Journal of Advances in Biology & Biotechnology

23(8): 44-57, 2020; Article no.JABB.57170
ISSN: 2394-1081

Screening of Laccase Producing Fungi Using Agro-Wastes under Different Cultural Conditions

O. G. Ndochinwa[1*], O. C. Amadi[1], T. N. Nwagu[1], G. N. Okpala[1], C. I. Nnamchi[1], and A. N. Moneke[1]

[1]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author OGN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OCA and TNN managed the analyses of the study. Author ANM managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2020/v23i830174
Editor(s):
(1) Dr. Anil Kumar, School of Biotechnology Devi Ahilya University, India.
Reviewers:
(1) Surachai Rattanasuk, Roi Et Rajabhat University, Thailand.
(2) Mary Obodai, CSIR-College of Science and Technology, Ghana.
(3) Deborah Murowaniecki Otero, Universidade Federal da Bahia, Brasil.
Complete Peer review History: http://www.sdiarticle4.com/review-history/57170

Data Article

Received 10 September 2020
Accepted 17 November 2020
Published 08 December 2020

ABSTRACT

This study evaluated the potential of fungal isolates for laccase production. Fungi cultures were screened for laccase production on plate assay using 2' 2' – azinobis-(-3-ethyl benzthiazoline -6-suphonate) (ABTS), and by submerged fermentation. Result obtained from the plate assay showed the formation of green halo after 2-4 days of incubation due to oxidation of 2' 2' – azinobis-(-3-ethyl benzthiazoline -6- suphonate) (ABTS) which is as a result of lignolytic enzymes production Utilization of selected agro-wastes residues (sawdust, plantain and banana peels) for laccase production was evaluated. Fungal isolates were identified based on their cultural characteristics according to standard mycology methods. Light microscopy was performed on cultures and the fungal isolates were identified by their different morphological and colonial characteristics after which they were confirmed using fungal atlas. Total protein content was also determined using the Bradford method Five out of twelve isolates were positive for oxidation of 2' 2' – azinobis-(-3-ethyl benzthiazoline -6- suphonate) (ABTS) which signifies laccase enzyme activity and identified as *Geotrichum spp, Cephalosporium spp, Trichoderma spp, Trametes spp* and *Fusarium spp*The highest enzyme activity was observed using *Trichoderma spp* at 57.1U/l, *Trametes spp* 51.99 U/l,

**Corresponding author: E-mail: Obinna.ndochinwa.pg83511@inn.edu.ng, obinna.ndochinwa.pg83511@unn.edu.ng;*

Fusarium spp had 29.2 U/l. *Geotrichum spp* and *Cephalosporium spp* were least at 28.04 U/l and 9.72 U/l respectively. *Trichoderma spp* was used for further studies. Effect of carbon sources, inoculum size, pH, total protein and shaker speed on laccase production was evaluated. Sawdust gave the highest yield of laccase enzyme 151.17U /l, followed by plantain peel with 62.49U/l of enzyme, banana peel recorded the least of laccase enzyme 54.94 U /l. Protein content obtained from sawdust medium was 45.8mg/ml, plantain peel medium had 29.2 mg/ml while banana peel was the lowest with 16.8mg/ml all at the sixth day of the fermentation process. Optimum pH for sawdust, banana peel and plantain peel was 5.95, 5.94, and 5.83 respectively. Using shaker incubator (150 rpm) with sawdust as carbon source, laccase yield of (310.45U/l) was obtained at pH 6, temperature 25°C and inoculum size of 10^5 spores per ml. Thus, sawdust can be categorically stated to be safe, cheap and could be suggested for prospective application of higher production of laccase enzyme in various industries.

Keywords: Agro-waste; ABTS; submerged cultures.

1. INTRODUCTION

Enzyme application in biotechnological and environmental processes is of increasing interest owing to their efficiency; selectivity and most importantly their being environmentally friendly health wise, but very expensive and applications require large volumes of enzymes. Laccases (E. C. 1.10.3.2, p-diphenol: dioxygen oxidoreductase Riva 2006) are a collection of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water [1]. Laccases finds wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants, and they have potential applications in detoxification of lignocellulosic biomass after thermochemical pretreatment [2,3,4,5,6,7].

In food processing, laccases is sometimes used in processes that improve or modify the colour appearance of food or beverage [8]. Some laccase products are commercially available for paper, textile, food and other industries [9], Rodríguez-Couto, 2012.

In nature laccases function is to degrade lignin in order to gain access to the carbohydrates in wood (cellulose and hemicellulose). This makes its application in the production of bio-fuel and value-added chemicals more relevant. Laccases from fungi represent proper candidates in processing of lignocellulosic biomass. Over 60 fungal strains, belonging to various classes such as *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes*, have been demonstrated to produce laccase [10]. The majority of laccases characterized so far have been derived from efficient lignin degraders such as white-rot fungi [11,12,13]. Laccases of fungi origin have been associated with lignin degradation, and are well known as component of fungal enzyme systems for lignin degradation.

Trichoderma spp are well known for their ability to grow on decayed wood and other agro-lignocellulosic biomass materials and can decompose lignin by secreting enzymes such as cellulase and laccases. Based on this, and the fact that *Trichoderma spp* can produce high amount of laccase in liquid cultures, laccases from this fungus may promote degradation/modification of lignin or lignin-derived components [14,15] and therefore represent proper candidates in processing lignocellulosic biomass.

The production of laccase by *Trichoderma spp* is very interesting due to combined production of laccases and cellulases, which in the degradation of lignocellulosic materials [2,16].

In view of its importance in large scale application, the current study was to screen locally for highly efficient laccase producing fungi with ability to secrete copious amounts of the enzyme to reduce the cost of production. To address this issue, the present study reports the production of laccase by local isolates of *Trichoderma spp* using agricultural by-products as substrates by submerged fermentation.

