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DETERMINATION THE OPTIMUM CONDITIONS OF LACCASE PRODUCTION BY LOCAL ISOLATE OF *PSEUDOMONAS AERUGINOSA* SR3 USING LAB SCALE FERMENTER

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ABSTRACT
Eighty eight local isolates of *Pseudomonas* sp. which were previously isolated and identified subjected to the primary and secondary screening processes to select the active *Pseudomonas* sp. isolate for laccase production. Among the isolates screened, ten isolates with maximum zone of hydrolysis and biodegradation ability in primary screening (the ratio of Z/G more than 10 mm) were selected for secondary screening. It has been found that *Pseudomonas* SR3 had the highest productivity of the enzyme (1102 U/mg protein). The selected isolate with highest level of laccase activity was identified as *P. aeruginosa* SR3 according to PCR technique. The optimum conditions of laccase production by isolate *P. aeruginosa* SR3 using submerged fermentation were obtained in the mineral salt medium (MSM) with p-ansidine as the best production medium, 1% glucose as the best carbon source, 3% peptone as nitrogen source, 3:1 C:N ratio, temperature 30 °C and pH 7.5, after 27 hr of incubation period. Laccase production was induced at concentration of 4 mM of CuSO4 and 0.06% of xylene. The production of laccase from *P. aeruginosa* SR3 was conducted in lab scale fermenter with continuous stirred. It has shown from the results that maximum laccase production was observed after 24 hr of incubation, beyond that the specific activity was enhanced after 18 hrs as far as it reach to maximal value after 24 hrs of incubation, which reached to 6214 U/mg protein, whereas after 24 hrs the production of laccase was decreased with increasing the incubation time. The results also reviled that laccase production started when xylene concentration was nearly depleted from the medium in the bioreactors after 24 hr.

KEYWORDS: *Pseudomonas* sp., hydrolysis, biodegradation ability, peptone, bioreactors.

INTRODUCTION

Laccases (EC 1.10.3.2) is an extracellular, multicopper oxidases enzyme belonging to the group of the blue copper proteins that uses molecular oxygen to oxidize various aromatic and nonaromatic compounds by a radical-catalyzed reaction mechanism. One-electron oxidation, leading to generation cation radicals of the pollutants, is performed by the ligninolytic enzymes. Those radicals can initiate chemical reactions like cleavage of C-C bonds or hydroxylation, which can result in more hydrophilic derivatives. Those products are co-metabolized the strains in the presence of appropriate carbon source or carbon dioxide[1]. Laccase was first described by Yoshida in 1883 when he extracted it from the exudates of the Japanese lacquer tree *Rhus vernicifera*, from which the name laccase was derived[2]. Laccases are widely distributed and found in fungi, higher plants, bacteria and also in a lower proportion in insects. Bacterial laccase is multi-copper oxidases (LMCOs) probably still the most favored expression system for recombinant proteins, in particular for industrial biocatalysts. Various approaches for increasing the yield of active recombinant enzymes in bacteria have been described [3] and [4] Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin [5], classical substrates of laccases include various lignin-derived phenols and aromatic amines. Ortho-substituted compounds as (guaiacol, caffeic acid, gallic acid, dihydroxy phenyl alanine, pyrogallol, o-phenylene diamine) tend to be better laccase substrates than para-substituted compounds as (p-cresol, p-phenylenediamine), therefore laccases are suitable for use in biotechnological applications and have advantages over other enzymes. The commonly compounds used for the detection and measurement of laccase activities represented by syringaldazine, (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) ABTS, 2,6-Dimethoxyphenol (2,6-DMP), O-tolidine [6,7]. Laccases are applied in many industrial sectors such as paper processing, discoloration of wine, environmental pollutants detoxification and chemical production from lignin[8]. The demand for removal of synthetic dyes from the textile industrial waste using fungal and bacterial laccase is being increased tremendously. The Current study was aimed to collection and identification of local isolates of *Pseudomonas* sp., screening and evaluation the ability of local isolates for laccase production, determination the optimum conditions for laccase production from selected isolate and large scale production of laccase in optimized conditions using lab scale bioreactor under completely controlled system.

MATERIALS & METHODS

Chemicals

Nutrient agar, (o-tolidine), and all other reagent grand chemicals were purchased from Hi-Media and Sigma-Aldrich, India.

Samples collection and bacterial isolation

Eighty eight isolates of *Pseudomonas* sp. which were previously isolated from industrial location, places of electrical generator, sites contaminated with dyes, agriculture place, garden soil and oil sediments were obtained from the Biology and Biotechnology Department /College of Science/ Baghdad University, Iraq. The isolates were identified previously by Epi and biochemical tests, and maintained on cetrimide agar medium as a selective medium, these isolates were prepared for screening experiments. The isolates were screened for laccase production according to method described by Arunkumar *et al*. [9].

Screening the *Pseudomonas* sp. isolates for laccase production

Primary screening (Semi-quantitative screening)

Screening of isolates in methyl orange solid medium

Eighty eight isolates of *Pseudomonas* sp. were screened to select the best isolates produced laccase by plate assay using MSM solid medium containing (g/l) (glucose 1g, yeast Extract 2g, NaCl 0.1g, CaCO3 0.02g, CuSO4 0.001, MgSO4 0.002g, FeSO4 0.01g and ZnSO4 0.009) supplemented with 0.1 g/l methyl orange as a source of hydrocarbon. A single colony of bacterial isolate previously activated in nutrient broth was spotted to the middle of MSM with methyl orange agar plate and then incubated at 30°C for 24 hrs. Clear zone of methyl orange hydrolysis around the colony was an indication of laccase secretion.

Screening of isolates in o-tolidine solid medium

Fifteen isolates of *Pseudomonas sp*. were previously grown on MSM with methyl orange agar and formation the clear zone around the its growth were transferred to the surface of MSM agar medium containing 2mM of o-tolidine then the isolates were incubated at 30°C for 24 hrs. Clear zone around the colony was an indication of laccase production. The radius of a clear zone was measured using electronic ruler in mm.

Secondary screening (Quantitative screening)

Preparation of bacterial inoculum suspension

Isolates from a cetrimide agar stocks were inoculated into nutrient broth and incubated for 24 hrs. at 30°C before using for tests. After incubation period, the cells in the suspension were counted by heamocytometer and the cell suspension was diluted using the same broth until 3×10^8 cell / ml was obtained.

Enzyme production using submerged culture

Fifteen isolates with maximum productivity based on primary screening were selected and cultivated on MSM with p-ansidine as inducer. A 250 ml flask containing 50 ml of MSM with p-ansidine medium was inoculated with 2.0 ml of overnight culture of isolates 3 $x10^8$ cell/ml. The flasks were incubated at 30◦C in the rotary shaking incubator (150 rpm) for 24 hrs. After 24 hrs incubation, enzyme of each flask was extracted by centrifugation at 8,000rpm for 10min. 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 run in duplicate.

