied from 150 to 355 µm. The desired particle size of studied substrates was obtained by sieving (using Indian standard sieves) the air

dried substrates. Unsieved substrate that enclosed particles of different sizes was also included in the study. Samples containing 5 moisture levels (30 %, 45 %, 50 %, 65 % and 70 %) were prepared by moistening 5 g of studied substrates with salt solution (Niladevi *et al.,* 2007).

Optimization of inoculum size for SsF & SmF

10 ml of sterilized distilled water was added to a sporulated 2 days old Bennet's slant culture. Serial dilution method using salt solution was applied for getting five different range of inoculum. Total 5 ranges of inoculum concentration ($3.0 \times 10^{2,}$ 2.6×10^{3}, 1.5×10^{4}, 1.3×10^{5}, and 7.0×10^{6}) were studied for Laccase production. Inoculum size for SmF was decided from the result of SsF, through which volume of inoculum varied from 5 % to 20 %.

Optimization of pH and temperature for SsF & SmF

Initial pH of the moistening solution was varied from 5 to 9 and the incubation temperature was studied between 25-40 °C in SsF as well as in SmF (Niladevi *et al.,* 2007).

Optimization of carbon supplements for SsF & SmF

Supplementation with additional carbon sources [dextrose at 1% w/v while sucrose, and soluble starch at 0.5 % w/v] were also carried out.

Optimization of nitrogen supplements for SsF & SmF

Supplementation with a variety of organic nitrogen sources [yeast extract, peptone, and tryptone at a concentration of 0.2 % w/v], inorganic nitrogen sources [ammonium sulphate, ammonium chloride, and potassium nitrate at a concentration of 0.1 % w/v] were carried out.

Effect of inducers for SsF and SmF

To study the effect of inducers on laccase production, various aromatic compounds such as p-anisidine, ortho anisidine, ferulic acid, guaiacol, pyrogallol, cupric sulphate and vanillic acid were incorporated in the fermentation medium. All the inducers were added at a concentration of 1 mM. Cupric sulphate and vanillic acid were dissolved in sterile water while all the other aromatic inducers were dissolved in 50 % alcohol. The inducers were added to the flasks just before inoculation.

Basel Medium and cultural conditions for submerged fermentation (SmF)

The basal medium used for laccase production had the following composition (g/L): Casein enzyme hydrolysate; - 2.0, Yeast Extract;- 3.0, NaCl;- 0.1, Dextrose;-5.0, $CaCO_3$; - 0.02, $CuSO_4$; -0.001, pH -7.5 and 1 ml of trace elements solution. The trace elements solution contained 0.l % $FeSO_4$, 0.09 % $ZnSO_4$ and 0.02 % $MnSO_4$, pH 7.5. Adequate aeration was provided by agitation at 7.17 relative centrifugal force (rcf) at 30 °C for 3 days. The culture grown under the same conditions for 48 h was used as the inoculum for enzyme production.

Optimization of aeration-agitation rate & oxygen solubility for SmF

To investigate the effects of dissolved oxygen solubility in shaken flasks on the behavior of *S.chartreusis* and on production of Laccase, a range of oxygen transfer rates was obtained by varying the volume of medium per flask. The relationship between the dissolved oxygen supply and production of Laccase (Esfahani *et. al*. 2004) fermentations were carried out with 25, 50 and 100 ml volumes of medium, inoculated with the same cell concentration in standard 250-ml Erlenmeyer flasks. The range selected for study agitation was between 2 rcf to 7 rcf. Oxygen solubility for improving the rate of dissolved oxygen were studied using Alumina of 0.01 %,0.02 % and 0.03 % concentration with 100 ml of basal media in 250 ml Erlenmeyer flask.

Time course study

Time course up to 120 hours were studied in both the fermentation process.

RESULTS AND DISCUSSION

Substrate screening in SsF and SmF

Among the different substrates screened, rice bran (39.6 U/gm) was the most suitable substrate for laccase production, followed by wheat bran (25U/gm, Figure 1A) in SsF. While in the submerged fermentation

rice straw (2.3 U/ml) was found suitable (due to it contains up to 12-17 % of lignin), after rice bran (2.6 U/ml) instead of wheat bran (Figure 1B). The selection of suitable substrate is one of the most important factors that decide the success of solid-state fermentation process. SsF was usually performed with filamentous fungi due to their ability to penetrate and colonize the solid substrate particles. In the present study, the filamentous nature of *S. chartreusis* was observed as an added advantage which facilitated the penetration of bran particles and utilization of the nutrients; a characteristic usually appreciated in solid-state culture conditions. Rice bran is a by-product of the rice milling process, and it contains various antioxidants that impart beneficial effects on human health. The major rice bran fraction contains 12%–13% oil and highly unsaponifiable components (4.3%). It also contains 4-hydroxy-3-methoxycinnamic acid (ferulic acid), which is also a component of the structure of non-lignified cell walls. The present study has proved the utility of rice bran, which is an inexpensive and easily available raw material, as a suitable substrate for laccase production in SSF as well as SmF. The presence of lignin related compounds in rice bran and rice straw are a factor that favours ligninolytic enzyme production. Laccase production has been carried out from rice bran in SmF and SSF using the basidiomycete fungus *Coriolus versicolor* and it was observed that rice bran was a better substrate for laccase production in both SmF and SSF as compared to other substrates like wheat bran and rice straw (Chawachart *et al.*, 2004). When the levels of those carbon sources decrease, laccase synthesis was induced by phenolic compounds containing in rice bran, leading to increasing of laccase production.

