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PRODUCTION OF LACCASE BY *BACILLUS SUBTILIS* AND *ASPERGILLUS NIGER* FOR TREATMENT OF TEXTILE EFFLUENT

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ABSTRACT

Evaluation of laccase produced by *Bacillus subtilis* and *Aspergillus niger* isolate for treatment of textile effluent was investigated in the study. The bacteria and fungi isolate (*Bacillus subtilis and aspergillus niger*) strains were tested for laccase producing ability through plate test method. An increase in temperature and pH was revealed to have a significant effect on *Aspergillus niger* and *Bacillus subtilis* at (0.61±0.02 and 0.51±0.15) and (0.27±0.15 and 0.39±0.14) respectively. Although *Aspergillus niger* had high resistant to change in temperature and pH compared to *Bacillus subtilis.* The findings further revealed that at different quantity

Introduction:

The treatment of textile effluent containing dye has been carried out by various physical and chemical methods over the last two decades for the removal of color from waste water. These methods have limited applicability as they are expensive and lead to the production of solid waste. The treatment processes are based on the microorganisms capable of decolorizing or degrading these recalcitrant compounds (Khehra et al., 2005). These biological processes are environmentally friendly

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(10 mL, 20 mL and 30 mL) of application of laccase produce by *Aspergillus niger* and *Bacillus subtilis*, there are significance difference of the absorbance rate with average of 0.46±0.13 and 0.49±0.11. The physical chemical properties such as pH, total dissolved solid, electrical conductivity of the textile effluent tend to varies significantly with the application laccase at different quantity. The researcher thereby concluded that laccase produced by *Aspergillus niger* and *Bacillus subtilis* are very good agent for treatment of textile effluent.

Keywords: Laccase, *Bacillus subtilis*, *Aspergillus niger*, textile effluents, enzyme

and can lead to complete mineralization of xenobiotic compounds. The studies of biodegradation of dyes and its derivate products are of environmental interest because of its recalcitrant nature, carcinogenicity, mutagenicity and toxic effects. Dyes never die, but some of the dyes when degraded biotically or abiotically, produce the end products that are more toxic than native dyes as a result of incomplete degradation. Two major sources of release of dyes into the environment are the textile and dyestuff manufacturing industries (Nigam *et al.,* 2000). These dyes are difficult to degraded and pose hazardous effect on aquatic life. In addition to dyes, textile effluent also contains high ionic strength, salt and high pH values as well. Over the past decade, many organisms capable of dye decolorization at laboratory scale have been reported, but there are few reports available on their exploitation in treatment processes. Efforts to isolate bacterial culture capable of degrading azo dyes started in the 1970s with reports of a *Bacillus subtilis* (Horitsu *et al*., 1997). Bacterial isolates from soil and sludge sample belonging to *Bacillus sp* was found to have high dye decolorization ability (Sharma *et al*., 2007). Laccase (p-diphenol

oxidase, EC 1.10.3.2) is a multi-copper oxidase, belonging to a family of enzymes, called the large blue copper proteins. The ranges of substrates which laccase can attack are any phenolic substrates such as p-diphenol (Thurston, 1994). The research therefore was designed to produce laccase by *Bacillus subtilis* and *Aspergillus niger* to treat textile effluent

Materials and Methods

The sampling site was Angwan saraki (SAS), Mechanic junction (SMJ) and Paida junction (SPJ) situated in Chanchaga Local Government Area in Niger State. with latitude 9°37' North and longitude 6°33' East (Figure 1)