Laccase activity

Laccase activity was estimated according to the method described by Kalral *et al*.[10] by using o-tolidine as a substrate, the oxidation of o-tolidine was detected by measuring the absorbance increase at 366 nm (ε366 = 27,600 M-1 cm-1) using a spectrophotometer. Protein concentration measured according to the method described by Bradford [11].

Identification of *Pseudomonas aeruginosa* isolate

Identification of selected *Pseudomonas* sp. which showed higher laccase activity followed by using sequences of the 16S ribosomal RNA, DNA extraction, polymerase chain reaction (PCR). The universal bacterial primer set was used to amplify 16S rRNA from the genomic DNA extracted 341F and 907R.The PCR reaction mixture was prepared as follows: 1 × PCR buffer, 3 µM MgCl2, 200 µM of each deoxynucleoside triphosphate (Promega Corporation, Wisconsin), 0.5 µM of each primer, 1U of Taq DNA polymerase (Promega Corporation, Wisconsin) and DNA templates in a final volume of 50 µl. All amplification reactions were conducted using a Rapid Cycler (Idaho Technology, Idaho). The PCR cycling conditions for the set were a touchdown approach of 30 cycles as follows: genomic DNA was denatured at 94°C for 5 min, followed by 5 cycles of 92°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 sec, 5 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec, and 20 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 sec with a final extension step of 72°C for 7 min. A 2-min incubation at 72°C was added to the end of PCR program. The positive control of *P. aeruginosa* and nuclease-free water as a negative control was included in every procedure.

Optimum Conditions for Laccase Production

Effect of fermentation media

The influence of fermentation media on the production of laccase was tested by cultivating of *Pseudomonas* sp. isolate in different culture media include (MSM with Para-ansidine, Nutrient broth with P-ansidine, Soya meal media, Yeast extract media, Starch media). Erlenmeyer flasks (250ml) containing 50ml of each tested medium in duplicates were sterilized and inoculated with 2% of overnight culture (3×10^8cell/ml) of the isolate. Flasks were incubated in shaker incubator (150 rpm) for 24 hr at 30 ºC. After the incubation, supernatant from each flask were filtered by centrifugation and the filtrate was taken for the determination the enzyme activity, protein concentration and the specific activity [12].

Effect of Carbon Sources

Fifty ml of optimum fermentation medium was prepared in (250 ml) Erlenmeyer flasks, pH was adjusted to 7.0. These flasks were sterilized by autoclaving at 121˚C for 15 min., then each sugar solution (fructose, maltose, sucrose, glucose, lactose, ribose, galactose, and starch)) was added in each flask separately. The flasks were inoculated with 2% of overnight culture of the isolate *Pseudomonas* sp. ($3x10^8$cell/ml) and incubated in shaker incubator (150 rpm) at 30ºC for 24 h. After the incubation, the cultures were centrifuged and the filtrate was taken for the

determination enzyme activity, protein concentration and the specific activity were measured [12].

Effect of Nitrogen Sources

The laccase production medium containing optimum carbon source was supplemented with (2 g/l) of different nitrogen sources (tryptone, yeast extract, urea, peptone, NH_4Cl and KNO_3) and the pH was adjusted to 7.0. After sterilization, the flasks were inoculated with selected isolate; the medium was incubated in shaker incubator (150rpm) at 30ºC for 24h. After the incubation, a supernatant were filtered from each flask and the filtrate was used for the enzyme activity, protein concentration and the specific activity were measured [13].

Effect of Carbon to Nitrogen Ratios (C: N)

In order to investigate the best carbon: nitrogen ratio that gives the maximum production of laccase, different C: N ratios were studied. Fifty ml of optimum fermentation medium was prepared in 250 ml Erlenmeyer flasks, each contained the best carbon and nitrogen source at different C: N ratios (1:0.5, 1:1, 1:1.5, 1:2, 2:1, 2:1.5, 2:2 and 3:1). After sterilization, the flasks were inoculated with 2% of overnight grown isolate and incubated in shaker incubator (150 rpm) at 30ºC for 24h[14]. After the incubation, supernatant were taken from each flask the enzyme activity, protein concentration and the specific activity were measured.

Effect of pH value

To determine the influence of the initial pH value of the culture medium on enzyme production, Erlenmeyer flasks (250 ml) containing 50 ml of the selected fermentation medium were adjusted using 0.1N HCL or 0.1N NaOH to give different pH values ranging from 5 to 10 (5, 6, 6.5, 7, 7.5, 8, 9 and 10), then the culture medium was inoculated with the selected isolate and incubated in shaker incubator (150 rpm) at 30ºC for 24h[15]. After the incubation, supernatant were taken from each flask and the enzyme activity, protein concentration and the specific activity were measured.

Effect of temperature

Laccase production was achieved at different temperatures (25, 30, 37and 42). After sterilization, the flasks were inoculated with 2% of overnight grown isolate and incubated in shaker incubator (150 rpm) at pH 7.5 for 24hr [16]. After the incubation, supernatant was taken from each flask for the determination the enzyme activity, protein concentration and the specific activity.

Effect of incubation periods

In order to determine the optimum incubation time for laccase production, the time course for enzyme production was followed (0, 2, 21, 24, 27, 30, 33, 48, 51, 55, 70 and 72)hr. The production medium was prepared and inoculated with selected isolate and incubated in shaker incubator (150 rpm) at pH 7.5 and at 30 ºC [15]. After each incubation time, supernatant were taken from each flask for the determination the enzyme activity, protein concentration and the specific activity.

Effect the concentration of Copper Sulfate

The effect of copper sulfate as inducer for maximum laccase production was determined by using laccase production medium containing different concentrations of CuSO4 (0.5, 1, 2, 4, 6, 8, 10) mg/l without p-ansidine. The medium was inoculated with the selected isolate and in shaker incubator (150 rpm) at 30ºC for 24h [17]. The separate medium with p-ansidine and CuSO4 at concentration 1 mg/ml was used as a control, the enzyme activity, protein concentration and the specific activity were measured.

Effect of enzyme inducers

Various hydrocarbons as inducers were used to find their effect on the production of laccase by *P. aeruginosa* SR3. The production medium containing optimum concentration of carbon source, nitrogen source, copper sulfate and was supplied with different inducers (Guaicol, Xylene, Toluene, O-Tolidine, benzoic acid, Phenol, Phenanethrene, P-ansidine, Resorcinol and Vanillic acid) at concentration of 0.04% and inoculated with the selected isolate [16]. Finally, the medium was incubated at 30 ^{o}C and pH 7.5 for 27 hr. After the incubation, supernatant were taken from each flask and the enzyme activity, protein concentration and the specific activity were measured.

Effect of xylene concentrations

The best concentration of xylene for maximum laccase production was determined by using production medium containing different concentrations of xylene (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.5, and 1)%. The medium was inoculated with the selected isolate after sterilization and incubated in shaker incubator (150 rpm) at 30ºC and pH 7.5 for 27h. Then the filtrate was taken to measure the enzyme activity, protein concentration and the specific activity.