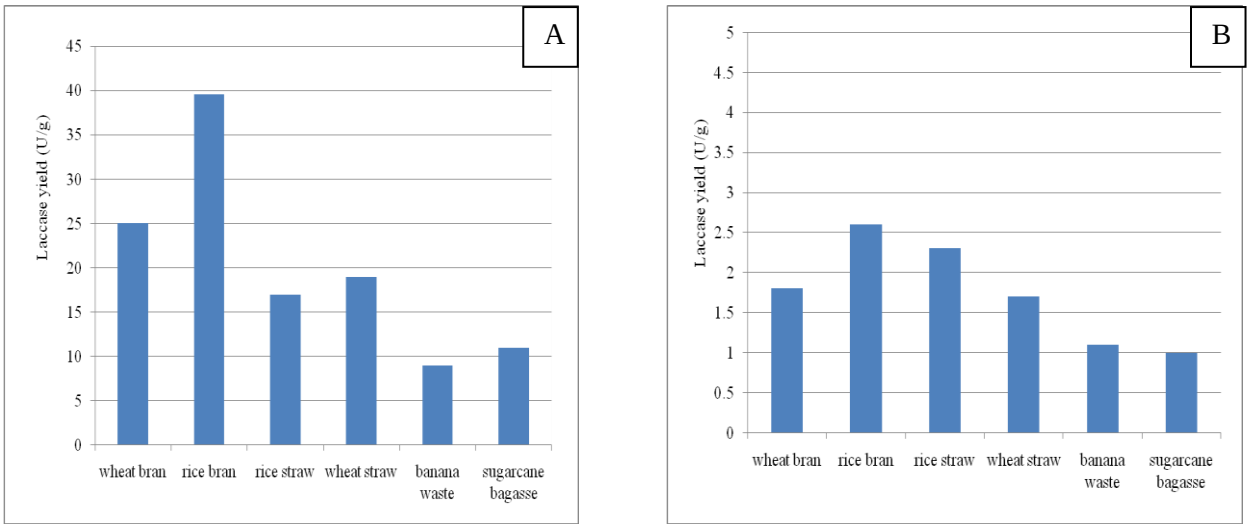


Figure 1: Screening of agro waste for Laccase production by *Streptomyces chartreusis*
A. Laccase production in U/g during Solid state fermentation.
B. Laccase production in U/g during Submerged fermentation.

Effect of substrate weight for SsF

Surface-to-mass ratio of solid substrate was one of the important factors in SsF, as it was directly related to the surface area available for the growth of cells. In this direction three variations in solid substrate loading (3 g, 4 g and 5 g of solid substrate) were studied to enumerate its role on the laccase yield in SSF. It was found from the results that 5 g of rice bran was yielded effective laccase expression (Figure 2). This phenomenon might be attributed to the presence of relatively higher availability of lignin related compounds in rice bran favours ligninolytic enzyme production.

Effect of particle size and initial moisture content in SsF

The adherence and penetration of microorganisms (Figure 3A & 3B) toward the solid substrate as well as enzyme action on the substrate clearly depend upon the physical properties of the substrate such as the

accessible area, surface area, porosity and particle size, etc of which particle size plays a major role because all other physical properties of the substrate depends on it. In the present study particle size in the range of 355 µm mixed with unsieved particles (Usp) was the optimum for laccase production (Figure 4).