2. MATERIALS AND METHODS

10 grams of each samples (decomposing tree stump, plantain and banana peel respectively) were appropriately diluted by 10 fold serial dilution to 10^{-1} cfu/ml and inoculated on Sabouraud dextrose agar (SDA) and Potato

dextrose agar (PDA) plates incubated at 25°C for at least 7 days. The pure cultures obtained were screened for the prensence of laccase using plate assay that contained 2' 2' – azinobis-(-3-ethyl benzthiazoline -6- suphonate) (ABTS), as indicator and synthetic medium with the following composition (g/l): peptone 3.0, glucose 10.0, K_2PO_4 0.6, $ZnSO_4$ 0.001, K_2HPO_4 0.4, $FeSO_4$ 0.0005, $MnSO_4$ 0.05, $MgSO_4$ 0.5, agar 20.0 and 0.02% 2' 2' – azinobis-(-3-ethyl benzthiazoline -6- suphonate) (ABTS) (adopted from Viswanath *et al.,*2008). The development of an intense greenish colour was considered as positive for laccase activity. The positive cultures were maintained on PDA slant. The basal medium comprised of the following in (g/l)peptone 3.0, glucose 10.0, K_2PO_4 0.6, $ZnSO_4$ 0.001, K_2HPO_4 0.4, $FeSO_4$ 0.0005, $MnSO_4$ 0.05, $MgSO_4$ 0.5, for quantitative screening of laccase enzyme (adopted from Viswanath *et al.,*2008). A total of 12 different fungal cultures were isolated.

2.1 Culture Conditions and Enzyme Production under Static Condition

The positive result of isolated fungi were cultivated on basal medium as described above. Cultivation was carried out in 250 ml Erlenmeyer flasks containing 50 ml basal medium, four (4) agar plugs (5 mm diameter of cork borer) of 7day old culture was used as inoculum. Fermentation was carried out under static condition for 10days, culture liquid was filtered using Whatman No 1 filter paper and filtrate was collected and used for enzyme assay. Fungal biomass, enzyme activity and pH of the medium was monitored at two days intervals.

2.2 Determination of Fungal Biomass

Mycelia of cultured fungi was separated using filtration. The filter paper along with the mycelia mat was dried at 60°C in an oven until constant weight. The initial weight of the filter paper was subtracted from the final weight to obtain the weight of the biomass [17].

2.3 Determination of Protein Content

An aliquot of the culture filtrate was used for the estimation of total protein concentration using the (Bradford 1976) method and Bovine serum albumin (BSA) as standard reagent.

The reaction mixture consisted of 2 ml of Bradford reagent (BSA and Comassie blue G) and 0.2 ml of the culture filtrate mixed gently. Colour development was measured at 595 nm within 2 minutes to 1 hour of mixing using Shimadzu UV – 1800 spectrophotometer (Tokyo Japan). A reagent blank containing deionized H_2O in place of the culture filtrate was also prepared.

2.4 Laccase Assay

The laccase activity was determined using 2,2' azinobis-(3- ethyl benzthiazoline-6-sulfonic acid) (ABTS) as the substrate. The laccase assay reaction mixture contained 0.5ml 0f 0.45mM ABTS, 1.2 ml of 0.1M phosphate buffer (pH 6.0) and 0.5 ml of culture filtrate to give final reaction volume of 2.2 ml. Water was used as blank condition. The oxidation of the substrate (ABTS) was monitored by increase in the absorbance at 420nm using Shimadzu UV- 1800 spectrophotometer (Tokyo Japan) over 90s at 30°C (± 2°C) using molar extinction co efficient $\xi_{420}=3.6 \times 10^4\ cm^{-1}M^{-1}$. Enzymatic activity was expressed as 1U = 1Ɲ mol of ABTS oxidized per min at 25°C (adopted from Das et al. [18]).

2.5 Identification of Fungal Isolates

Fungal isolates were identified based on their cultural characteristics on Potato dextrose agar PDA and Sabouraud dextrose agar SDA according to standard mycology methods by Barnett and Hunter, [19]. Light microscopy of isolates was performed on cultures on Sabouraud dextrose agar SDA and Potato dextrose agar PDA. The fungal isolates were identified by their different morphological and colonial characteristics after which they were confirmed using fungal atlas.

2.6 Slide Culture Techniques

The method of Crowley et al., (1969) and Dugan, (2006) was used whereby 1 cm^2 of Potato dextrose agar PDA and Sabouraud dextrose aga SDA was cut from a plate approximately 2 mm deep and placed on a sterile glass slide. Fungal isolates was inoculated into four vertical sides using a sterile needle. A sterilized cover slip was applied so that it over lapped the medium on all sides and the preparation was placed on suitable support in petri dish containing blotting paper soaked in 20% glycerol in water. The preparation was kept moist at 26°C until adequate growth developed. After removing the medium the fungus adherent to both cover slip and slide was

examined. A drop of alcohol was added followed by a drop of lactophenol blue and the preparation suitably covered and examined under the power of objectives microscope (× 40).

2.7 Optimization of Laccase Production

2.7.1 Effect of different agricultural wastes on laccase production under static condition

Agro-waste materials including saw dust, plantain and banana peel were obtained from processing unit and Market in Nsukka, Enugu State, Nigeria. They were sun dried until constant weight was maintained, homogenised and sieved (2 mm mash). The agro waste materials were used for laccase production, the waste that elicited the highest laccase production was used for further studies (adopted from Patel et al. [20].

2.7.2 Effect of varying inoculum size on laccase production under static condition

The effect of different inoculum sizes was studied over a range of 10^4-10^9; haemocytometer was used to determine spore concentration adopted from Patel et al. [20].

2.7.3 Effect of various initial pH range (3-7) on laccase production under static condition

The effect of initial pH 3-7 in different buffers (sodium acetate pH 3–5 and sodium phosphate pH 6–7) and the optimum inoculums size on laccase enzyme production was determined [21].

2.7.4 Production of laccase using shaker culture

The optimum inoculums size and two best pH values (pH 5 and pH 6) were used in this study. The flask were incubated in an electronic shaker (at 150 rpm) or six days. The total protein content [22, 23].

3. RESULTS AND DISCUSSION

3.1 Isolation of Microbes

Fig. 1 shows positive plate test of a laccase producing organism. Slight change in colour of the media from colourless to green due to oxidation of the indicator compound ABTS by day two and increased by day four.

Figure two shows the result of various fungal biomass obtained in this study, *Geotrichum spp* had the highest fungal biomass 0.41 g followed by *Cephalosporium spp* 0.40 g and *Trichoderma spp* 0.38 g. The lowest biomass was observed in *Fusarium spp* and *Trametes spp* with biomass of 0.35 g and 0.33 g respectively.

Fig. 3 shows total protein content of different fungi isolates during laccase production. It was observed that the highest protein concentration was recorded at the 6th day for all the isolates and *Geotrichum spp* produced the highest total protein content of 20 mg/ml followed by *Cephalosporium spp* with 19.6 mg/ml. *Trichoderma spp* had 16 mg/ml. The lowest protein content was observed in *Fusarim spp* and *Trametes spp* with 15.2 mg/ml and 14.8 mg/ml respectively.