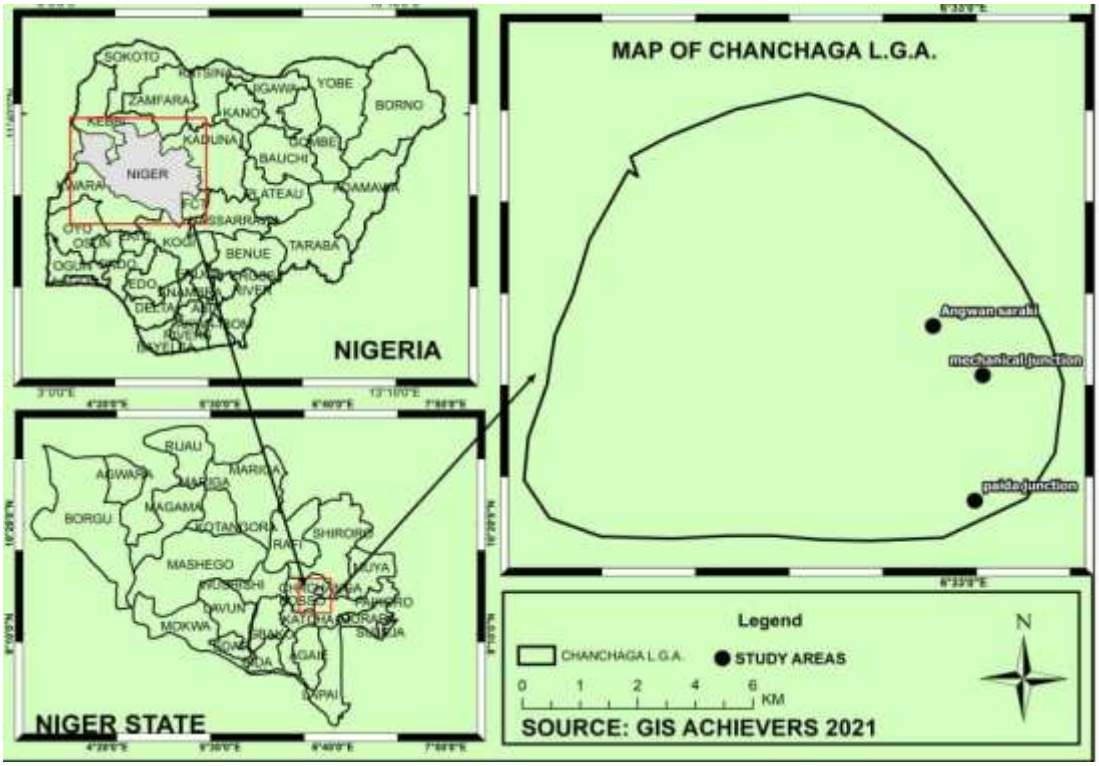

Figure 1: Study area map.

Samples Collection

The soil contaminated with textile effluents was randomly collected from dyeing workplace at Angwan saraki (SUS), Mechanic junction (SMJ) and

Paida junction (SPJ) Minna, situated at Chanchaga Local Government Area, Niger State. Soil samples were collected in sterilized polythene bags from a depth of 10 –15cm below the earth's surface, with clean hand trowel and was transported to Microbiology Laboratory, Federal University of Technology, Minna for further analysis. All the samples were kept at 4^{o}C until used.

Isolation characterization and Identification of *Bacillus subtilis* and *Aspergillus niger* from Dye Contaminated Soil

Dilution plate methods were carried out for the isolation of *Bacillus subtilis* from the soil sample. One gram of the soil samples was transferred to 9ml of 0.85% sterile saline water. Tenfold serial dilution was performed by transferring one milliliter of aliquot from each of the samples to 9ml of 0.85% saline water. From the diluted samples 0.1ml of the diluent was plated on nutrient agar and nutrient agar supplemented with Tween 80. The Plates were incubated for 24 - 48h, at 37°C for bacteria and fungi were plated on sabouraud dextrose agar (SDA) and incubated for 72 h at 28$\pm$2 ^{o}c. Colonies with zone of clearance on NA was picked and stored in a sterile nutrient agar slant for further studies (Mobarak-Qamsari *et al.*, 2011). The obtained pure culture was identified via the microscopic, morphological and biochemical characteristics.

Morphological and cultural characters of isolated fungi

The isolated fungi were cultured on sabouraud's dextrose agar medium by inoculating a fungal isolate on a plate. The inoculated media plate was incubated at 28 $\pm$ 2°C. Cultural observations of the colonies were observed on fourth day after inoculation and the cultural appearances, colony colour, and aerial hyphae on SDA, (Revati *et al.,* 2019). Colony colours were determined by using a pictorial atlas for identification of fungi by

Watanabe (2002) was equally used in the description of colony morphology.