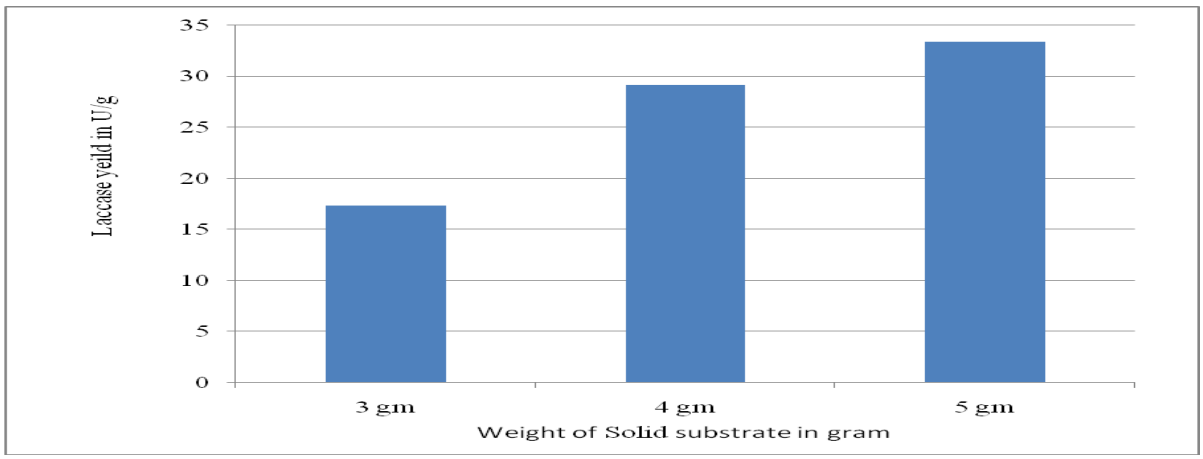


Figure 2: Solid substrate weight (Rice Bran) in Laccase production with *Streptomyces chartreusis*

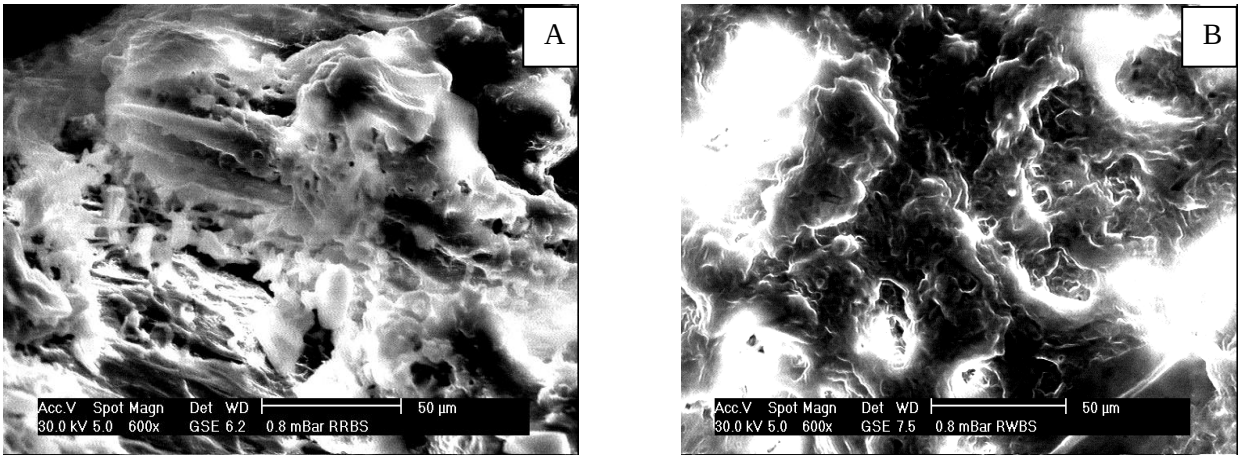


Figure 3: Appearance of *Streptomyces chartreusis* with solid substrate using scanning electron microscopy

A. Appearance of Rice bran in 600 X scanning electron microscopy using solid state fermentation
B. Appearance of Wheat bran in 600 X scanning electron microscopy using solid state fermentation

The enzyme yield was low in the case of substrates with lower and higher particle size, which was in congruence with the general concept that lower particle size results in substrate agglomeration, enhanced channeling problems and decreased heat transfer while larger particles reduce the production due to limited surface area for microbial attack (Pandey *et al.*, 2000). When unsieved substrates containing different particle size were used, the enzyme production was found better. Moisture (Estahani *et al.*, 2004) is another key parameter to control the growth of microorganisms and metabolite production in solid-state fermentation. Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer while decrease in initial moisture level results in reduced solubility and low availability of nutrients to the culture. An initial moisture content of 65 % was the optimum for laccase production by *Streptomyces. chartreusis* (Figure 5).