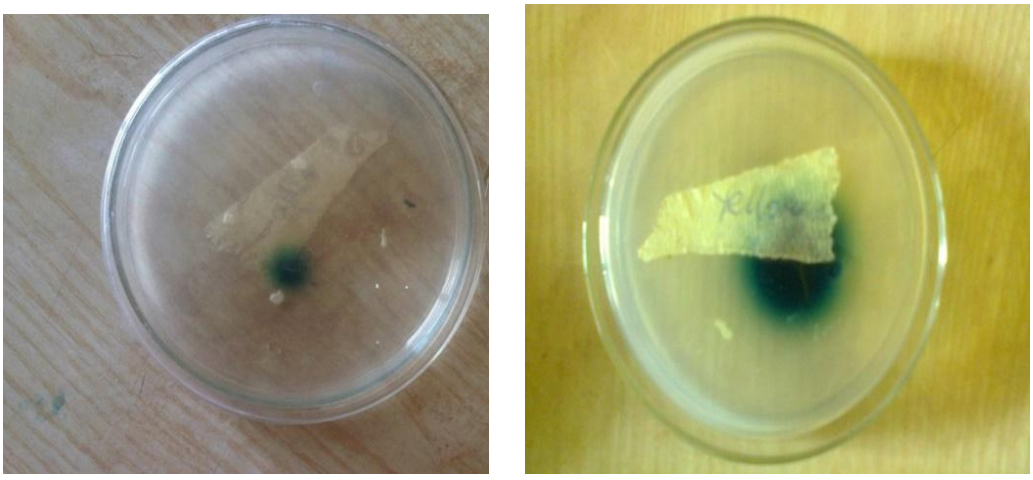


Fig. 1. Positive plate test of a laccase producing organism

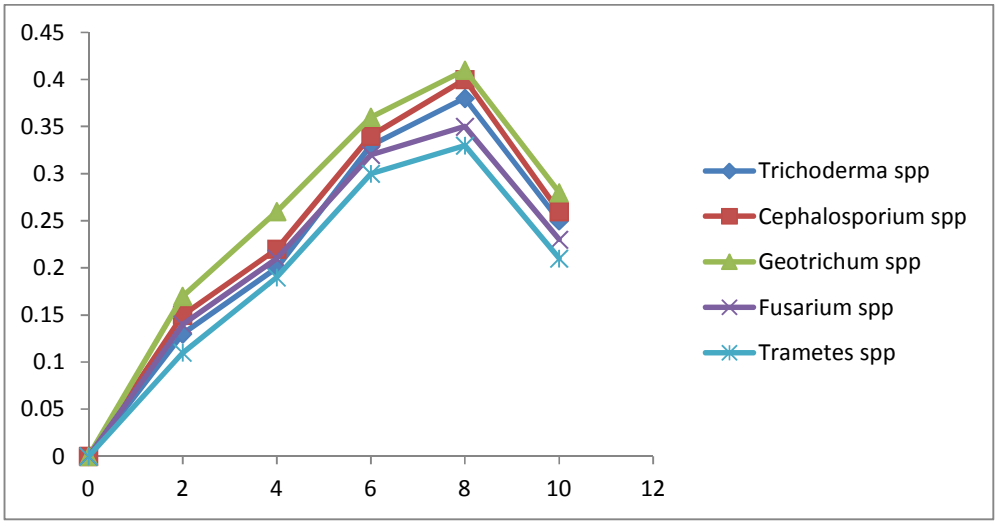


Fig. 2. Result of various fungal biomass obtained

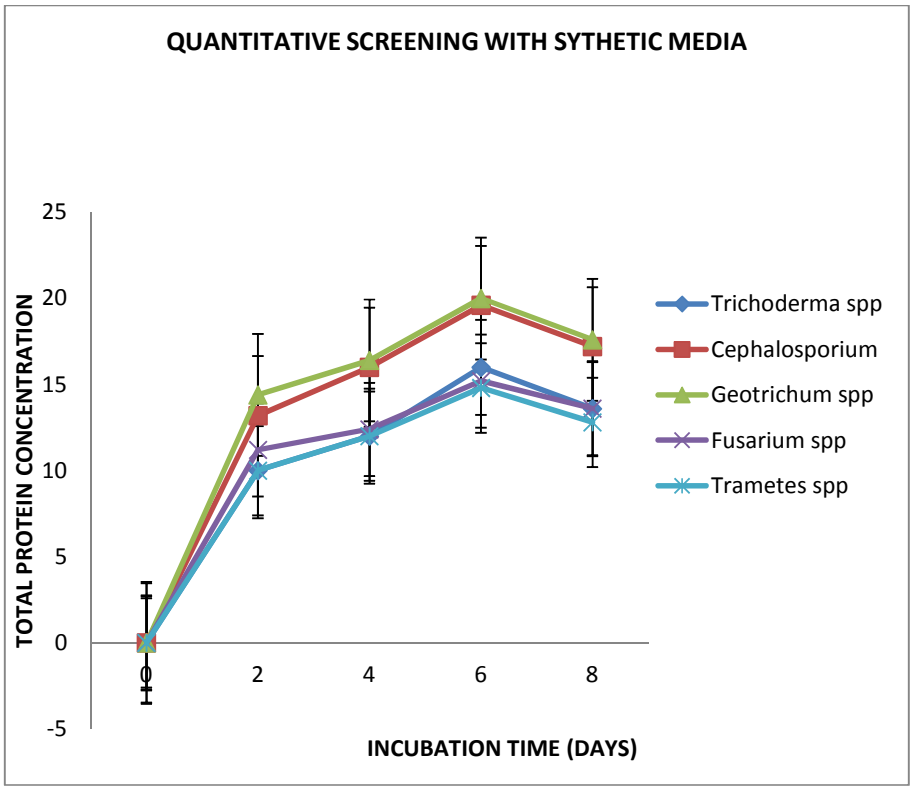


Fig. 3. Total protein content of different fungal isolates during laccase production using synthetic medium in submerged fermentation

Fig. 4 shows the result of various enzyme activity of the different fungal cultures used in this study. Enzyme activity of different fungal cultures revealed maximum activity for all isolates to be on day 4. *Trichoderma spp* had the highest enzyme activity of 57.1 U/l. *Geotrichum spp* and *Cephalosporium spp* were lower with 28.04 U/l and 9.72 U/l respectively. The isolate (*Trichoderma spp*) with the highest enzyme yield was used for further studies.