Micro-morphological characterization

A drop of Lactophenol cotton blue (LCB) were placed on the center of a clean slide, using a sterile needle, a small portion of the pure fungal colony in the given culture were transferred to the drop of LCB slide. It was teased apart with dissecting unneeded, was covered with a cover slip and carefully placed on the prepared slide to avoiding air bubbles. The microscopic observations were carried out to study the morphological characters of the isolated fungi, were visualized under high power in order to identify the fungus hyphae presence or absence of septa in order to determine their taxonomic classes.

Screening *Bacillus subtillis* and *Aspergillus niger* for Laccase Enzymes Production.

Bacteria and fungi isolate (*Bacillus subtilis* and *Aspergillus niger)* strains were tested for laccase producing ability through plate test method. The ability of the bacterial and fungal strains to produce laccase wee vrisualized according to the method of (Kiiskinen *et al.,* 2004).

Fungi (*Aspergillus niger*) was separately inoculated on PDA agar plates supplemented with guaiacol (0.02%) (wt/wt), 1 naphthol (5mM), and tannic acid (0.5%) (wt/wt) and inoculated with *Aspergillus niger* cultures was used for screening of laccase production. The culture was incubated at 25^{o}C. The bacterial strain (*Bacillus subtillis*) was inoculated in nutrient agar and grown at 37 °C. Laccase secretion was monitored by visual color change in the plates, due to oxidation of screening agents, for several days (More *et al.,* 2011, Kiiskinen *et al.,* 2004). A reddish-brown color was formed when secreted laccases react with guaiacol, a deep purple color

was formed when they react with 1-naphthol, and a brown color were formed when laccases react with tannic acid.

Preparation of Inoculum

Bacterial inoculum was prepared by the method described by Gangadharan *et al.*, (2006). A volume of 50 ml of nutrient broth were inoculated with a loopful of cells from a 24h old slant and kept at 37°C and 25°C for *Aspergillus niger* in a rotary shaker (100 rpm). After 18h of incubation, 1 ml of this nutrient broth and sabouraud dextrose broth culture of *Bacillus subtillis* and *Aspergillus niger* were used as the inoculum for solid state fermentation.

Production of Crude laccase under SSF Condition

Exactly, 2g of rice bran were taken in different flasks and moistened with 100mL of Mineral Basal Salt Solution (MBSS) containing (g/L^{-1} peptone 3, dextrose 1g, K_2HPO_4 0.4, KH_2PO_4 0.6, $MnSO_4$ 0.5, $FeSO_4$ 0.0005, and $ZnSO_4$ 0.01). The initial moisture level in the medium was 20% (w/v) and alpha napthol (Pointing 1999). The culture medium was adjusted to pH before sterilization by autoclaving at 121°C for 15minutes. The flasks were sterilized and were allowed to cool at room temperature, fermentation was carried out at pH, and temperature of B*. subtilis* and agitation was at 10,000rpm. And with the aid of a sterile bottle cork, a 5mm disk of fungal (*Aspergillus nigger*) isolates was separately incubated into the cultivation medium. incubation was carried out at pH, 27 $\pm$ 2°C for 5days in a shaker incubator operated at 10,000rpm for 10min. Hundred milliliter sterile production media containing 2% carbon and nitrogen source were prepare with optimize pH according to the composition giving by Unyayar *et al.*, (2005).

Laccase is extracellular enzymes, so its recovery at 10,000rpm for 10minutes to separate the cell. The supernatant obtained were used as the crude enzyme extract and was stored in vials for further used (Aslam and Asgher, 2011).

Optimization of Culture Condition for Laccases by *Bacillus subtilis* and *Aspergillus niger*

The various process parameters that influence the enzyme production during SSF were optimized over a wide range. The strategy adopted for standardization of process parameters to evaluate the effect of an individual parameter and to incorporate it at standardized level before standardizing the next parameter.