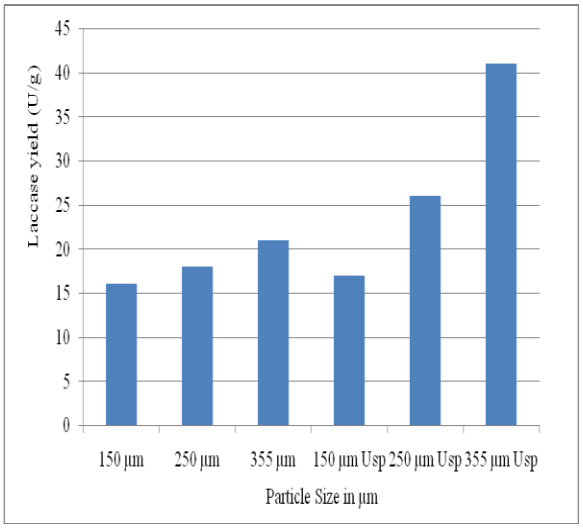


Figure 4: Effect of Particle Size

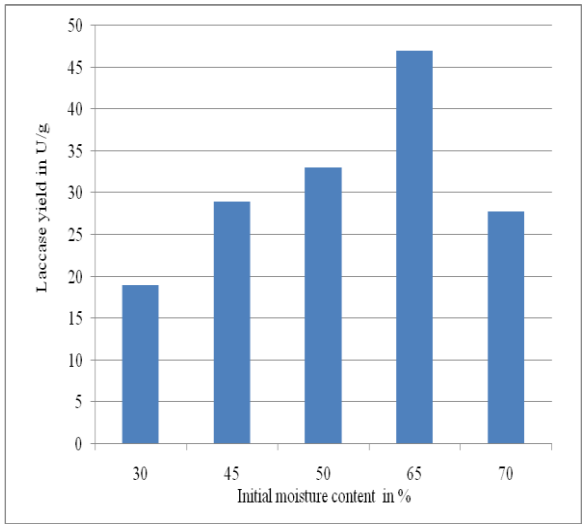


Figure 5: Effect of Initial Moisture

Optimization of inoculum size for SsF & SmF

The enzyme yield was reduced at lower and higher inoculum levels (Muhammad *et al.,* 2012). A very low inoculum size was found to be inadequate for enzyme production, while the inoculum level above optimum reduced the yield probably due to the competition for nutrients. Where all five inoculum concentration defines as series 1 to 5. In which series 3 stands for 1.5 x 10^4 CFU yielded maximum (42.6 U/g) Laccase production (Figure 6A) in SsF. The optimization of inoculum size revealed that an inoculum size of 12 % yielded maximum (5.0 U/ml) was the optimum for laccase production in SmF (Figure 6B).

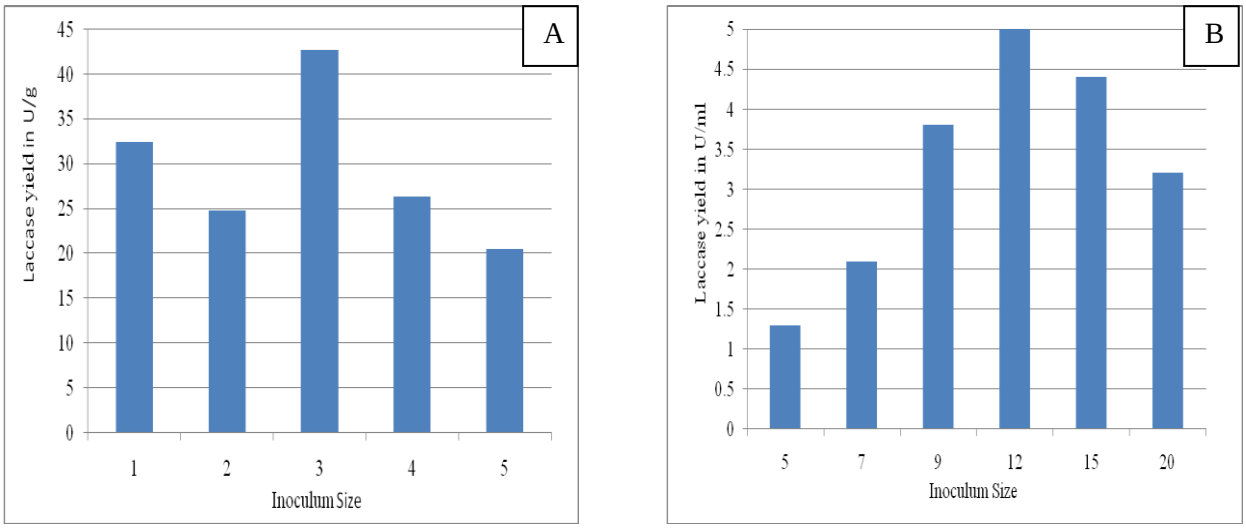


Figure 6: Inoculum size of Streptomyces chartreusis

A. Optimization of inoculum size on Laccase production in U/g by solid state fermentation

B. Optimization of inoculum size on Laccase production in U/ml by submerged fermentation

Effect of pH and temperature in SsF and SmF

The optimum pH for maximal laccase production during SsF was pH 8 (38.9 U/g, Figure 7A). In submerged fermentation same pH gives higher laccase production. The particle size 355 µm with unsieved particles (Usp), initial moisture content was 65 % and inoculum size was 1.4 x 10^7 were used to

carry out the experiments in both the fermentation process. The current results confirmed that the enzyme production was favored by neutral to alkaline pH range, where as the acidic pH decreased the enzyme yield considerably (Figure 7A, 7B).

