

DETERMINATION THE OPTIMUM CONDITIONS OF LACCASE PRODUCTION FROM LOCAL ISOLATE OF *STREPTOMYCES SP.* USING SOLID STATE FERMENTATION

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ABSTRACT

Six different bacterial and fungal isolates included *Pseudomonas sp.*, *Bacillus sp.*, *Candida sp.*, *Klaveromyces sp.*, *Aspergillus sp.* and *Streptomyces sp.* were tested to produce laccase enzyme by using submerged fermentation, *Streptomyces sp.*, *Candida sp.*, and *Pseudomonas sp.* were chosen as being the heaviest in terms of enzyme production, and were tested for laccase production by solid state fermentation using different supports such as a solid media including sawdust with 0.06% xylene, sawdust and bran mixture (2:1) with 0.06% xylene. By using a combination of sawdust and bran (2:1), the productivity of laccase produced from *Streptomyces sp.*, *Candida* and *Pseudomonas sp.* reached 177.8, 144.3 and 103.46 U. g⁻¹ respectively. *Streptomyces sp.*, isolate was chosen for laccase production. Optimum conditions for laccase production was determined from the selected isolate *Streptomyces sp.* by using an amalgamation of sawdust and bran (2:1) in pH 5.5 and the medium was incubated at 30°C for 14 days. This specific activity for laccase enzyme reached 1315 U.mg⁻¹. The maximum removal efficiency of textile dyes such as (yellow, red and black) by crude laccase was reached 87%, 43% and 74% respectively after a period of 3 hours.

Keywords: Laccase Enzyme, *Streptomyces sp.*, Solid State Fermentation

حسين وأخرون

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تحديد الظروف المثلى لانزيم اللاكيز المنتج من العزله المحليه لبكتريا *Streptomyces sp.* بوساطه تخمرات الحاله الصلبه

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المستخلص

اختبرت ستة عزلات محليه من البكتيريا والفطريات لانتاج انزيم اللاكيز شملت *Pseudomonas sp.* و *Bacillus sp.* و *Candida sp.* و *Klaveromyces sp.* و *Aspergillus sp.* و *Streptomyces sp.* باستعمال تقنيه المزارع المغموره. تميزت العزلات *Streptomyces sp.* و *Candida sp.* و *Pseudomonas sp.* بانتاجيتها العاليه لانزيم اللاكيز، ودرست قابليتها على انتاج الانزيم بوساطه تخمرات الحاله الصلبه باستغلال دعائم الوسط الصلب المتمثله بنشاره الخشب المدعم بنسبه 0.06% زليلين اوخليط من نشارة الخشب ونخاله الحنطه بنسبه 1:2 المدعم بالزليلين بنسبه 0.06% لكل وسط. لوحظ ان انتاجيه اللاكيز المنتج من بالعزلات الثلاث بوساطه خليط من نشاره الخشب ونخاله الحنطه بنسبه 1:2 قد وصلت الى 177.8، 144.3 و 103.46 وحدة. غم¹ على التوالي. حددت الظروف المثلى لانتاج انزيم اللاكيز من العزله المحليه *Streptomyces sp.* باستعمال خليط من وسط نشارة الخشب ونخاله الحنطه بنسبه 1:2 ، اذا كان الاس الهيدروجيني الامثل 5.5 والحضن عند درجه حرارة 30 م° لمدة 14 يوم وبلغت الفعاليه النوعيه للانزيم 1315 وحدة. ملغم⁻¹ بروتين. بلغت كفاءه تفكيك صبغات معامل الانسجه (الصفراء والحمراء والسوداء) اقصاها باستعمال المستخلص الخام للاكيز وكانت 87% و 43% و 74% بعد ثلاث ساعات على التوالي .