Production of laccase in lab scale fermenter

Lab scale fermenter (5L) was carried out with optimum conditions result from batch experiment. Approximately 3.5 liter (working volume) of fermentation medium was prepared with addition of xylene instead of p- ansidine according to the results of optimum inducer. Then the fermentation media was autoclaved in-situ within the bioreactor after covering and taping of sensitive parts of the bioreactor at 121ºC for 15 min. After sterilization, the bioreactor was transferred to the safety biological cabinet and the different probes and autoclaved parts were reconnected to the bioreactor. The sterilized medium was inoculated with 2% (v/v) *P. aeruginosa* SR3 isolate and the bioreactor temperature was set on 30ºC with agitation at 400 rpm at a regular interval of 24h during operation, the fermentation was extended for three days. Sample was taken at (0, 3, 18, 21, 45, 48, and 66) hr and analyzed for laccase activity, protein concentration, specific activity and biomass whereas xylene concentration was estimated using Gas chromatography (GC) technique under conditions [Column Reprosil 100, C18 dimension (25 x0.46) cm , mobile face (Acetonitrile–water) flow rate (1.2 ml/min.), column temperature (30 C°) and wave length (245nm)] (Analysis was performed in diplicates) [18].

RESULTS & DISCUSSION

Screening of *Pseudomonas* sp. isolates for laccase production

Primary screening of laccase producing isolates (Semi-quantitative screening)

Primary screening using methyl orange solid medium

A simple method of screening was followed to determine laccase producing bacteria on MSM with methyl orange agar medium. The efficiency of bacterial isolates was

compared by estimating the ratio between the colorless zone diameter around bacterial cultures (Z) and the growth zone (G). Among all *Pseudomonas* isolates were screened, among them fifteen isolates were laccase producer, whenever clear zone around the MSM with methyl orange plate well was formed (fig. 1). The clear zones ratio of isolates was ranged from (5.6-16.3) as shown in (table 1). The isolates were further screening to select an active *Pseudomonas* sp. isolate for lacccase production. Shah [19] reported the ability of *Pseudomonas* sp. to decolorize and degrade methyl orange at concentration up to 500 mg/l.

Primary screening using o-tolidine solid medium

Another screening method was applied to select laccase producing bacteria using MSM with o-tolidine plate medium, fifteen bacterial isolates with high productivity, were cultivated on o-tolidine plate. Results recorded in table (1) shows that all isolates were laccase producer through the formation of the clear zone around the MSM with o-tolidine (fig. 2).The isolates were gave different clearance zone ratios ranged from (1.5-30.1) mm. The isolates were chosen for further secondary screening (Quantitative screening). Devasia and Nair [20] found that the different microbes have the ability to degradation and tolerance of different hydrocarbons for up to 0.04% when growing in MSM include (Catechol, Hydroquinone, Pyrogallol, 2,6 dimethoxy phenol, Guaiacol, ABTS, Syringaldazine, Catechin, p-cresol, p-aminophenol, p-phenylene diamine and tropolone) at pH 5.6.

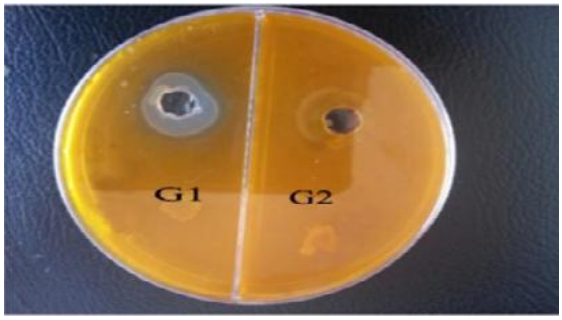

FIGURE 1: Laccase activity of *Pseudomonas* sp. on methyl orange solid medium. G1: Positive result (*Pseudomonas* isolate has a laccase activity on methyl orange agar medium), G2: Negative result (*Pseudomonas* isolate doesn't have a laccase activity on methyl orange agar medium)

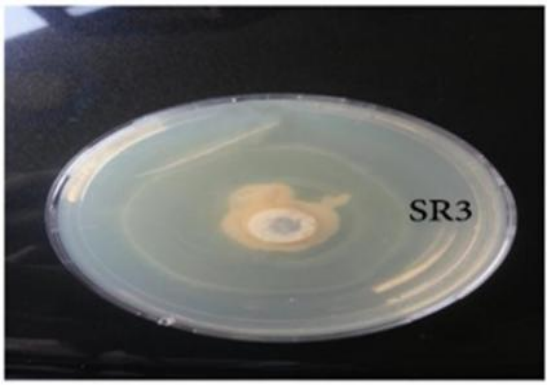

FIGURE 2: Laccase activity of *Pseudomonas* sp. on O-tolidine solid medium

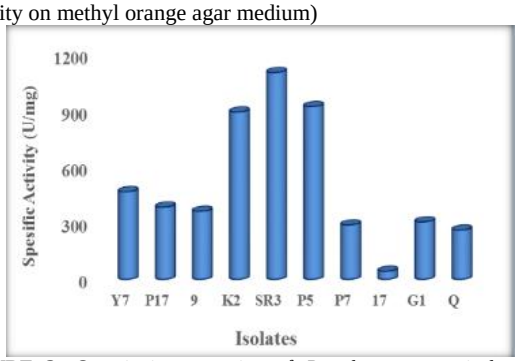

FIGURE 3: Quantitative screening of *Pseudomonas* sp. isolates for laccase production using submerged fermentation medium

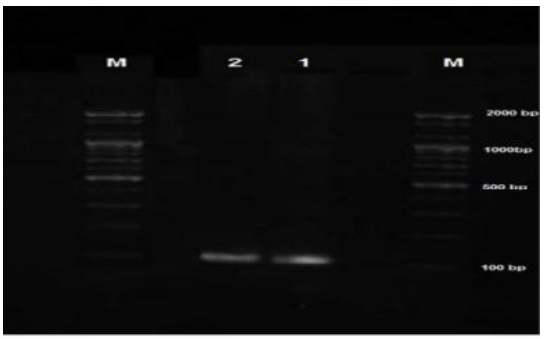

FIGURE 4: *Pseudomonas* isolate with 341F and 907R primers set (The first and the second lines are positive *P. aeruginosa* isolate, M: marker)

Secondary screening (Quantitative screening)

Ten isolates of *Pseudomonas* sp. with maximum zone of hydrolysis and biodegradation ability in primary screening (the ratio of Z/G more than 10) were screened again for their enzymatic activity by cultivation it in MSM supplemented with 0.04% p-ansidine. Among ten isolates, the isolate (SR3) gave the highest enzymatic activity, laccase specific activity in crude supernatant was reached to 1102 U/mg protein, whereas the laccase specific activity for the other isolates were ranged between 43 and 920.8 U/mg protein (fig. 3). In accordance with these results, the isolate Pseudomonas SR3 which had the higher specific activity was selected for remaining studies. The variation between members of the same species in ability for laccase production may be due to the genetic variation, the type and sources of isolates and the conditions of cultivation, such as media components, temperature, pH and aeration and stirring, which helped to increase the ability of SR3 isolate to produce the enzyme in a liquid medium [21] and [22].