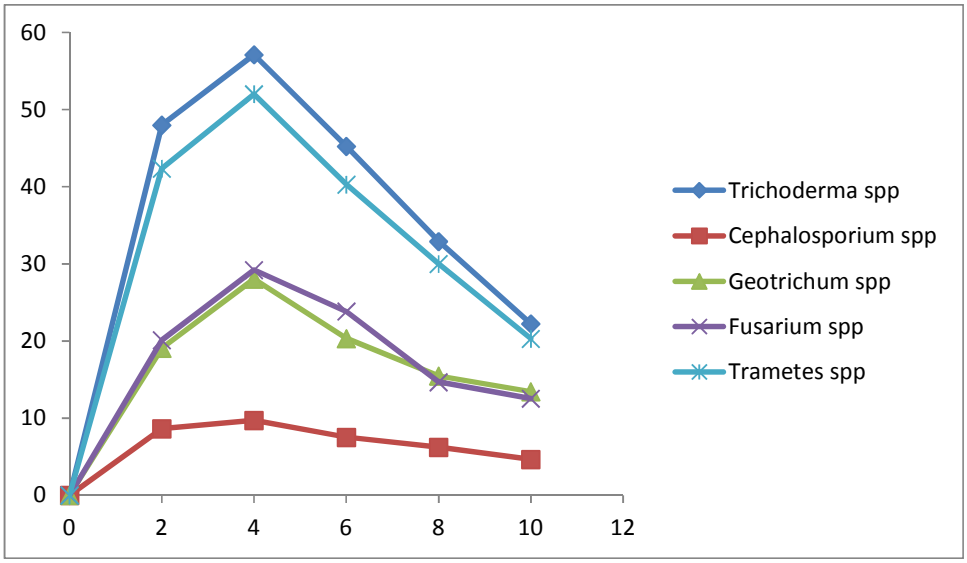


Fig. 4. The result of various enzyme activity of the different fungal cultures

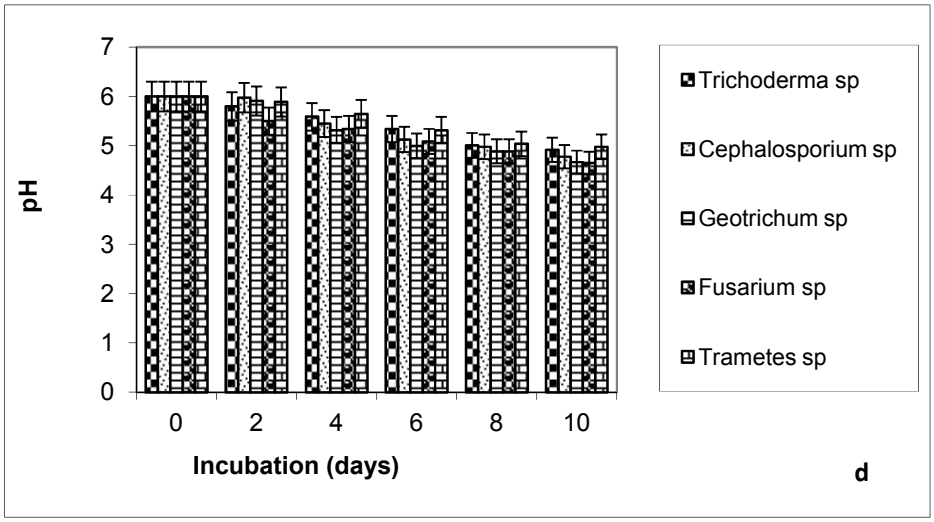


Fig. 5. The pH of medium evaluated at two days intervals

Fig. 5 shows the pH of medium evaluted at two days intervals. Slightly decrease in pH was observed for all the medium.

The ability of *Trichoderma spp* to utilize different agro waste as carbon source for laccase production was evaluated (Fig. 6). Results revealed the fungus ability to utilize the different substrate but with varying yields. Maximum enzyme activity was observed by sixth day of fermentation for the entire agro-waste materials used with saw dust displaying the highest enzyme activity of 151.71 U/l followed by plantain peel with 62.49 U/l and finally banana peel 54.92 U/l. But with control enzyme activity was observed by fourth day with 57.1 U/l.

Fig. 7 shows the pH of medium evaluted at two days interval. It was observed that there was a decrease in pH for all the medium except the control that maintained its pH 5 for 10 days.

Fig. 8 shows the results of laccase production by *Trichoderma spp* with spore inocula ranging from 10^5 to 10^9 spores/ml. The results revealed formation and release of laccase to be related to

spore inoculum level. At day 2 laccase was formed in all inoculum level. However optimum yield of 162.49 U/ml was noticed in fermentation inoculated with10^5 spores/ml, higher inoculum gave (10^8 and 10^9 spores/ml) gave lower yield of laccase 133.37 U/l and 113.77U/l respectively but increased protein content. In all inoculum level pH of medium dropped with time.