كلمات مفتاحيه: انزيم اللاكيز، بكتريا الستربتومايسس، تخمرات الحاله الصلبه

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2.) is a multicopper blue oxidase capable of oxidizing ortho and para-diphenols as well as aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical (10). Laccases can catalyse the oxidation of many substances coupled to the reduction of molecular oxygen to water (13). Laccases are widely found in fungi and higher plants as well as in a lower proportion of insects and bacteria, more than a hundred laccases have been isolated and characterised with the majority derived from fungi, including specifically white rot basidiomycetes, plant and bacteria (22). In contrast to fungal laccases, only a few bacterial laccases have been the subject of study. More recent and rapid progress in whole genome analysis suggests that these enzymes were widespread in bacteria (33). Bacterial laccases have advantageous properties compared to fungal laccases with respect to industrial applications. Compared to fungal laccases, they were highly active and much more stable at a high temperature and high pH value (33). Laccase production was carried out using two types of fermentation: submerged fermentation (SMF) and solid-state fermentation. Solid-State Fermentation (SSF) is a fermentation process conducted in the absence of free-flowing water, using either a natural support or an inert support as a solid material (21). The selection of an appropriate solid material for performing solid state fermentation is very important as it has a strong influence on the process (12). The current study focuses on the production of laccase enzyme by a local isolate of *Streptomyces* sp. as well as determining the optimum conditions for enzyme production.

MATERIALS AND METHODS

Chemicals Cetrimide agar, Nutrient agar, Nutrient broth, Potato dextrose agar (PDA), Potato dextrose broth (PDB), soya bean, Mannitol, o-tolidine, xylene and all other reagent grand chemicals were purchased from Hi-Media and Sigma Aldrich, India.

Mineral Salt Medium (MSM) The medium was prepared from the following components (per litre of distilled water): Yeast Extract 0.5g, NH_4NO_3 0.1g, CaCO_3 0.5g,

$\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$ 0.02g and $(\text{NH}_4)_2\text{SO}_4$ 0.002g (26).

Microbial Isolation Sources

Six different bacterial and fungal isolates included *Pseudomonas* sp., *Bacillus* sp., *Candida* sp., *Klaveromyces* sp. *Aspergillus* sp. and *Streptomyces* sp. were previously isolated from industrial locations, included electrical generators, contaminated places with dyes, agricultural locations, garden soil and oil sediments. Each of these were obtained from the Biotechnology Department, College of Science at University of Baghdad. Identified previously by an Epi-test and biochemical tests, these isolates were prepared for screening experiments of laccase production according to methods described by Arunkumar et al. (4).

Quantitative Screening of laccase production by local isolates: Submerged culture (Liquid Fermentation)

Preparation of Microbial Inoculum

Suspension :Isolates from cetrimide agar, potato dextrose agar and soya bean agar were inoculated into nutrient broth, potato dextrose borth and soya bean broth separately. Thereafter, they were incubated at 30°C for 24 hours to develop bacterial isolates and for 7 days for fungal isolates. This process was conducted separately before using the results for further tests.

Enzyme Production in Submerged Fermentation

Three types of media (50 ml) were used for the preparation of laccase production according to the type of microorganism for producing laccase; A: nutrient broth medium with 0.06% xylene, B: PDB with 0.06% xylene and C: soya bean broth medium with 0.06% xylene were inoculated with 2.0 ml of fresh culture of isolate separately. The flasks were incubated at 30°C in the rotary shaking incubator (150 rpm) for 24 hours for bacterial isolates and for 7 days for fungal isolates. After incubation time, the enzyme of each flask was extracted by centrifugation at 8000 rpm for 10 min. The clear supernatant was considered as a crude enzyme and it was assayed for laccase activity and protein concentration. The enzyme activity and protein concentration were applied simultaneously.