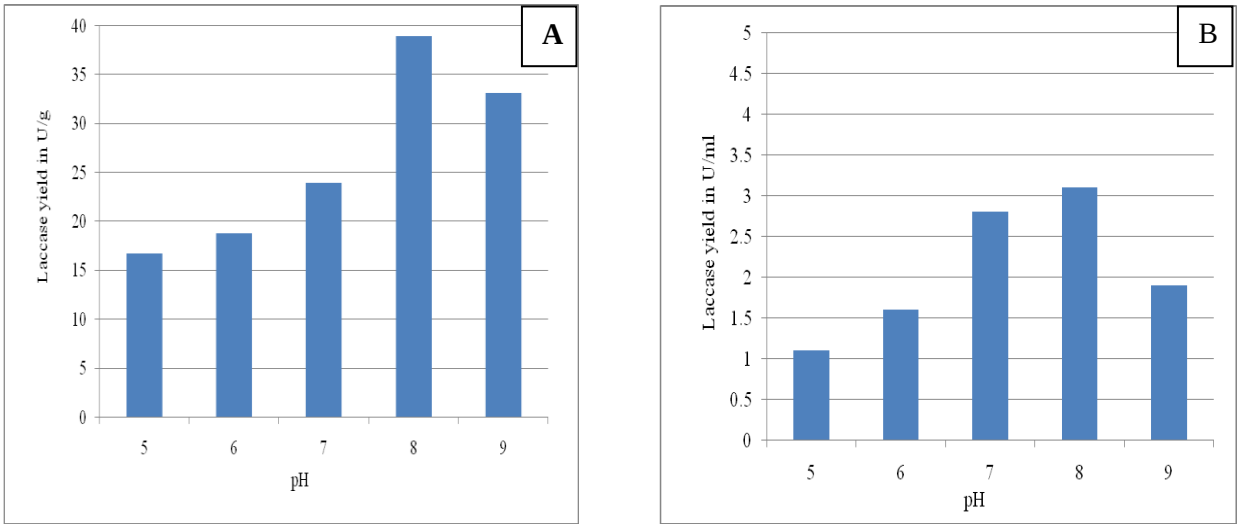


Figure 7: Screening for pH study in solid state fermentation and submerged fermentation
A. Effect of pH in Solid state fermentation with *Streptomyces chartreusis*
B. Effect of pH in Submerged fermentation with *Streptomyces chartreusis*

Results of the present study suggested that an incubation temperature of 30 °C (Figure 8A & 8B) was the optimum for Laccase production using rice bran as a substrate. Similar results on the effect of temperature were observed in submerged fermentation hence it can be fulfilled that temperature exerts a similar effect on growth and Laccase production irrespective of the mode of fermentation.

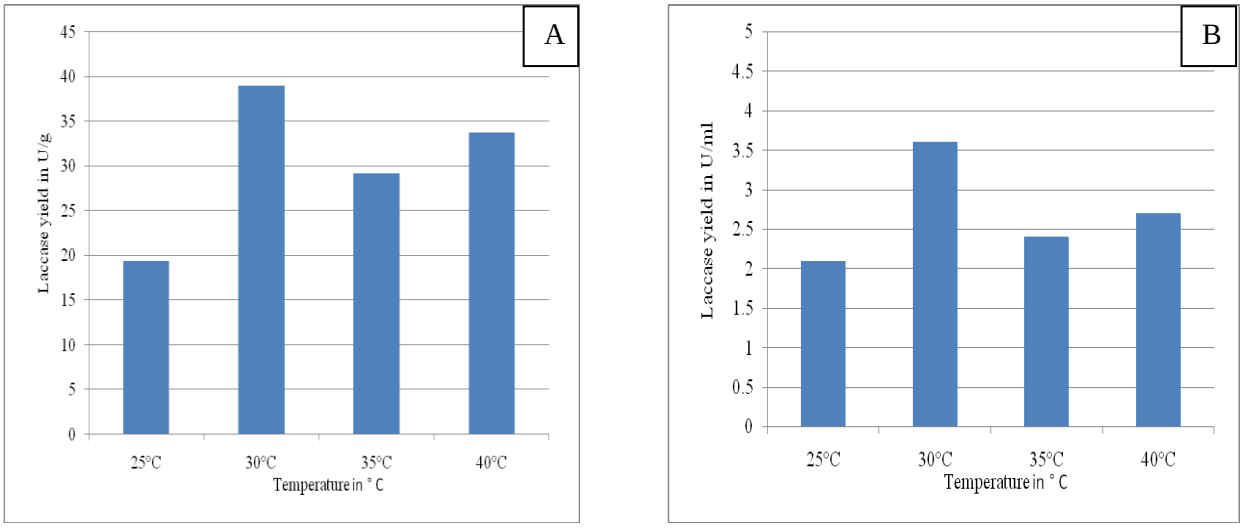


Figure 8: Screening for Temperature study in solid state fermentation and submerged fermentation
A. Effect of Temperature in Solid state fermentation with *Streptomyces chartreusis* in U/g of Laccase
B. Effect of Temperature in Submerged fermentation with *Streptomyces chartreusis* in U/ml of Laccase

Effect of supplemental carbon and nitrogen source for SsF & SmF

Nature and type of carbon and nitrogen sources are among the most important factors for any fermentation process (Pandey and Radhakrishnan, 1992). Among the different supplemented carbon sources used, dextrose was comparatively less repressive for Laccase production, which yielded 40.1 U/g and 4.6 U/ml (Figure 9A, 9B), while all the other carbon sources reduced the enzyme yield considerably.