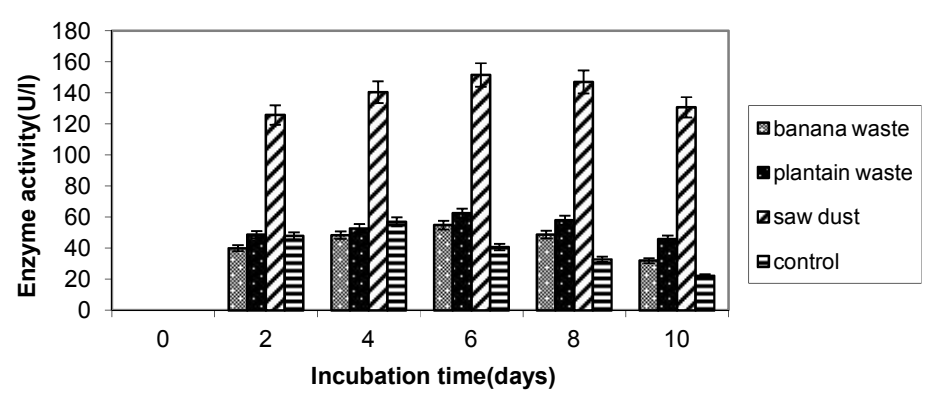


Fig. 6. The ability of *Trichoderma spp* to utilize different agro waste

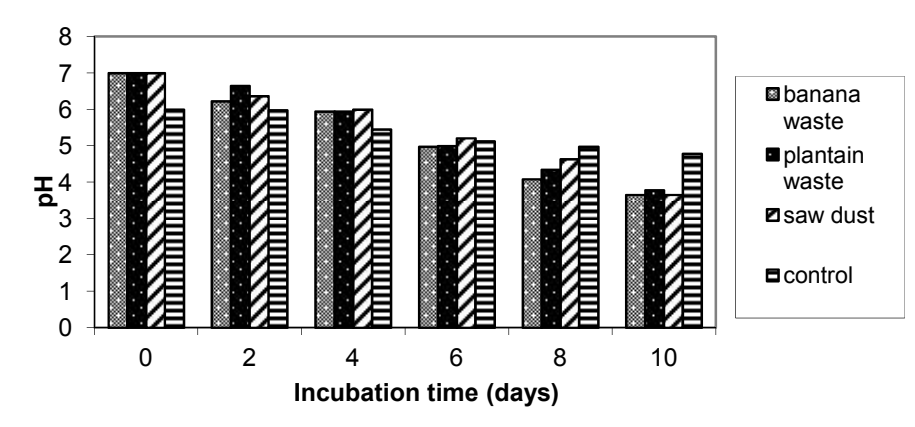


Fig. 7. The pH of medium evaluated at two days interval

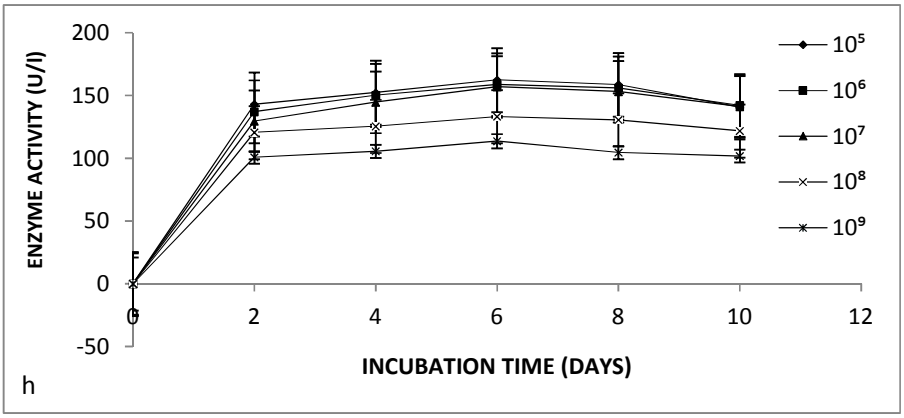


Fig. 8. The results of laccase production by *Trichoderma spp* with spore inocula ranging from 10^5 to 10^9 spores/ml

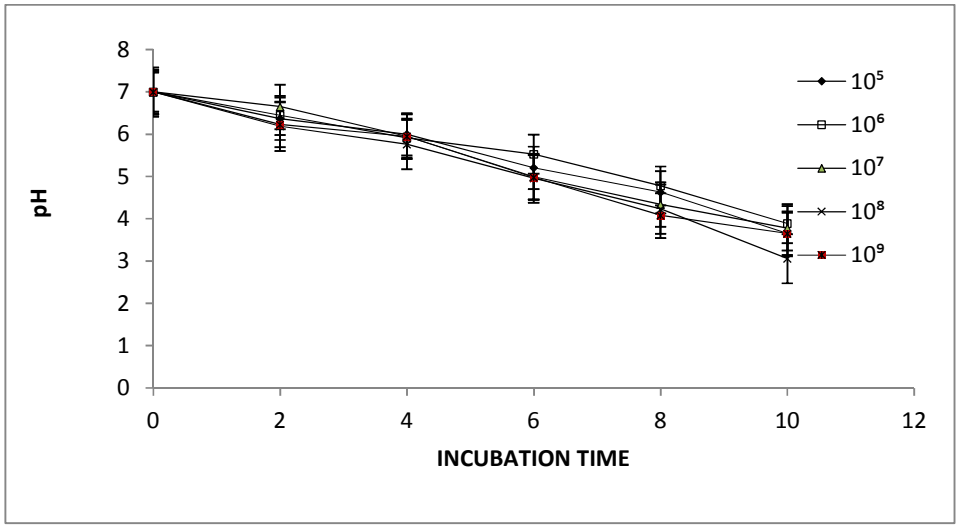


Fig. 9. The p H of the medium evaluated at two days

Fig. 9 shows the pH value of the medium evaluated at two days. A sharp decrease in pH was observed for all the medium.

Effect of initial pH 3-7 on process parameters was evaluated (Fig. 10). Initial medium pH of 6 had the highest enzyme activity of 173.65U/l; laccase production was reduced at lower (acidic) initial pH 3.

Fig. 11 shows the pH of all the medium evaluated at two days interval. A slight decrease in pH was observed for all the medium.

Effect of shaker and static conditions on laccase production was investigated. Results revealed shaker conditions to have influenced enzyme yield (Fig. 12a and 12b). Under shaker conditions enzyme activity of 310.57 U/ml and 301.2U/ml was obtained for both initial pH 6 and 5 respectively by day 4 of fermentation. Lower enzyme activity of 171.85 U/l and 160.56 U/l (pH6, 5) respectively was observed in static culture. Total protein for both pH was over 80 mg/ml in shaker. Medium pH tended towards acidity.