Solid State Fermentation (SSF)

Three isolates with maximum productivity, based on submerged fermentation, were selected and cultivated on two types of solid state fermentation media. These included A: sawdust medium with 0.06% xylene and B: sawdust: bran (2:1) media with 0.06% xylene. Flasks containing 5gm of each media separately and wetted with MSM were inoculated with 2.0ml of fresh culture of isolates. The flasks were incubated at 30°C for 24 hours for bacterial isolate and 7 days for fungal isolate. After the incubation period, laccase enzymes of each flask were extracted by using 0.1M of phosphate buffer pH 7.0, followed by gauzes then by centrifugation at 8000 rpm for 10 minutes. The clear supernatant was considered as a crude enzyme and it was assayed for laccase activity and protein concentration (4).

Laccase Assay

According to the method described by Kalral et al. (17), laccase activity was estimated by using O-tolidine as a substrate. Using a spectrophotometer, the oxidation of O-tolidine was detected by measuring the absorbance increase at 366 nm ($\epsilon_{366} = 27,600 \text{ M}^{-1} \text{ cm}^{-1}$). Protein concentration was measured according to the method described by Bradford (6).

Optimum Conditions for Laccase Production

Effect of Fermentation Media: The influence of the solid media on the production of laccase was checked by cultivating the isolate, *Streptomyces* sp. in two types of culture media as mentioned above. Erlenmeyer flasks (250ml) containing 5gm of each tested medium in duplicates and wetting with M.S.M. were sterilised and inoculated with 2% of fresh culture from the isolate. Flasks were incubated for 7 days at 30 °C. After the incubation, an enzyme from each flask was extracted by using 0.1 M of phosphate buffer pH 7.0, followed by gauzes then by centrifugation at 8000 rpm for 10 minutes. The filtrate was taken for the determination of the enzyme activity, protein concentration and the specific activity (2).

Effect of Temperature

Laccase production was achieved in an optimum fermentation medium pH 7.0 at different temperatures including 25°C, 30°C,

35°C and 40°C. After sterilisation, the flasks were inoculated with 2% of the fresh grown isolate and incubated for 7 days. After the incubation period, the enzyme was extracted from each flask and determine the enzyme activity, protein concentration and the specific activity (28).

Effect of Initial pH at Media

To determine the influence of the initial pH value of the medium on enzyme production, Erlenmeyer flasks containing 5gm of the selected solid fermentation medium were dampened by M.S.M. enhanced with 0.06% xylene with a range of different pH values ranging from 4.5 to 10 were established. Thereafter, the post sterilisation culture medium was inoculated with the selected isolate and incubated at 30°C for 7 days. After the incubation period, supernatant was taken from each flask and the enzyme activity, protein concentration and the specific activity were measured (20).

Effect of Moisture Ratio

Mineral salt medium enhanced with 0.06% xylene was added to the culture media (5gm) at different ratios: 1:1, 1:2, 1:3, 1:4 and 1:5 w/v. The humidified medium was placed in Erlenmeyer flasks and autoclaved at 121°C for 15 minutes. The autoclaved medium was inoculated with 2 ml of selected bacterial inoculum and incubated for 7 days at 30 °C. Flasks without inoculation were used as control (23).

Effect of Incubation Period

Different incubation times were examined to determine the suitable incubation period for laccase production. The selected medium was humidified with M.S.M enhanced with 0.06% xylene at the optimum pH, inoculated with 2.0 ml of 24-hour old culture isolate and incubated at 30 °C for different incubation periods of 1, 2, 7 and 14 days. Flasks without inoculation were used as control (16).

Laccase Application

Dyes Decolourisation : For decolourisation experiment, the dyes, textile black, textile red and textile yellow, obtained from Al-diwanayah textile factory, were used in the present study. The reaction mixture for the degradation of dyes contains 10ml of 30mg/l for each dye and 1ml of (1265 U. ml⁻¹) of each individual crude enzyme solution in a

modified form, as referred to by Alam (1). The reaction mixture was incubated in a shaker incubator at 120 rpm. Instead of enzymes, distilled water was used in the control experiment. The degradation of a specific dye was calculated in different incubation timeframes of 0, 1, 2 and 3 hours. The percentage of removal efficiency for each dye was calculated by the absorbance at λ max according to Zhang et al. (38).