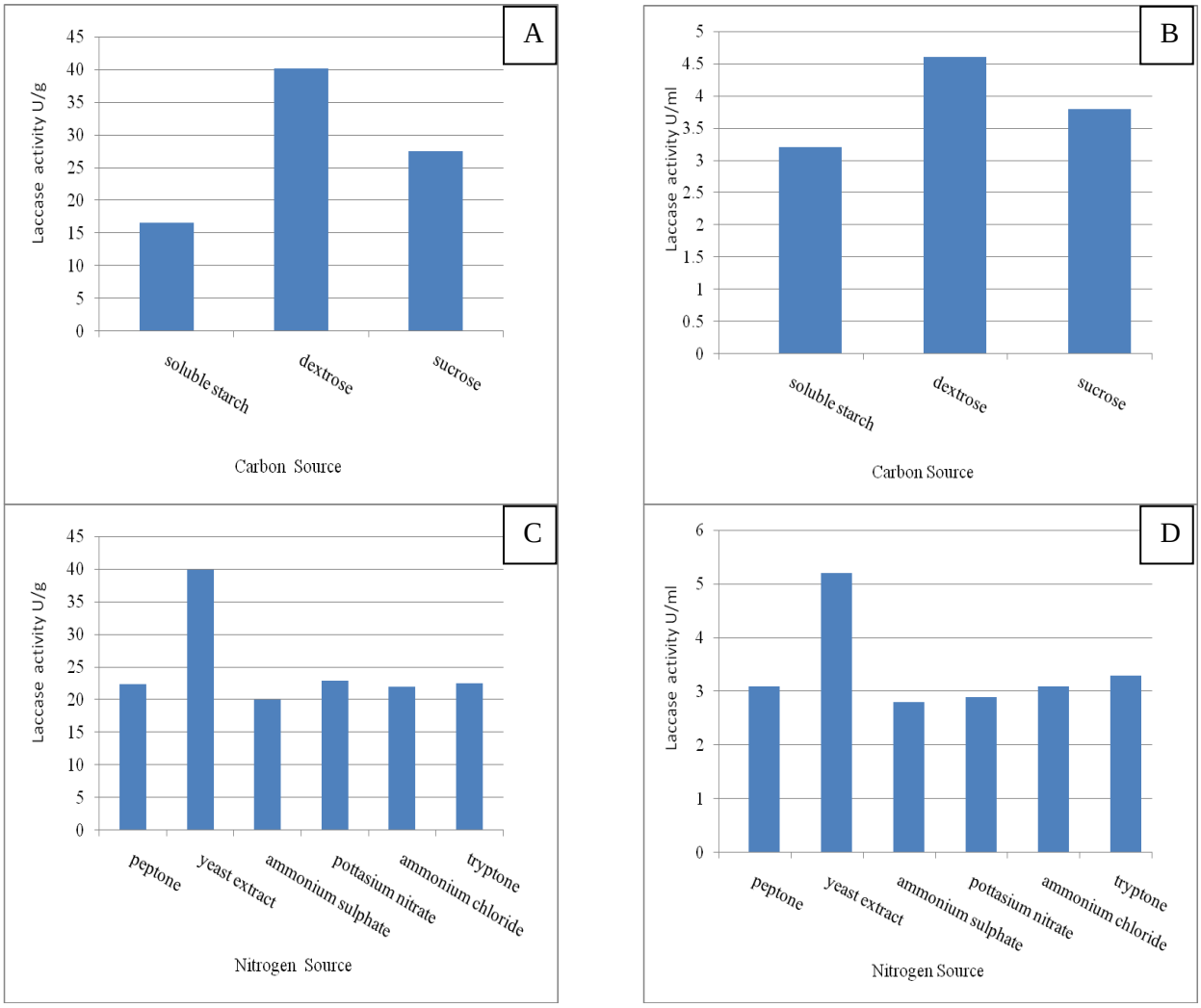


Figure 9: Screening for supplemental Carbon & Nitrogen source for Laccase production by *Streptomyces chartreusis*

A. Effect of supplemental Carbon source for Laccase production by Solid state fermentation in U/g
B. Effect of supplemental Carbon source for Laccase production by Submerged fermentation un U/ml
C. Effect of supplemental Nitrogen source for Laccase production by Solid state fermentation in U/g
D. Effect of supplemental Nitrogen source for Laccase production by Submerged fermentation un U/ml

This was probably due to the reason that dextrose was a readily utilizable substrate which would promote the biomass production. Replacement of yeast extract with peptone failed to elicit laccase production. This confirmed the suitability of yeast extract as the nitrogen source (39.8 U/g and 5.2 U/ml) for laccase

production (Figure 9C,9D) by *S. chartreusis* and similar result has also been reported from *S. psammmoticus* (Nila Devi *et al.* 2007). Same way in the SmF system the organic source were added at a concentration of 4 gm/L and inorganic nitrogen source were included in such a way to contain 0.002 % Nitrogen. The results with yeast extract and dextrose were found satisfactory as same as in SsF. The time course of laccase production indicated that the maximum enzyme yield was achieved at 96 h of incubation.