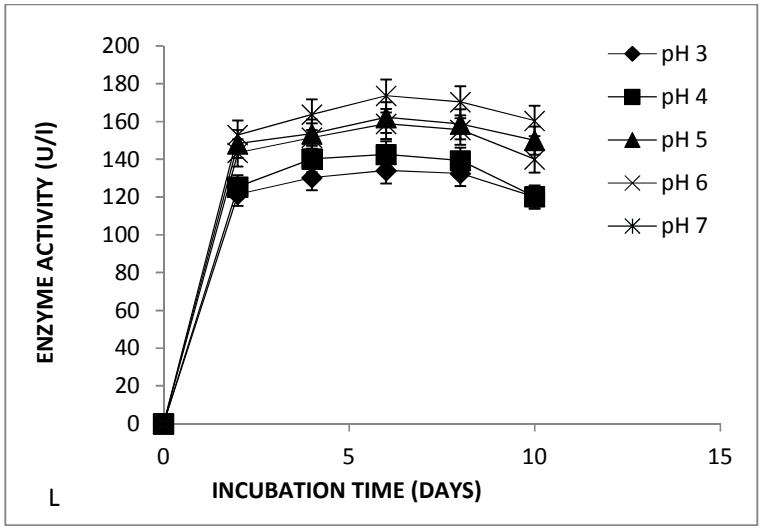


Fig. 10. Effect of initial pH 3-7 on process parameters was evaluated

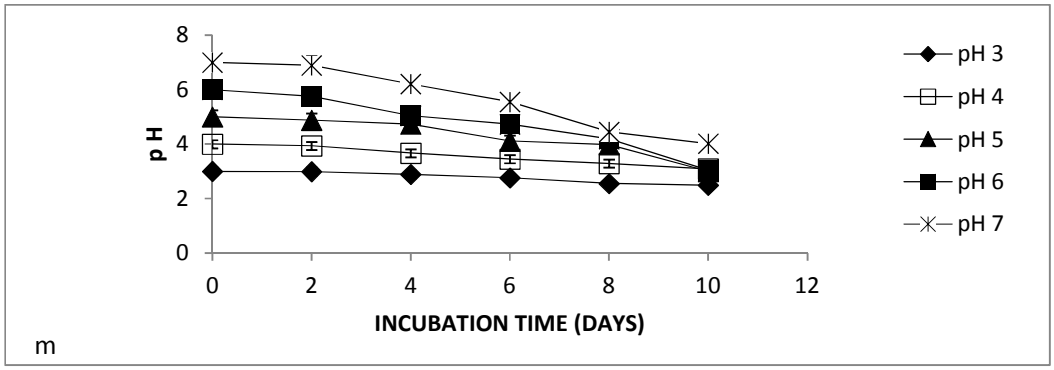
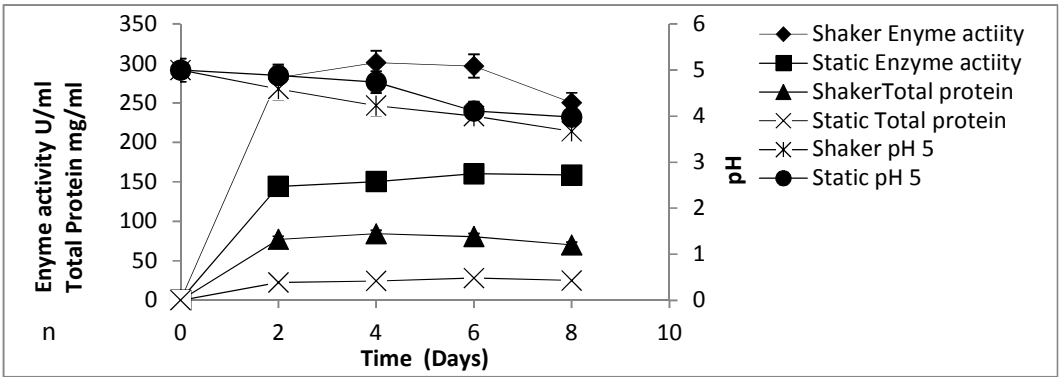


Fig. 11. The pH of all the medium evaluated at two days interval.



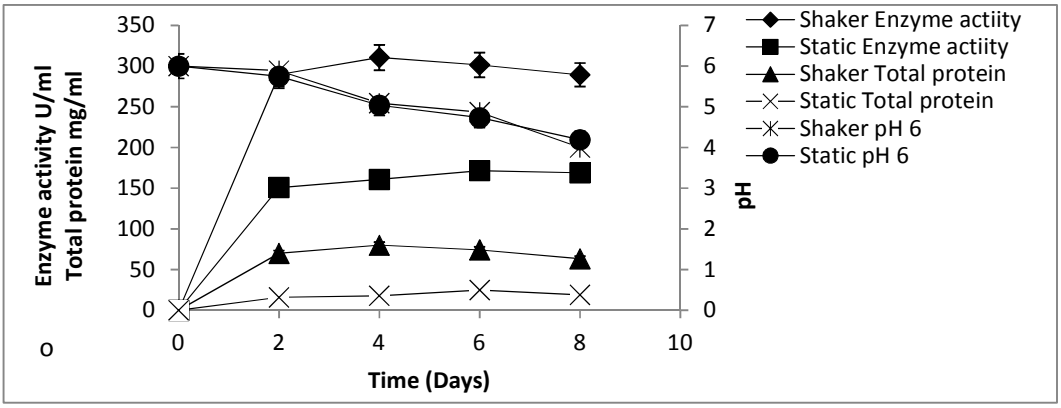


Fig. 12a, b. Effect of shaker and static conditions on laccase production was investigated

3.2 Discussion

The use of coloured indicator enabled visual detection of lignolytic activities and presents simple method of screening with no measurement [24]. Previous studies [25,26] have reported plate-assay as an efficient and simple method for bio-prospecting fungi with novel lignolytic enzymes for industrial application purposes. The green halo formation observed in the plate was as a result of oxidation of coloured compound due to lignolytic enzyme production [26,1]. Also it is an evidence of multi-enzymatic actions that could be applied in xenobiotic biodegradation studies as well as a suggestion of the physiological conditions of basidiomycetes during bioremediation process (Machado et al. 2005). The five fungi isolates produced appreciable level of laccase in the presence of both glucose-peptone medium and agro waste materials. The growth and laccase production by these fungi isolates was significantly influenced

by agro waste materials used (about 65%) and this is consistent with the view of Mendoza et al., [27] that used bagasse and orange peel medium to grow *Galerina spp* HCL strain which resulted to increase in the enzyme activity by 4 fold. This can be attributed to the inducer inclusion mechanism of fuelling genes because most fungi species posses' extensive reservoir of genes encoding catabolic enzymes directed against dozens of compounds of nutritional value. This principle states that when growing a cell on single substrate as sole carbon source as an energy source, the cell requires high level of the enzymes that can metabolize that substrate and feed the catabolic product into the central fuelling pathway because all metabolic pathway in cell must follow from the metabolites produced by these enzymes unlike the basal medium where glucose is already provided for direct ultilization. Fungi species are able to sense the appropriateness of each catabolic pathway in a given circumstance and regulate gene expression. Stajic et al., [28] had a contrary opinion after analyzing many other carbon sources during pretreatment with *Pleurotrus eryngii* 616 and *Pleutrotus ostreatus* 493 and 494 cultures and reported laccase activity was maximum when glucose is supplied than in any other carbon source in the medium. Soya bean meal supplementation enhanced the secretion of laccase involved in the degradation of cellulose, hemicelluloses and lignin [29] studies on use of shaker incubation revealed low pH and increase in inoculum size led to excessive growth of mycelium and this affected the production yield of the enzyme due to mass transfer and metabolic rate limitation. Galhaup et al., [30] reported excessive growth affected the mechanical set up of the reactor. *Penicillium martensii* NRC 345 under shaking condition produced more laccase than under static condition as reported by Elshafei et al., [23] and this corresponds with the data obtained from this study but contradicts the report of Tavares et al., [31] that reported agitation did not play a role in the production of laccase by *Trametes versicolor.* However [32] reported that the effect of aeration varies between fungi species as growth of some fungi is highly favoured with aeration while others can suffer stress caused by oxygen.