RESULTS AND DISCUSSION

Quantitative Screening of Microbial Isolates Screening the Isolates in Liquid Media

(SMF): In this study, six microbial isolates were obtained and screened for their enzymatic activity according to the medium type of each one. Bacterial isolates were cultivated in nutrient broth with 0.06% xylene, fungal isolates were cultivated in PDB with 0.06% xylene and *Streptomyces* isolate was cultivated in soya bean broth with same ratio of xylene. Among six isolates, *Streptomyces* sp., *Pseudomonas* sp. and *Candida* sp. illustrated the highest enzymatic activity and laccase specific activity in crude supernatant reached 3464 U. ml⁻¹ protein from *Streptomyces* sp., 4057 U. ml⁻¹ protein from *Pseudomonas* sp. and 4670 U. ml⁻¹ protein from *Candida* sp. However, the laccase specific activity for the other isolates ranged between 68.5 and 593.1 U. ml⁻¹ protein (Fig. 1). In accordance with these results, the isolates *Streptomyces* sp., *Pseudomonas* sp. and *Candida* sp., which had the higher specific activity, were selected for using in SSF. The variation between members of the same isolated for laccase production may be due to the genetic variation and the type and sources of isolates. In addition, the conditions of cultivation, such as media components, temperature, pH and aeration and stirring, have contributed to the increased ability of the isolates to produce the enzyme in a liquid medium (8).

Screening the Isolates in Solid State Fermentation

For more detection and meticulous selection of efficient microbial isolates to produce laccase, three isolates of *Streptomyces* sp. *Pseudomonas* sp. and *Candida* sp. From SMF media with highest specific activity were screened again for their enzymatic activity.

Cultivation in SSF media including sawdust, sawdust and bran mixture (2:1) were supplemented with 0.06% xylene. Among three isolates, the isolate *Streptomyces* sp. produced the highest enzymatic productivity, laccase productivity in crude extract reached 177.8 U.g⁻¹ in mixed medium with 80.4 U.g⁻¹ in sawdust medium fig. (2A), *Pseudomonas* sp. has 103.46 U.g⁻¹ productivity in mixed medium and with 69.2 U.g⁻¹ in sawdust medium fig. (2B). However, laccase productivity for *Candida* sp. reached 144.3 U.g⁻¹ in mixed medium and in sawdust medium, the productivity reached 35.5 U.g⁻¹ fig. (2C). According to these results, *Streptomyces* sp. was considered the best isolate for laccase production and the mixed substrate sawdust: wheat bran (2:1) was selected for improving laccase production by a solid substrate. This may be due to its depth and diversity in nutrient value, such as proteins, vitamins, functional compounds and lipids (34). The supply of compounds also stimulates the production of required biomolecules and increases its metabolic activity (9). Sawdust /wheat bran is a substrate and a simple which is composed of co-products of coarse agricultural origin containing all the nutrients required for growth of micro-organisms (24). Conclusively, the agriculture waste media did not support the production of the enzyme for any of the above isolates. The selection of an appropriate solid material for performing solid state fermentation is very important, as it has a strong influence on the process (12). Selection is dependent on several factors which are primarily related to cost and availability and, as such, may involve screening of several agro-industrial residues. Moreover, the utilisation of this support mechanism helps to solve issues of pollution caused by their disposal (31). Laccase production proved to be reliant on the medium and improved enzyme production in the medium can be attributed to the availability of a complete pool of amino acids, carbon and nitrogen sources as well as other supplements (35). Niladevi with co-workers (25) identified rice straw as a suitable substrate for laccase production from *Streptomyces psammoticus* in a solid-state fermentation (17.3 U.g⁻¹), followed by coffee

pulp (U.g^{-1}) (24). Evidence also illustrated that among six substrates using *Plerotus ostreatus*, wheat bran was the best substrate for laccase production with maximum specific activity at 410.30 U.mg^{-1} .