Effect of inducers

A result of our study directs that inducers play a significant role in enhancing the production of laccase. After getting results related to particle size, pH, temperature, initial moisture content, effect of carbon and nitrogen source studies in the flask level experiments revealed that rice bran and wheat bran used as a solid substrate is enough to study the effect of inducers in the Laccase production.

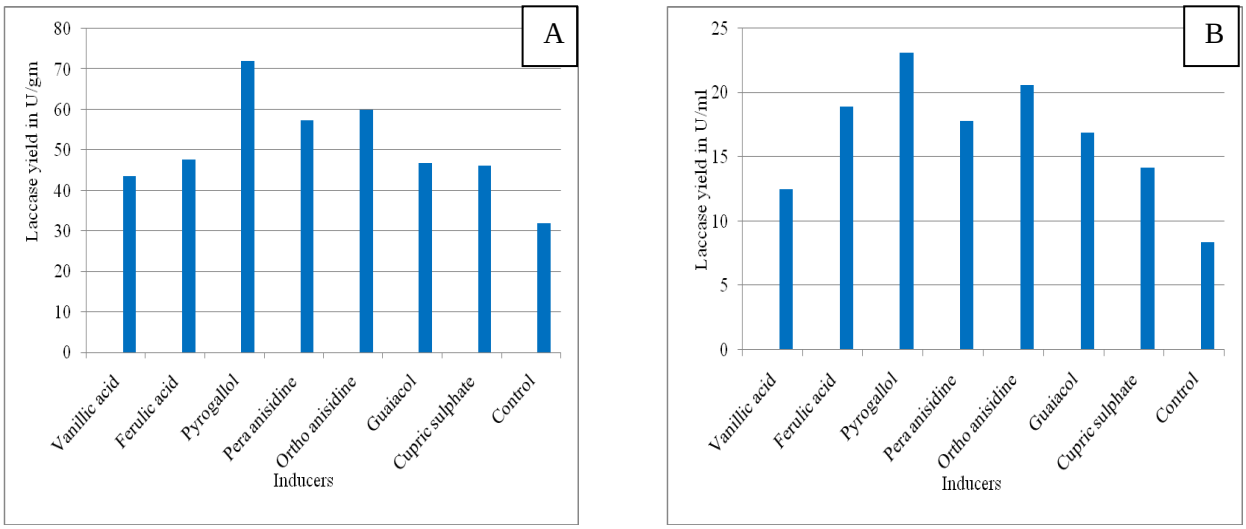


Figure 10: Screening of Inducers for Laccase production by *Streptomyces chartreusis*

A. Effect of Inducers for Laccase production by solid state fermentation in U/g

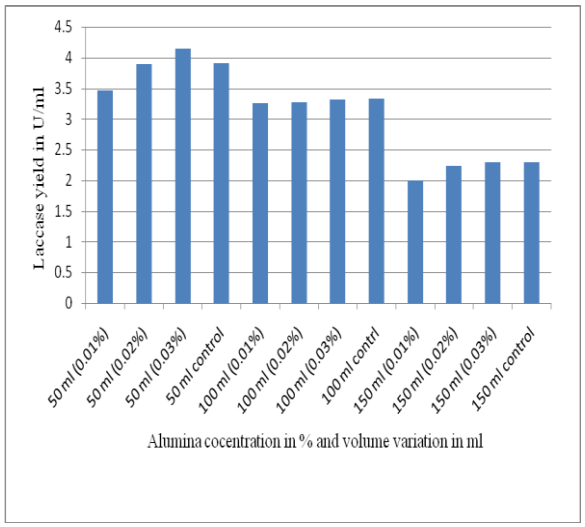
B. Effect of Inducers for Laccase production by submerged fermentation in U/ml

Ortho anisidine is the most efficient inducer for laccase production by *S. chartreusis*, followed by pyrogallol. Ortho anisidine enhanced the laccase production and giving a yield of (60 U/g & 20.6 U/ml) against the control (32 U/g & 8.4 U/ml) while increase in laccase production was achieved with pyrogallol (72 U/g & 23.1 U/ml) in both SsF (Figure 10A) and SmF (Figure 10B). Other inducers like ferulic acid, cupric sulphate, guaiacol, venilic acid and para anisidine were also found to be enhancing the laccase yield. Aromatic inducers and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (Leonowicz *et al.*, 2001) and the nature of the compound that induces laccase production differs greatly with the species. However, it remains a general practice to select the inducers in such a way that they are either polyphenols or lignin related structures.

Effect of aeration-agitation rate & oxygen solubility for SmF

The rate of aeration was not influencing the enzyme production drastically. But 50 ml of medium volume in 250 ml of flask gave less enzyme production at 0.01 % alumina, while higher enzyme production was found with 0.03 % alumina. Although the amount of enzyme production do not affect too much during aeration study. Here the main influence was the correlation between the amount of alumina added and volume variation of flask. The control flask having the absence of alumina, but with three different volume variation higher enzyme productions was found at 50 ml of medium volume, it means higher volume variation with lower concentration alumina gives good yield (Figure 12). The agitation rate

influenced the enzyme production in such a way that the yield increased with increase in agitation up to 200 rpm although the effect was not drastic (Figure 11). The enhancement in enzyme production at higher agitation rate was due to the better aeration in the well agitated flasks that was essential for the growth and enzyme production by *Streptomyces chartreusis*.