4. CONCLUSION

In the view of this enzyme importance in large application and the limitation of this application due to lack of capacity to produce large volumes of highly active laccase at affordable cost with identifying inexpensive raw material for the enzyme production. This study has successfully isolated five different species of fungi with (*Trichoderma spp*) showing the ability to produce copious amounts of laccase from inexpensive and easily available agro industrial (sawdust, plantain peel and banana peel) under submerged fermentation. Optimization of fermentation process (cultural parameters and operational conditions) has also resulted in higher laccase production. These substrate are safe, cheap and could be suggested for higher production of enzyme.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kiiskinen L, Kruus K, Balley M, Yiosmaki E, Siika-aho M, Saloheimo M. Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology. 2004;150:3065-3074.
2. Rodriquez CS, Herrera JLT. Industrial and biotechnological applications of laccases: A review. Biotechnology Advances. 2006;24:500-513.
3. Bagewadi K, Mulla I, Ninnekar H. Purification and immobilization of laccase from Trichoderma harzianum strain HZN10 and its application in dye decolorization. Journal of Genetic Engineering and Biotechnology. 2017;15(1):139-150.
4. Abd El Monssef A, Hassan A, Ramadan M. Production of laccase enzyme for their potential application to decolorize fungal pigment on aging paper and parchment. Annals of Agricultral Science. 2016;61(1):145-154.
5. Giacobbe S, Pezzella C, Lettera V, Sannia G, Piscitelli A. Laccase pretreatment for agro food wastes volarization. Bioresource Technology. 2018;265:59-65.
6. Weichuan Q, Hai L. Enhanced decolorization of malachite green by a magnetic graphene oxide-CotA laccase composite. International Journal of Biological Macromolecules Article in Press; 2019
7. Fathali Z, Rezaei S, Faramarzi M, Habibi-Rezaei M. Catalytic phenol removal using entrapped cross-linked laccase aggregates. International Journal of

Biological Macromolecules. 2018;122: 359-366.

8. Verma A, Shirkot P, Dhiman K, Rana N, Joshi V. Bacterial laccase mediated inhibition of enzymatic browning in apple juice and its sensory evaluation. International Journal Curr Microbiology Applied Science. 2018;7(1):3371-3381.
9. Osma J, Toca-Herrera J, Rodriguez-Couto S. Banana skin: A novel waste for laccase production by *Trametes pubescens* under solid state condition. Application to synthetic dye decolouration. Dye Pigment. 2007;75:32-37.
10. Gianfreda L, Xu F, Bollag JM. Laccases: A useful group of oxidoreductive enzymes. Bioremediation. Journal. 1999;3:1-26.
11. Rico A, Rencoret J, Del Rio JC, Martinez AT, Gutierrez A. Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock. Biotechnology for Biofuels. 2014;7:6.
12. Eggert C, Temp U, Dean J, Eriksson K. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett. 1996;391:144-148.
13. Niku-paavola ML, Karunen E, Kantelinen A, Viikari L, Lundell T, Hatakka A. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus P*hlebia radiata*. Journal of Biotechnology. 1990;13:211–221.
14. Chakroun H, Mechichi T, Martinez MJ, Dhouib A, Sayadi S. Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: Application on bioremediation of Phenolic compounds. Process Biochem. 2010;45:507–513.
15. Khambhaty Y, Anath S, Sreeram K, Rao J, Nair B. Dual utility of novel, copper enhanced laccase from *Trichoderma aureoviridae*. Int J Biol Macromol. 2015;29(81):6975.
16. Gochev VK, Krastanov AI. Fungal laccases review. Bulgarian Journal of Agricultural Science. 2007;13:75-83.
17. Rasera K, Ferla J, Dillon P, Riveiros R, Zeni M. Immobilization of laccase from *Pleurotus sajor-caju* in polyamide membranes Desalin. 2009;245:657-661.
18. Das N, Sengupta S, Mukherjee M. Importance of Laccase in vegetative growth of Pleurotus florida. Appl. Environ. Microbiol. 1997;63:4120-4122.
19. Barnett HL, Hunter BB. Illustrated genera of imperfect fungi (4th edition). The American phytopathological society. St paul Minnesota, USA. 1999;218.
20. Patel H, Gupte A, Gupte S. Effect of different culture conditions and inducers on production of laccase by a Basidomycete fungal isolate *Pleutrotus ostreatus* HP 1 under solid state fermentation Bio Resources. 2009;4(1):268-284.
21. Heinzkill M, Beach L, Halkier T, Schneider P, Anke T. Applied and Environmental Microbiology. 1998;64:1601.
22. Bradfford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytica Biochemistry. 1976;72:248-254.
23. Elshafei A, Hassan M, Haroun B, Othman A. Optimization of laccase production from Penicillium martensii NRC345. Advances in Life Science. 2012;2:31-37.
24. Okino LK, Machado KMG, Fabris C, Bononi VLR. Ligninolytic activity of tropical rainforest basidiomycetes. World J Microbiology Biotechnology. 2000;16:889-893.
25. Masalu R. Lignin degrading enzymes from mycelia cultures of wild Tanzanian mushrooms. Master of science thesis University of Daressalaam Tanzania. 2004;91.
26. Rodriquez CS, Rivela I, Sanroman A. *In vivo* decolourization of the polymeric dye poly R-478 by corn cob cultures of Phanerochaete chrysosporium. Acta. Biotechnology. 2000;20:31-38.
27. Mendoza L, Mamo G, Flores N, Gimenez A, Hatti-kaul R. Laccase mediator system for activation of agarose gel application for immobilization of proteins. Journal of Molecular Catalysis B-Enzymatic. 2011;68:270-274.
28. Stajic M, Persky L, Friesem D, Hadar Y, Wasser S, Nevo E, Vukojenik J. Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* spp" Enzyme Microbiology Technology. 2006;38:65-73.
29. Shashirekha M, Rajarathnam S, Bano Z. Enhancement of bioconversion efficiency and chemistry of the mushroom, *Pleurotus sajor-caju* (Berk and Br.) Sacc. produced on spent rice straw substrate,