Optimum Conditions for Laccase Production

Different bioprocess conditions, affecting laccase production by *Streptomyces* sp. under solid state fermentation, were optimised for maximum enzyme production. A number of factors were identified as influencing the growth and production of laccase such as culture media, initial pH, temperature, incubation time and wetting ratio. The impact of these factors, in relation to a combination of media components, also influences the growth and metabolite production (29). Hence, optimisation of the fermentation process facilitates the reduction of fermentation cost and helps to obtain a high yield of laccase enzyme.

Effect of Temperatures on Laccase Production

Results in fig. (3) highlighted the capability of isolate *Streptomyces* sp. to grow and produce laccase at wide range of temperatures including 25°C , 30°C , 35°C and 40°C . Laccase production was found to be maximum at 30°C with specific activity of 757.3 U.mg^{-1} . The specific activity of laccase at 25°C , 35°C and 40°C was 457.3, 754 and 344 U.mg^{-1} respectively. Temperature fluctuations are key influencers on the rates of biochemical reactions, either by inducing or repressing enzyme production (36). Hynes and Tagg (15) indicated that the production of laccase enzyme was inhibited in low temperatures. The temperature was affected on microbial enzyme production by influencing the solubility of oxygen in the media, the vibration energy of molecules and speed of enzymatic reactions in the cells that reflect positively or negatively on enzyme production (7). According to (19), temperature influences the growth in solid state fermentation, the production enzyme and their metabolites. (25) found that the optimum temperature of laccase production from *Streptomyces psammoticus* in solid-state fermentation was 32°C (27.6 U.g^{-1}).

Effect of Initial pH on Laccase Production

To study the effect of the initial pH on laccase production, *Streptomyces* sp. was cultivated in the production medium with different pH values. The pH of the medium was adjusted to 4.5, 5, 5.5, 6, 7.0, 7.5, 8 and 10. As can be seen in figure (4), the highest laccase specific activity, 1292 U.mg^{-1} was obtained at pH 5.5. However, the increase or decrease of pH values above or below 5.5, can lead to a reduction in enzyme activity. Generally, the effect of pH in enzyme production is attributed to its role in the solubility of the medium nutritional substances, its influence on the substrate ionization and its availability for the micro-organism, in addition to its influence on the enzyme stability (28). From another perspective, pH of the culture media often influences the fermentation course and enzyme production rate. pH of the medium drastically affects the conformation of the plasma membrane which, subsequently, affects the membrane bound ribosomes involved in protein synthesis (30). The pH of the culture significantly influences many enzymatic processes and transports the compounds across the cell membrane (32). (25) reported that the optimum pH of laccase production from *Streptomyces psammoticus*, in solid-state fermentation for moistening the rice straw medium, was 8.0.

Moisture Ratio

Moisture is another key parameter to control the growth of micro-organisms and metabolite production in solid state fermentation. Five gm of sawdust: wheat bran (2:1) was humidified by distilled water and enhanced with 0.06% xylene with different moisture ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 w/v. These treatments were tested to select the optimum moisture ratio for laccase production. The best moisture ratio was 1:4 w/v which gave specific activity 850 U.mg^{-1} . The ratios 1:1, 1:2, 1:3 and 1:5 resulted in 330, 540, 680, and 820 U.mg^{-1} respectively (Fig. 5). The moisture level in SSF has a great impact on the physical properties of the substrate. Solid substrates used in SSF are insoluble in water. Therefore, water will need to be absorbed on the substrate particles, which could be used by the micro-organisms for growth and metabolic activity. Thus, the degree of hydration of the substrate

plays an important role on the growth of the fungi and, subsequently, the enzyme production (27). Higher initial moisture in SSF leads to sub-optimal product formation due to reduced mass transfer and a decrease in initial moisture level results in reduced solubility and low availability of nutrients to the culture (14). (25) identified that the initial moisture content of laccase produced by *Streptomyces psammoticus* was 65% (26.8 U.g^{-1}). Humidified banana stalks with a moisture ratio of 60% were used to produce laccase from *Schizophyllum commune* IBL-06 and the laccase specific activity was 40 U.mg^{-1} (16).