Identification of *P. aeruginosa* isolate

In order to undertake the molecular analysis, DNA was extracted from the putative *P. aeruginosa* candidates isolated using both complementary methods. In studies of bacterial populations associated with economic pollution, *Pseudomonas* derived DNA, which is readily amplified with general bacterial primer sets, can be easily detected following DNA extraction and subsequent PCR amplification.

TABLE 1: The Z/G ratio of Pseudomonas sp. In methyl orange and o-tolidine solid medium

No.	Isolated symbol	Ratio (Z/G) in methyl orange mm	Ratio (Z/G) in o-tolidine mm
1	G1	10.3	14.2
2	P5	11.9	10.7
3	SR3	12.3	30.1
4	P7	13.3	22.6
5	Q	12.8	19
6	K14	5.7	1.5
7	P17	16.3	16.9
8	17	10.2	26.2
9	9	14.2	17
10	K2	10.7	29.2
11	71	6.3	18.3
12	S	5.6	4.2
13	Y7	16.2	21.5
14	M	14.4	7.3
15	A	9.5	2.4

Z: Inhibition zone diameter, G: Growth culture diameter

TABLE 2: Laccase production by P. aeruginosa SR3 in a bioreactor under the optimum conditions

Time (hr.)	pH	Specific Activity U/mg	Xylene Con. mg/l (ppm)	Biomass g/L
Initial Time	7.3	53.8	2250	0.1
3	7.5	130.8	662	0.5
18	7.5	3990	54	2.7
24	7.5	6214	0	4.5
42	7.5	4850	0	2.9
48	7.5	1489	0	1.7
66	7.5	992	0	0.86

Here, using such general primers (341F/907R), bands were detected confirming the identity of the putative isolate as members of the genus *Pseudomonas* therefore the isolate was designated as *P. aeruginosa* SR3 and selected for the remaining studies fig. (4). *P. aeruginosa* has the genome size of about 5.2 to 7 million base pairs (Mbp) with 65% Guanine + Cytosine content. The genetic repertoire of *P. aeruginosa* reflects the lifestyle of this ubiquitous bacterial species. *P. aeruginosa* strains are found in various environmental habitats as well as in animal and human hosts, where they can act as opportunistic pathogens. The colonization of this broad spectrum of habitats goes along with the ability to exploit many different nutrition sources and high potential for adaptation to new (or changing) environmental conditions[23]. The metabolic versatility is provided by genes encoding not only the enzymes participating in metabolic pathways, but also by a very high number of transcriptional regulators and two component regulatory systems. More than 500 regulatory genes were identified in the genome of *P. aeruginosa* [24].

Optimum conditions for laccase production

Effect of fermentation media

The influence of fermentation media on the production of laccase was tested by cultivating the isolate *P. aeruginosa* SR3 in five different media, namely A: Mineral Salt (MS) with p-ansidine, B: Nutrient broth with p-ansidine, C: Soya meal media, D: Yeast extract media and E: Starch media. After incubation, enzyme activities of culture filtrates were determined. Among the five media used, the mineral salt supplemented with p-ansidine was found to be the best medium for laccase production as indicated by specific activities. The highest specific activity of laccase observed was 1234U/mg while, the specific activity of laccase using nutrient broth with p-ansidine, starch media, yeast extract media and soya meal media with were decreased to (989, 265, 487 and 504) U/mg protein respectively as shown in fig. (5). Therefore, this medium was selected to optimize the other fermentation parameters. Laccase production proved to be very much dependent on the medium and better enzyme production in the medium can be attributed to availability of a complete pool of amino acids, carbon and nitrogen source and other supplementation [25].

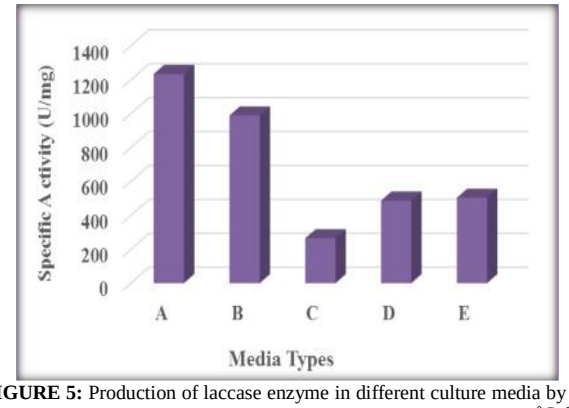

FIGURE 5: Production of laccase enzyme in different culture media by *P. aeruginosa* SR3 using submerged culture in shaker incubator at 30°C for 24 hrs. A: Mineral salt medium with p-ansidine, B: Nutrient broth with p-ansidine, C: Soya meal medium, D: Yeast extract medium, E: Starch medium

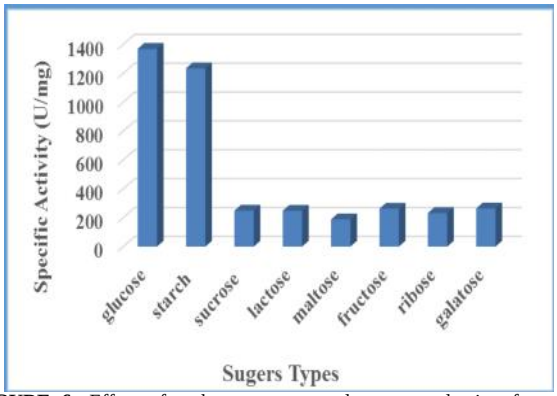

FIGURE 6: Effect of carbon sources on laccase production from *P. aeruginosa* SR3 using submerged culture system pH 7 at 30°C and for 24 hrs.

Effect of Carbon Sources

Laccase production was tested in the presence of different carbon sources incorporated in to the production medium with concentration of 1% (w/v). Among eight different carbon sources glucose supported good laccase production (fig. 6).Higher laccase specific activity was reached to 1370 U/mg compared to the lower specific activity with maltose containing medium 189 U/mg. The effect of carbon source on enzyme production was reported in bacterial laccase production, glucose showed the highest potential for the production of laccase because glucose is monosaccharide which is easily broken down and rapidly utilized by the microorganism [26].

Effect of Nitrogen Sources

The effect of different nitrogen sources were evaluated at optimum carbon source. Peptone was the best source of nitrogen that supported the growth and enzyme production from *P. aeruginosa* SR3, the laccase specific activity was increased to 1525 U/mg compared with other nitrogen sources (fig.7). Nitrogen plays key role in laccase production, the nature and the concentration of nitrogen in the culture medium for growing the organism are essential for laccase production. Medium containing peptone showed the highest laccase activity as enzymes are substrate specific. Peptone is the simplified source of protein and can be readily uptake by the microorganism [27].