Effect of Temperature on laccase activity and stability

To investigate the effect of temperature on *Bacillus subtilis* and *Aspergillus niger*. 50mL of MBSS and 2g of rice bran were introduced into 250mL Erlenmeyer flasks. The flasks were sterilized, cooled to room temperature, and inoculated with B*. subtilis* and *Aspergillus niger* were incubated at different temperatures (25, 30, 35, 40, 45, and 50◦C) under optimum incubation. The content soft the flasks were centrifuge at 10,000rpm for 10min at 4◦C and the supernatant was used to assay the enzyme activity at 420nm.

Effect of pH on Laccase Activity and Stability

To investigate the effect of hydrogen ions on biological activities is similar to their hydrogen ion concentration on enzyme activity of *Bacillus subtilis* and *Aspergillus niger*, 50mL of mineral basal salt solution and 2g of rice bran and wheat bran were taken in 250mL Erlenmeyer flasks and pH was adjusted in each of the flasks between 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0,

respectively, by addition of 1N/HCl and 1N/NaOH. The flasks were sterilized, cooled to room temperature, and inoculated with B*. subtilis* and incubated at optimized and temperatures for rice bran). The contents of the flasks were centrifuge at 10,000rpm for 10min and the supernatant was used to assay the enzyme activity at 420nm.

Measurement of Laccase Activity

Laccase enzymes activity was performed according (kalra *et al.,* 2013). A crude enzyme extract solution of 1ml was added to a 3ml sodium acetate buffer solution.

The mixture was added with 1ml of 2mM guaiacol, homogenized by using a vortex, and incubated for 15 minutes at room temperature. The reduction of guaiacol concentration was measured using a spectrophotometer at a wavelength of 450nm. For the blank solution 1ml of aquadest, 1ml of 2mM guaiacol and 3ml of sodium acetate buffer were mixed then incubated by using the Lambert-Beer equation, with guaiacol molar absorptivity of 12,100/M/cm. the enzymes activity is expressed in the unit. A unit of enzymes activity is equivalent to a reduction of 1nmol of guaiacol per minute from the treatment of 1ml of enzyme to quaiacol under normal conditions (U/ml)

Partial Purification of Laccase

Partial purification of laccase enzyme was done through Ammonium sulfate precipitation and Dialysis.

Dialysis

Pellet was dissolved in 0.01M phosphate buffer at optimized pH and was dialyzed against the same buffer overnight at 4ºC.

Physiochemical Analysis

The physiochemical parameters to be analyzed before and after treatment include pH, temperature, biological oxygen demand (BOD), chemical oxygen demand (COD), Total suspending solid (TSS), nitrogen electrical. (Mondal *et al.*, 2005).

Results

Cultural biochemical characteristics of isolate

Table 1: Cultural biochemical characteristics of isolate

Code	Gram RXT	Fermentation				Starch Hydrolysis	Catalase	Citrate	Coagulase	Methyl RED Rxt	Oxidase	Motility	Urease Production	organisms
		L	S	G	M									
SPJ[3] A	+VE Rod cocci	-	+	+	+	+	+	-	-	-	-	-	+	
SUS[3] A	+VE Rod in chain	-	+	+	-	-	+	-	-	-	-	+	+	
SMJ[2] A	+VE Rod cluster in chain	-	+	+	+	+	+	-	-	-	-	+	+	
SMJ[3] A	+VE Short rod cluster in chain	-	+	+	+	+	+	+	-	-	+	+	-	

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Optimization of temperature for Culture Condition of Laccases by *Aspergillus niger* and Bacillus subtilis