Incubation Time

In order to optimise the maximum incubation period for laccase production, *Streptomyces* was grown on sawdust/bran (2:1) for different incubation periods of 1, 2, 7 and 14 days. Results in fig. (6) show that a gradual increase in specific activity of laccase enzyme with increased incubation periods reached the maximum activity (1315 U.mg^{-1}) on the 14th day of incubation. This may be attributed to the change in conditions of culture during this period, such as diminishing oxygen and nutrients. Selected studies have demonstrated that production of laccase by *Plerotus ostreatus*, observed after 7 days of incubation in solid state fermentation medium, contain wheat straw as substrate (5). Whereas (23) identified the laccase production from *Bacillus subtilis* MTCC was 2414 after a 96-hour period.

Application of Laccase enzyme

The various textile dyes (textile yellow, textile red and textile black) degradation capability of laccase (crude) were studied at dyes concentration of 30 mg/l , after 3 hr as seen in fig.(7). Absorbance of each dyes was recorded at suitable wave length for each one. Results showed that the removal efficiency were reached to (87, 43 and 74) % of yellow, red and black dyes respectively. Also the result offered that the value of each absorbance was decreased through the incubation time increases and stabilized after 3 hr and, compared with absorbance of the control which didn't changed during 3 hrs ,these results indicated that laccase have ability to degrade different dyes. Kokol (18) observed that crude laccase without purification is

cheaper and more stability comparing to purified laccase. Nevertheless, using crude laccase would reduce the cost of enzymatic-based decolorization process in industrial scale. These dyes were not decolorized at the same extent that may be due to the difference of the redox potentials and the suitability of their steric structure with the active site of the enzyme (37). The extent of decolorization activity depends on the source of the enzyme and the chemical structure of the dye (11).

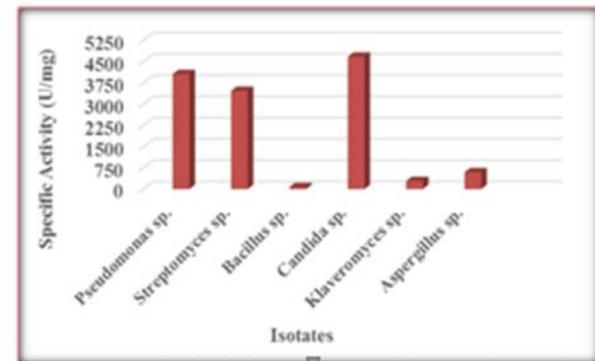


Figure 1. Quantitative screening of different isolates for laccase production using submerged cultures

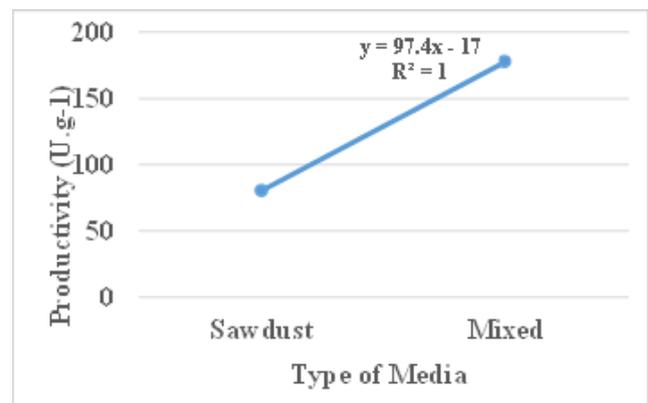


Figure 2 A. Effect of different media on laccase production from *Streptomyces* sp. isolates using SSF