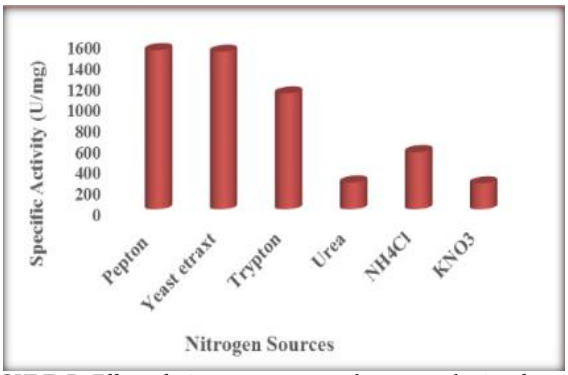

FIGURE 7: Effect of nitrogen sources on laccase production from *P. aeruginosa* SR3 in shaker incubator (150 rpm) pH 7 at 30 °C after 24 hr.

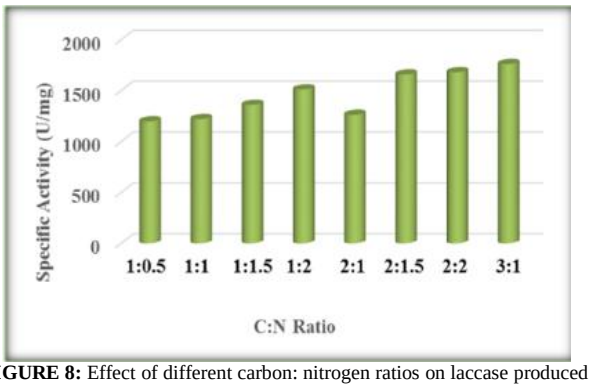

FIGURE 8: Effect of different carbon: nitrogen ratios on laccase produced by *P. aeruginosa* SR3 using submerged culture system in shaker incubator (150 rpm) at 30 ^{O}C and pH 7 for 24

Effect of Carbon to Nitrogen Ratios (C: N)

The isolate *P. aeruginosa* SR3 was found to produce laccase to a higher extent, increasing the glucose concentration. Maximum laccase specific activity were obtained at C: N ratio (3: 1) (fig. 8). The enzymatic production synthesis activation can also be explained by the balance between the presence of easily metabolized carbon as glucose for the microbial growth and the presence of inducers like cellulose and lignin. The requirements of carbon in living organisms are usually greater than nitrogen. Therefore, the balance between the concentrations of them in the fermentation medium is a crucial aspect as it can determine how microorganisms use these sources [28].

Effect of pH values

To study the effect of the initial pH on laccase production, *P. aeruginosa* SR3 was grown on production medium with different pH values (5-10). As can be seen in figure (9) higher laccase specific activity (1780) U/mg was obtained at pH 7.5, increase or decrease pH value above or below 7.5, lead to reduce 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 microorganism, in addition to its influence on the enzyme stability [29].

Effect of temperatures on laccase production

The results in fig (10) showed the capability if isolate *P. aeruginosa* SR3 to grow and produce laccase at wide range of temperatures include 25, 30, 37 and 42°C, laccase production was found to be maximum at 30 °C with specific activity of 1684 U/mg, while the specific activity of laccase at 25, 37 and 42 were 1157, 1212, 1175 respectively. Kiran and Vandana [26] found the highest laccase activity produced by *P. aeruginosa* at 40ºC. However Kuddus [15] found the optimal temperature of laccase production from *P. putida* was 30°C.

Effect of Incubation periods on laccase production

The optimum incubation period was studied to detect the periods of bacterial growth and enzyme production. From the results in fig. (11), it was evident that laccase production commenced during the first 18 hrs and the specific activity was enhanced after 24hrs as far as it reach to maximal specific activity after 27hrs of incubation, which recorded 1785 U/mg protein. Whereas after 72hrs of incubation, the specific activity was decreased with increasing the incubation time, this may be due to the change in the conditions of culture along this periods such as diminishing of oxygen, nutrients and accumulating of toxic metabolites which inhibit the bacterial growth. The incubation time plays an important role in the growth of microorganisms and enzyme secretion; some researcher indicated that the laccase production from *P. fluorescence* increases with time till 72 h after that enzyme production decreased due to depletion of macro and micronutrients in the production medium [26].

Effect the Concentrations of Copper Sulfate

The effect of different concentrations of $CuSO_4$ on laccase production was studied. The result in figure (12) showed that 4 mg/l of copper sulfate induced laccase production with specific activity (1479) U/mg, while the specific activity of laccase using $CuSO_4$ at (0.5, 1, 2, 6, 8 and 10) mg/ l were 1092, 1404, 1469, 1475, 1473, 1433 U/mg protein respectively as shown in (fig.12). However the

specific activity of the control (1 mg/l of $CuSO_4$ with 0.04% p-ansidine) was the highest. Therefore, these results suggested that the increase in the specific activity is resulted from the synergistic action of copper sulfate and p- ansidine. However, when the copper concentration was increased above 4 mg/ml it resulted in significant decrease in microorganism growth and laccase production. This may be attributed to an inhibitory effect of copper at higher concentrations. Bittin *et al*. [30] improved the levels of laccase activity when used $CuSO_4$ in conjunction with the aromatic compounds.

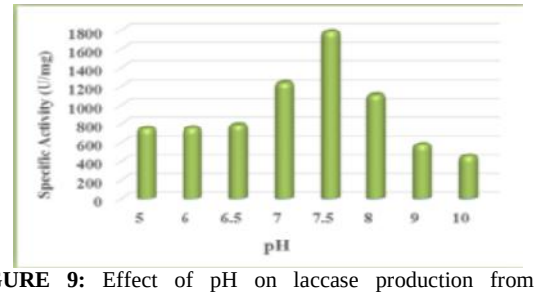

FIGURE 9: Effect of pH on laccase production from P. aeruginosa SR3 using submerged culture system in shaker incubator at 30C for 24hrs.

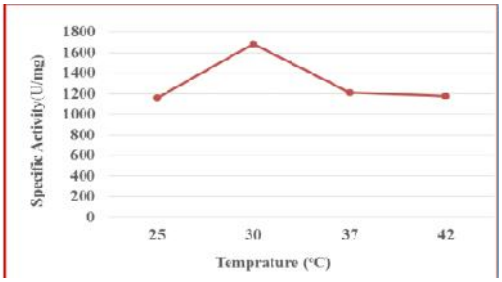

FIGURE 10: Effect of temperatures on laccase production from *P. aeruginosa* SR3 in shaker incubator (150rpm) at pH 7.5 after 24 hr.