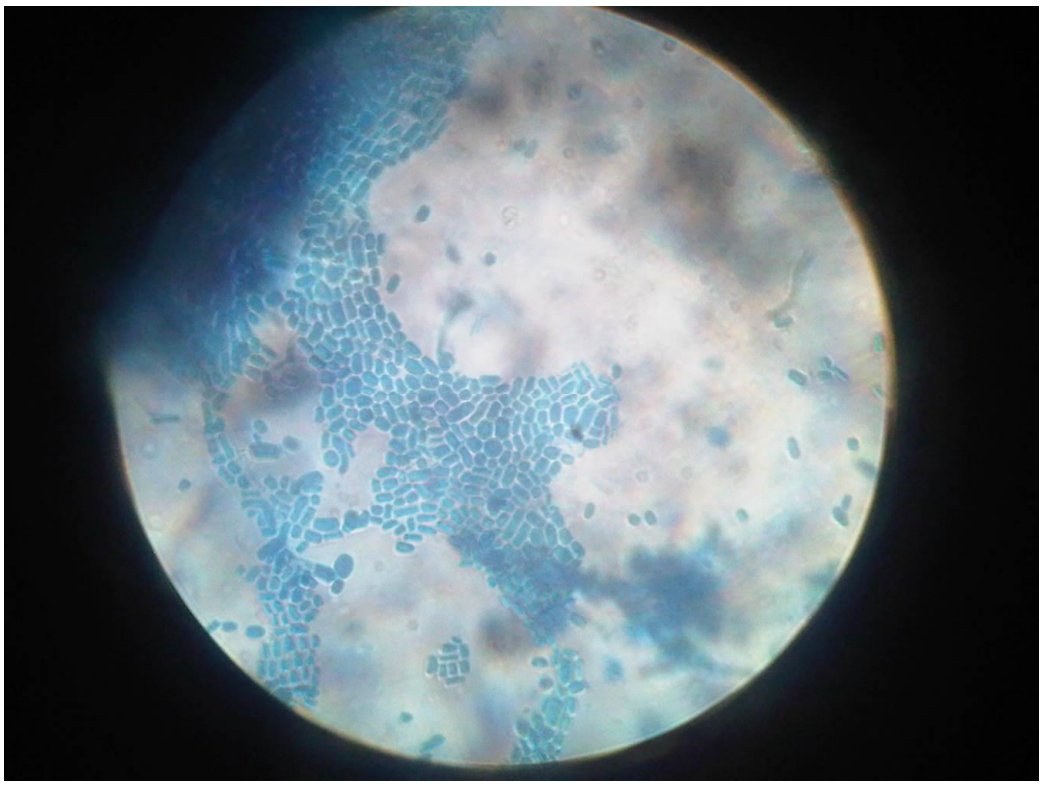
supplemented with oil seed cakes. Food Chemistry. 2002;76:27-31.
30. Galhaup C, Wanger H, Hinterstoisser B, Haltrich D. Increased production laccase by the wood-degrading basidiomycete Trametes pubescens. Enzyme Micro-biology Technology. 2002;30:529-536.
31. Tavares M, Coelho M, Agapito P, Coutinho P, Xaiver R. Optimization and modeling of laccase production by Trametes versicolor in a bioreactor using statistical experimental design. Applied Biochemistry and Biotechnology. 2006;134:233-248.
32. Brijwani K, Rigdon A, Vadlani P. Fungal laccases: Production, function and application in food processing. Enzyme Research. 2010;1-10.

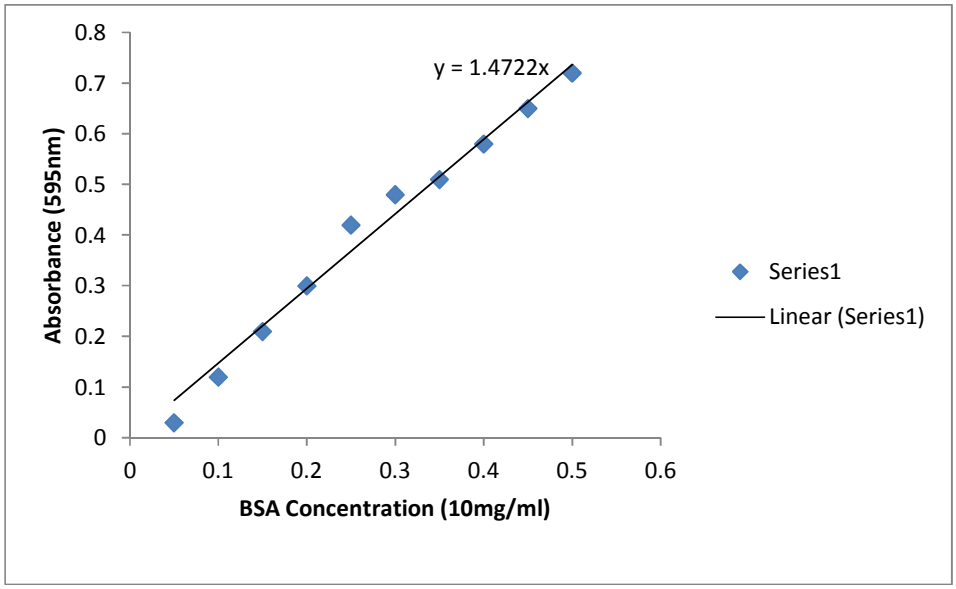
Appendix A

Preparation of BSA Reagent

The reagent was prepared by dissolving 100mg comassie blue G in 50ml of 95% ethanol. The solution was mixed with 100ml of 85% phosphoric acid and made up to 1 litre with deionized water. The reagent was filtered through whatman No 1 filter paper and stored in an amber bottle at room temperature. BSA standards in the concentration range 0 – 0.6 ug/ml was prepared with distilled water.


Yellow
colony
Black
colony





Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/57170