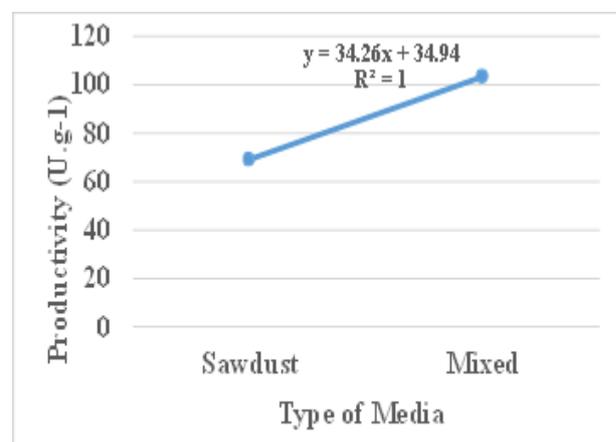


Figure 2 B. Effect of different media on laccase production from *Pseudomonas* sp. isolates using SSF

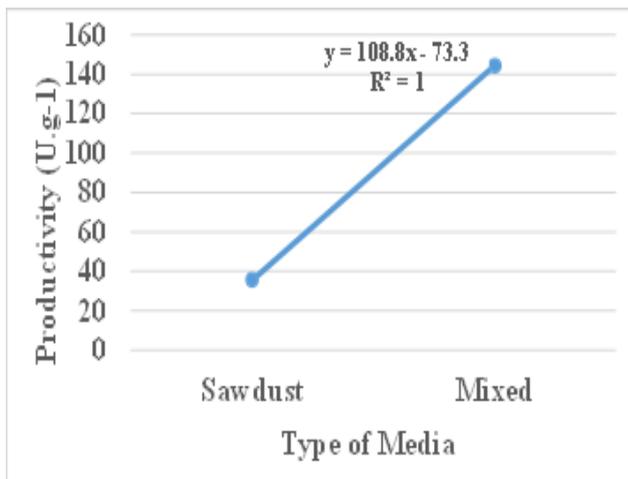


Figure 2 C. Effect of different media on laccase production from *Candida* sp. isolates using SSF

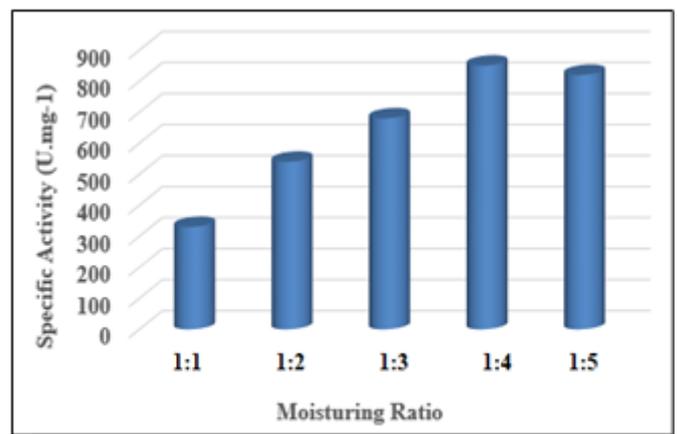


Figure 5. Effect of moisturing ratio on laccase production from *Streptomyces* sp. using solid state culture system in pH 5.5 at 30 °C after 7 days

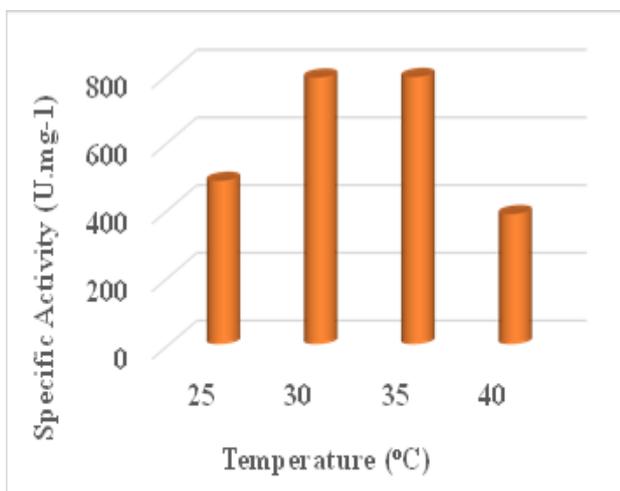


Figure 3. Effect of temperatures on laccase production from *Streptomyces* sp. in pH 7.0 after 7 days