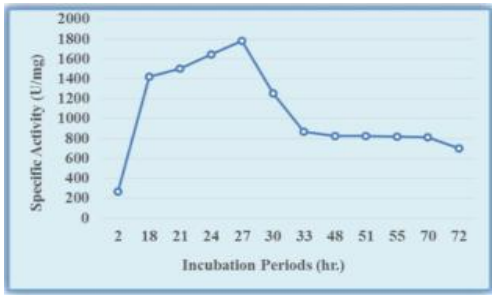

FIGURE 11: Effect of incubation period on laccase production from P. aeruginosa SR3 using submerged culture system pH 7.5 at 30°C and for 72hr

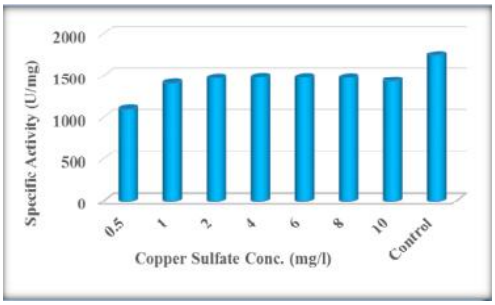

FIGURE 12: Effect of different concentrations of copper sulfate on laccase production by *P. aeruginosa* SR3 in production medium pH 7 at 30 °C and after 24 hr. of incubation

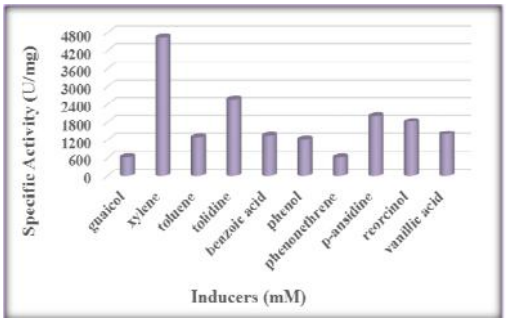

FIGURE 13: Effect of Inducers on laccase production from *P. aeruginosa* SR3 in production medium pH 7.5 in shaker incubator at 30 °C and after 27 hr.

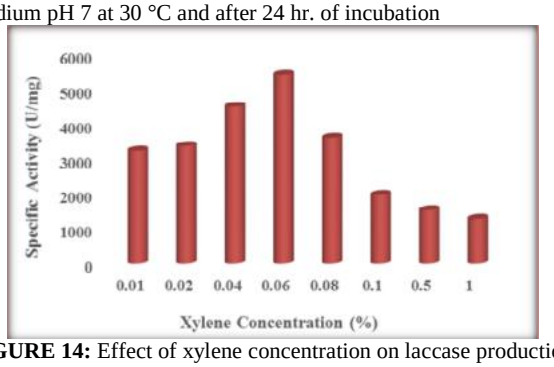

FIGURE 14: Effect of xylene concentration on laccase production from *P. aeruginosa* SR3 in production medium pH 7.5 at 30 °C and after 27 hr. of incubation

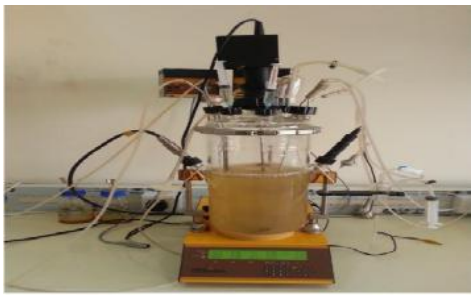

FIGURE 15: Growth *P. aeruginosa* SR3 in lab scale fermenter system (5 liter) under optimum conditions

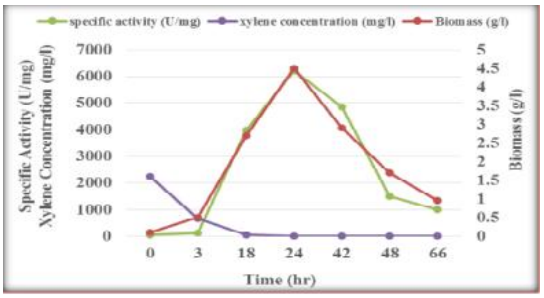

FIGURE 16: Laccase production by *P. aeruginosa* SR3 in a bioreactor under the optimum conditions (pH 7.5, 30 °C, 250 mg O2/min and 400 rpm for 66 hr.)

Effect of inducers on laccase production

Laccase production was induced in the presence of different phenolic and hydrocarbons inducers added to the production medium include (guaicol, xylene, toluene, o-tolidine, benzoic acid, phenol, phenonethrene, p-ansidine, resorcinol and vanillic acid) at concentration (0.04%). Some of used inducers were enhanced laccase production while, others are repressed the enzyme production.

Xylene, o-tolidine, p-ansidine and resorcinol are induced laccase production while enzyme production was repressed with guaicol, toluene, benzoic acid, phenol, phenonethrene, and vanillic acid fig. (13). One of the most effective approach to increase the yield of laccase is supplementation of the nutrient in the medium with an appropriate inducer, the most widely reported potent inducer of the laccase synthesis is xylene [31]. Aromatic compounds have been widely used to elicit and enhanced laccase production by different organisms [32].

Effect of Xylene Concentrations

The effect of different concentrations of xylene on laccase production was studied by addition different concentration of xylene included (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.5, and 1) % to the production media. It was evident from the results in the fig. (14) that laccase production commenced in 0.01% and the specific activity was enhanced with 0.04% as far as it reach to maximal specific activity 5432 U/mg protein at 0.06% in the fermentation media. Whereas, the specific activity was decreased with increasing the concentration of xylene above of 0.06%. At higher concentrations, the xylene had a reduced effect on laccase production probably due to toxicity. Many studies have shown that only lower concentrations of xylene induce laccase production[33]. The negative effect of xylene on the laccase activity of *A. blazei* strains may be at the concentrations of xylene (0.5-1.5 mM).

Production of laccase in lab scale fermenter

After determination the optimum condition for laccase production by *P. aeruginosa* SR3, the fermentation media was scaled up inside stirred tank fermenter at optimum condition observed in batch culture system to investigate the feasibility of achieving high activity level for laccase at large scale. Laccase production by *P. aeruginosa* SR3 was scaled up in a 5 L stirred tank fermenter with working volume of 3.5L. The fermenter was equipped with instrumentation for measurement and control of agitation, temperature, pH and stirrer speed, as shown in fig. (15). Laccase activity increased at the end of the logarithmic phase of growth and reached a maximum at the stationary phase. The specific activity was enhanced after 18 hrs as far as it reach to maximal specific activity after 24 hrs of incubation, which reached to 6214 U/mg protein.