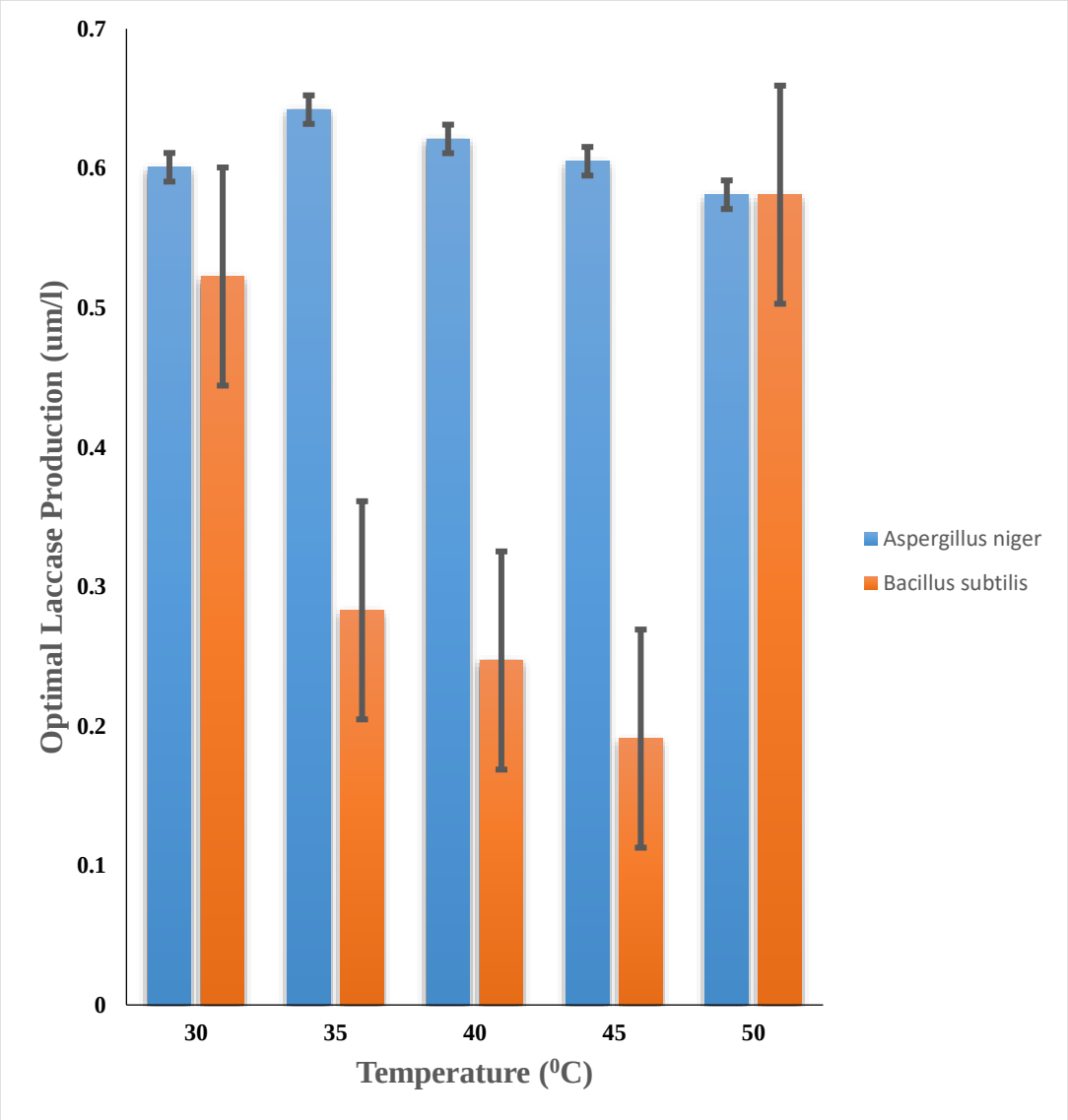

Figure 2: Optimization of temperature for Culture Condition of Laccases by *Aspergillus niger* and *Bacillus subtilis*

Figure 2 revealed that the optimal laccase of 0.642 U/ml by produce by *Aspergillus niger* at temperature of 35°C. to *Bacillus subtilis* produced maximum Laccase of 0.581 U/ml at increase temperature of 50°C. This

implies that to high extent *Aspergillus niger* produce more Laccase at temperature as low as 35°C compared to *Bacillus subtilis* that may required high temperature to produce large quantity of laccase