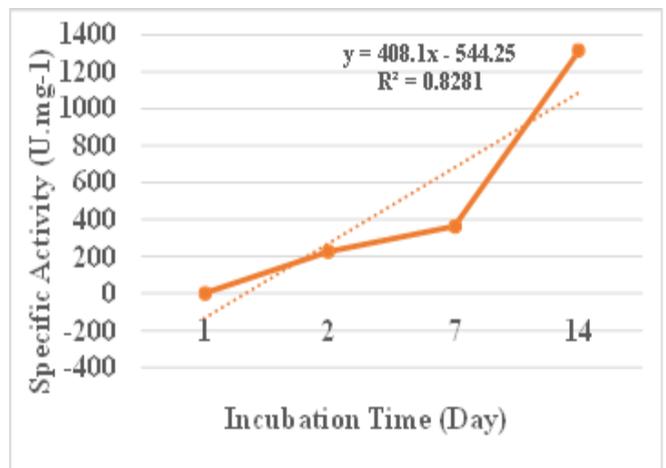


Figure.6 Effect of incubation time on laccase production from *Streptomyces* sp. using solid state culture system in pH 5 at 30 °C

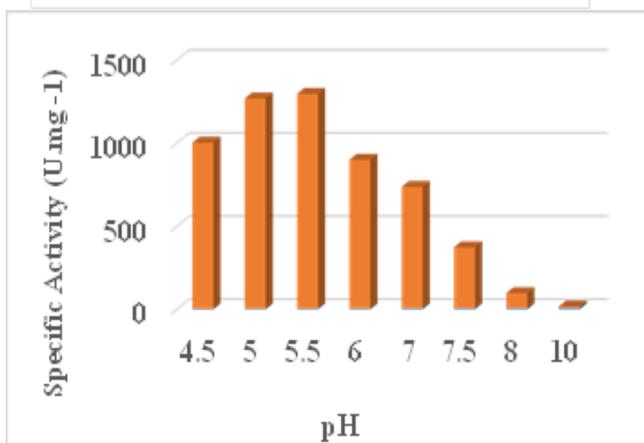


Figure 4. Effect of initial pH on laccase production from *Streptomyces* sp. using solid state culture system at 30 °C for 7 days

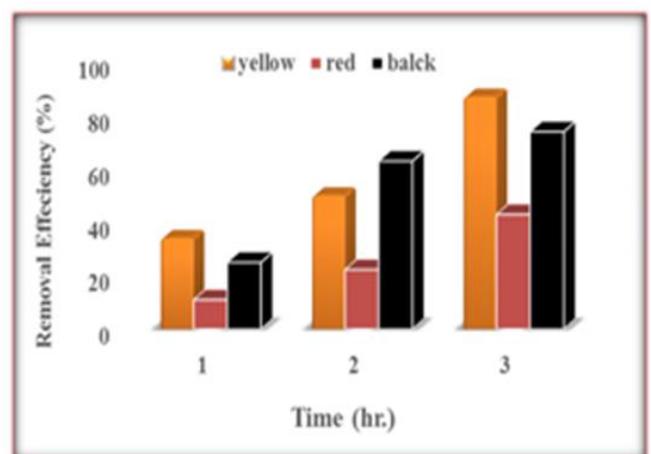


Figure 7. Dyes decolorization by crude laccase produced by *Streptomyces* sp. at a concentration of 30 mg/l after 3 hr

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