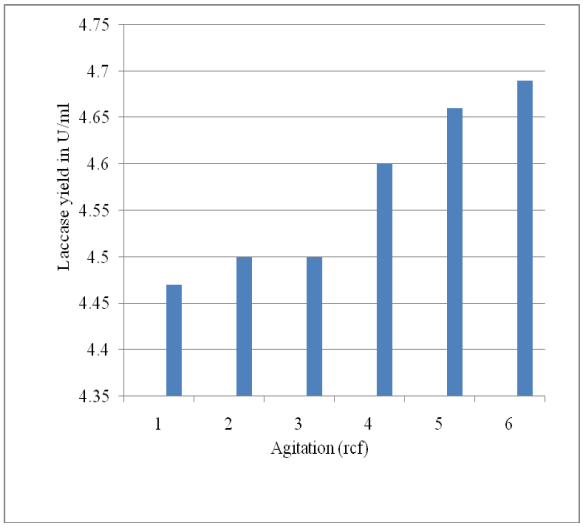
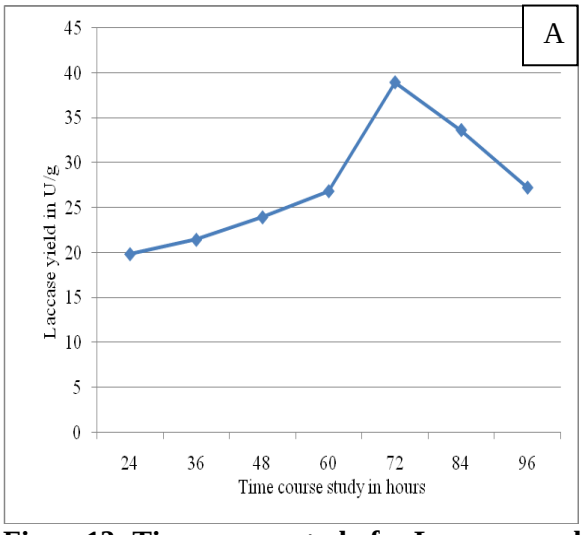


Figure 11: Effect of Alumina and Volume Variation Figure: 12 Effect of Agitation

Effect of Time Course Study

The result of time course of laccase production showed that the time of maximum laccase production remained same in submerged fermentation also (Figure 13A & 13B).



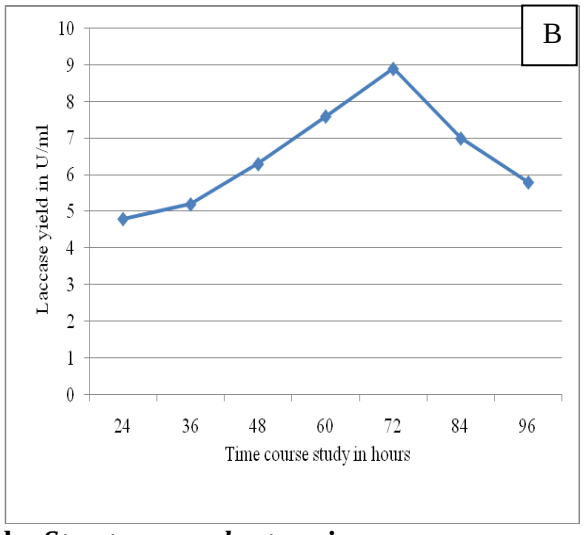


Figure13: Time course study for Laccase production by *Streptomyces chartreusis*

A. Time course study for Laccase production by solid state fermentation in U/g

B. Time course study for Laccase production by submerged fermentation in U/ml

Conclusion

This study revealed that laccase production by *Streptomyces chartreusis* was considerably higher in SsF than in SmF. Solid-state fermentation is generally regarded as more suitable for the fungal system. The production of 72 U/g of laccase from *Streptomyces* species, using rice bran as the substrate, is a promising

result and it suggests that solid-state culture is also functional with actinomycetes. Laccases are the most extensively studied group of enzymes among oxidases. The filamentous nature of these organisms might be favoring their growth on solid substrates. Optimization of the fermentation process by conventional procedures resulted in two-fold increase in laccase production. The study has confirmed the suitability of using inducers and additional carbon-nitrogen supplements for enhancing laccase production by this strain and it has also successfully evaluated the utility of rice bran, which is an inexpensive and easily available agro-industrial waste for laccase production under solid-state fermentation. These remarkable properties make this organism a best candidate for biotechnological applications especially in the areas where alkaline conditions are preferred. These significant properties make this organism a best candidate for biotechnological applications especially in the areas where alkaline conditions are preferred. In addition the organism produced laccases within a short incubation period of 72 h. The optimization yielded a twofold enhancement in laccase production than the un-optimized medium.

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