Whereas after 24 hrs of incubation the specific activity was decreased with increasing the incubation time, that's indicate an increase of specific activity of laccase enzyme increase with incubation hours to reach the optimum and then decrease with increase the incubation hours (fig.16). The biomass concentration, in cell dry weight (CDW), of *P. aeruginosa* SR3 inoculated culture was estimated, the result in fig. (16), dedicated that the biomass was conjugated with specific activity and increase with accordance to the incubation time, the specific activity of laccase reach to maximum 6214 U/mg protein when the biomass was reached to 4.5 g/l after 24 hr. of incubation and decrease with specific activity after 66 hr. to reach 0.96 g/ l when specific activity decreased to 992 U/mg protein, table (2) and fig. (16). Xylene was measured by (GC) on a C18 column and the results were showed in table (2) and detailed in fig. (17, 18, 19, 20, 21 and 22). From the results laccase production started when xylene concentration was nearly depleted from the medium inoculated bioreactors after 24 hr. During the initial laccase production in the fermenter, coinciding with xylene utilization period, laccase activity was low at 3 hr. When the consumption of xylene was nearly complete, laccase production increased considerably. The maximum laccase production was reached to 6214 U/mg in continuous stirred tank bioreactor fig. (16) in comparison to the shake flask level, it was 5432 U/ml. The increase in production at the fermenter level is because of enhanced aeration and agitation. It is important to produce the enzyme from inexpensive and optimized media on large-scale for the process to be made commercially viable and also to study the influence of various parameters on enzyme production[34]. The yield of extracellular enzymes like laccase is significantly influenced by physicochemical conditions. Figure (15) shows the growth of the *P. aeruginosa* SR3 in the bioreactor in control culture the production of laccase was proportionally increased with the bacterial biomass. The maximum production of laccase and biomass from *Pleurotus ostreatus* by bioreactor were obtained on 12th day of the incubation (620±1.5 U/l and 17.5±2.1 g/l respectively) at initial pH 6 [35].

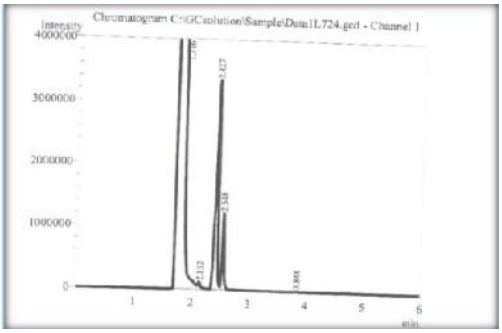

FIGURE 17: Chromatogram of xylene concentration at initial time within lab scale bioreactor (5 liter) for production *P. aeruginosa* SR3 laccase

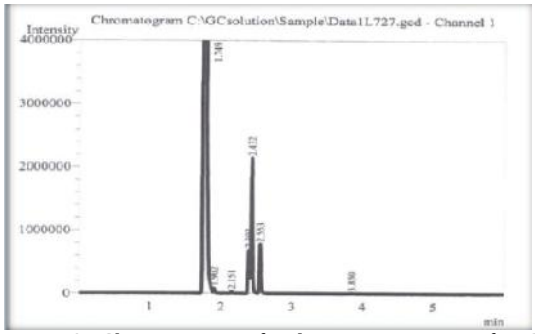

FIGURE 18: Chromatogram of xylene concentration after 3 hr within lab scale bioreactor (5 liter) for production P. aeruginosa SR3 laccase

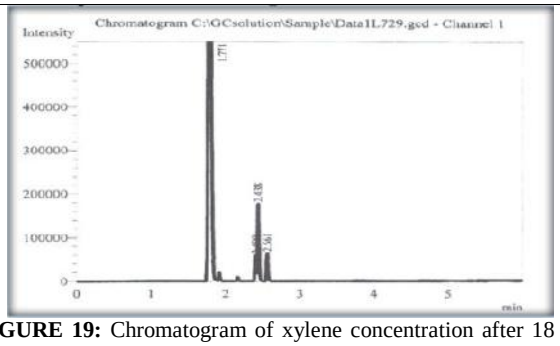

FIGURE 19: Chromatogram of xylene concentration after 18 hr within lab scale bioreactor (5 liter) for production *P. aeruginosa* SR3 laccase

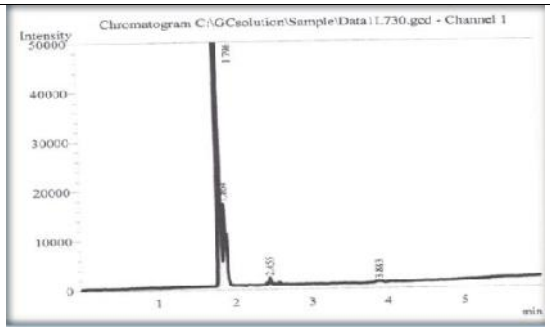

FIGURE 20: Chromatogram of xylene concentration after 24 hr within lab scale bioreactor (5 liter) for *production P. aeruginosa* SR3 laccase

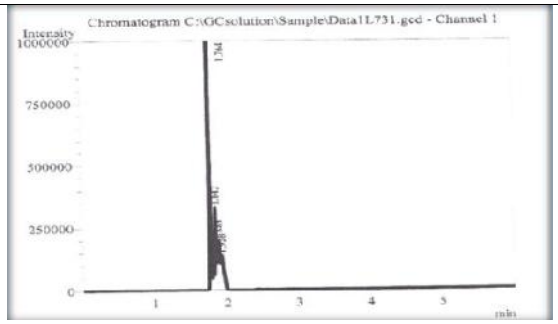

FIGURE 21: Chromatogram of xylene concentration after 42 hr within lab scale bioreactor (5 liter) for production *P. aeruginosa* SR3 laccase

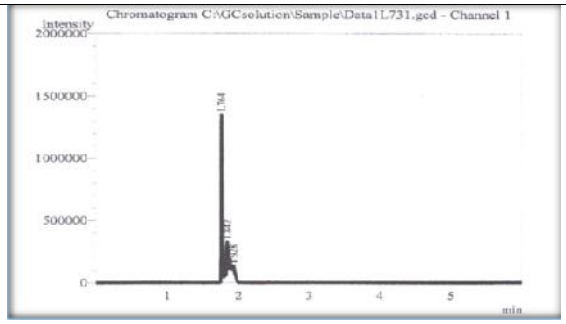

FIGURE 22: Chromatogram of xylene concentration after 66 hr within lab scale bioreactor (5 liter) for production *P. aeruginosa* SR3 laccase

REFERENCES

[1]. Mester, T. and Tien, M. (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. International Journal of Biodeterioration & Biodegradation. 46:51-59.

[2]. Thurston, C.F. (1994) "The structure and function of fungal laccases," Journal of Microbiology. 140 (1): 19–26.

[3]. Robert, V., Mekmouche, Y., Pailley, P.R. & Tron, T. (2011) Engineering laccases: in search for novel catalysts. Journal of Current Genomics. 12: 123-129.

[4]. [Sorensen, H.P. & Mortensen, K.K. (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. Journal of Biotechnology. 115: 113-128.

[5]. Youn, H.-D., Kim, K.-J., Maeng, J.-S., Han, Y.-H., Jeong, I.-B. ,Jeong, G., Kang S.-O. and Hah, Y.C. (1995) Single electron transfer by an extracellular laccase from the while rot fungus *Pleurotus ostereatus*. Journal of Microbiology.141: 393-398.

[6]. Solano, F., Lucas-Elio, P., L´opez-Serrano, D., Fern´ andez, E., & Sanchez-Amat, A. (2001) Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. FEMS Journal of Microbiology Letters. 16: 175–181.

[7]. Miller, R., Kuglin, J. and Gallagher, S. (1997) A Spectrophotometric Assay for laccase using O-Tolidine. Journal of Food Biochemistry. 21(1): 445–459.

[8]. Win, D.T., Than M.M. and Tun, S. (2003). Lead removal from industrial waters by water hyacinth. Assumption University Journal of Technology. 6: 187-192.

[9]. Arunkumar, T., Alex Anand, D. and Narendrakumar, G. (2014). Production and Partial Purification of Laccase from *Pseudomonas aeruginosa* ADN04. Journal of Pure & Applied Microbiology. 8(2): 727-731.

[10]. Kalral, K. Chauhan, R., Shavez, M. and Sachdeva, S. (2013). Isolation of laccase producing *Trichoderma* Spp. and effect of pH and temperature on its activity. International Journal of Chemistry and Technology Research. 5(5): 2229-2235.

[11]. Bradford, M. (1976)A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry Journal. 72: 248-254.

[12]. Arora, D.S. and Gill P.K. (2001) Effects of various media and supplements on laccase production by some white rot fungi. Journal of Bioresource Technology .77: 89-91.

[13]. Sivakami, V., Ramachandran, B., Srivathsan, J., Kesavaperumal, G., Smily, B. and Mukesh Kumar D. J. (2012) Production and optimization of laccase and lignin peroxidase by newly isolated *Pleurotus ostreatus* LIG19. Journal of Microbiology and Biotechology Research. 2 (6):875-881.

[14]. Holkar, S.K., Begde, D.N., Nashikkar, N.A., Kadam, T.A. and Upadhyay, A.A. (2013) Optimization of some culture conditions for improved biomass and antibiotic production by *Streptomyces spectabilis* isolated from soil. International Journal of Pharmaceutical Sciences and Research. 4 (8): 2980-2987.

[15]. Kuddus, M., Joseph, B., Ramteke, P.W. (2013) Production of laccase from newly isolated *Pseudomonas putida* and its application in bioremediation of synthetic dyes and industrial effluents. Journal of Biocatalyst and Agriculture Biotechnology. 2(4): 333-338.

[16]. Pointing, S.B., Jones, E.B.G., Vrijmoed, L.L.P. (2000) Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia Journal. 92(1): 139-144.

[17]. Sivakumar, R, Rajendran, R, Balakumar C, Tamilvendan C. (2010) Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma* Sp. International Journal of Engineering Science and Technology. 2(12):7133-7141.

[18]. Nawar, J., Khalil, I., and Abduljabbar, A. (2014) Separation and Determination of poly aromatic hydrocarbons in vegetables sample in Baghdad city using

HPLC technique . Ibn Al-Haitham Journal for Pure and Applied Science. 27 (1): 247-259.

[19]. Shah, M.P., Patel, K.A., Nair, S.S. and Darji, A.M. (2013) Microbial Decolorization of Methyl Orange Dye by *Pseudomonas* spp. ETL-M. Applied & Environmental Microbiology. 1(2):10.

[20]. Devasia, S. and Nair A.J. (2016) Screening of Potent Laccase Producing Organisms Based on the Oxidation Pattern of Different Phenolic Substrates. International Journal of Current Microbiology and Applied Science. 5(5): 127-137.

[21]. Rüchel, R., Tegeler, R. and Trost, M. (1982) Acomparison of secretory proteinases from different strain of *Candida albicans*. Sabouraudia Journal. 20: 233-244.

[22]. Cassone, A., De Bernardis, F., Mondello, F., Ciddia, T. and Agatensi, L. (1987) Evidence for a correlation between proteinase secretion and vulvoriginal candidsis. Journal of Infectious Disease. 156: 777 – 783.

[23]. Ramos, J.L. (ed.) (2004) Pseudomonas Volume 1: Genomics, Life Style and Molecular Architecture. New York: KluwerAcademics/Plenum Publishers.

[24]. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R.E., Lory, S. and Olson, M.V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Journal of Nature. 406:959–964.

[25]. Singh D.A. and Kaur, P.G. (2000) Laccase production by some white rot fungi under different nutritional conditions. Journal of Bioresource Technology. 73:283-285.

[26]. Kiran, P.J. and Vandana, P. (2014). Congo red dye decolorization by partially purified laccases from *Pseudomonas aeruginosa*. International Journal of Current Microbiology and Applied Science. 3(9) 105-115.

[27]. Vandana, P. and Peter, J.K. (2014) Application of partially purified laccase from *Pseudomonas fluorescens* on dye decolorization. International Journal of Advanced Technology in Engineering and Science. 2(8): 2348 – 7550.

[28]. Egli, T. and Zinn, M. (2003) The concept of multiple-nutrinet-limited growth of microorganisms and its application in biotechnological processes. Journal of advances Biotechnology. 22 (1): 35-43.

[29]. Bull, A.T. and Bushnell, M.E. (1976) Environmental control of fungal growth. In: The filamentous fungo. (ed. Smith, J.E. and Berry, D.R.). 2: 1-29. Edward Arnold. London.

[30]. Bettin, F., Montanari, Q., Calloni, R., Gaio, T. A., Silveira, M. M. and Dillon A. J. P. (2014) Additive Effects of CuSO4 and Aromatic Compounds on Laccase Production by *Pleurotus sajorcaju* PS-2001 using sucrose as a carbon source. Brazilian Journal of Chemical Engineering. 31 (2): 335 – 346.

[31]. Revankar M.S. and Lele, S.S. (2006) Enhanced production of laccase using a new isolate of white rot fungus WR- 1. Proceeding Biochemistry Journal. 41: 581588.

[32]. Leonowicz, A., Cho, N. S., Luterek, J., Wilkolazka, A., WotjasWasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D. and Rogalski, J. (2001) Fungal laccase: properties and activity on lignin. Journal of Basic Microbiology. 41:185–227.

[33]. Xavier, AMRB., Tavares, APM., Ferreira, R. and Amado, F. (2007) *Trametes versicolor* growth and laccase induction with byproducts of pulp and paper industry. Electronic Journal of Biotechnology. 10: 444-451.

[34]. Seeta Laxman, R., Sonawane, A. P., More, S. V., Rao, B. S., Rele, M. V. and Jogdand, V. V. (2005) Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. Journal of Process Biochemistry. 40: 3152-3158.

[35]. Muhammad, N. B.-S. (2013) Fermentation and kinetic studies on laccase production by *Pycnoporus sanguineus*. Dissertation submitted in fulfillment of the requirement for the degree of Master of Science. Institute of biological science faculty of Sciences University of Malaya Kualalumpur.