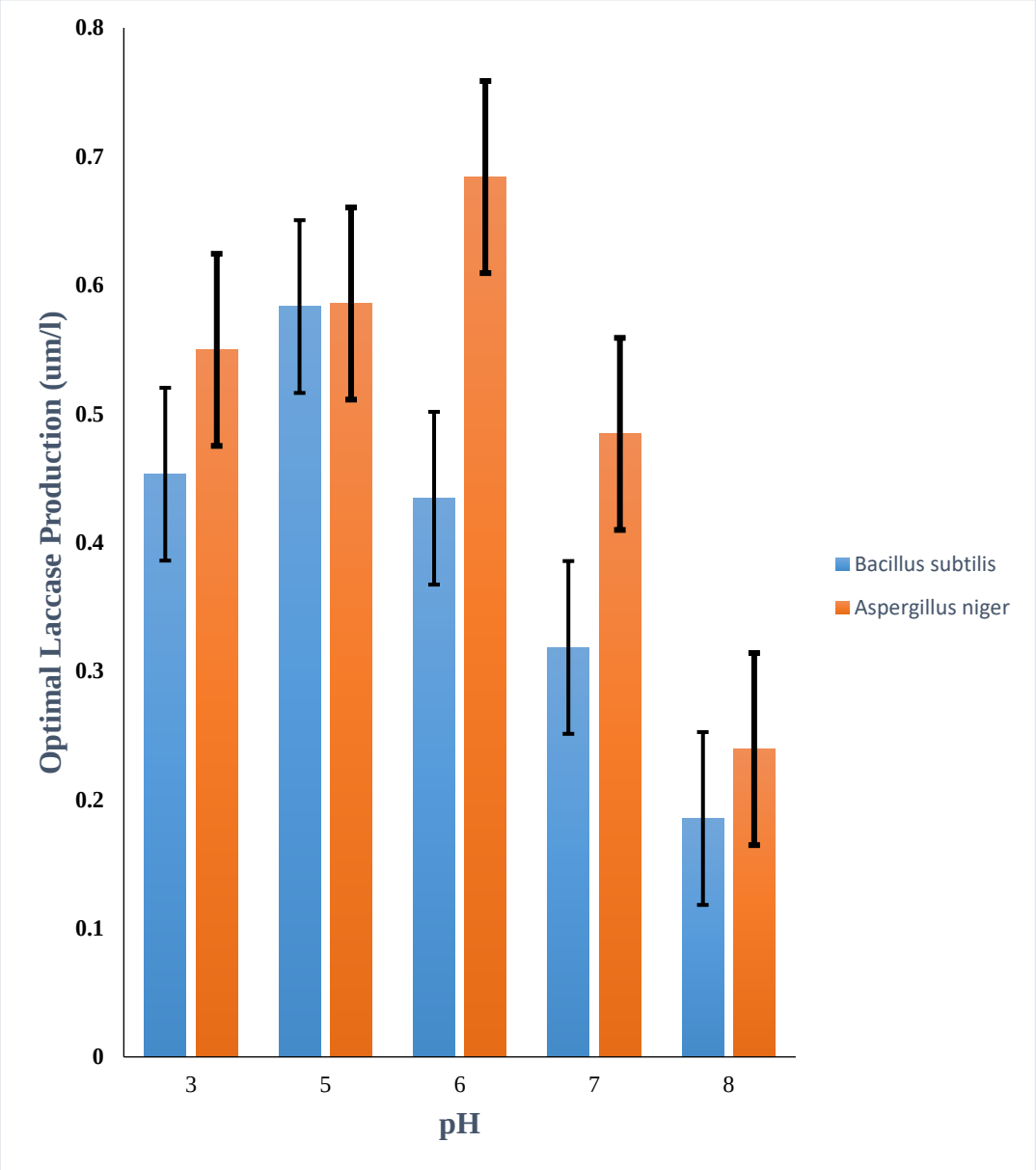

Figure 3: Optimization of pH for Culture Condition of Laccases by *Aspergillus niger* and *Bacillus subtilis*

In Figure 2, the optimal production of Laccases by *Aspergillus niger* and *Bacillus subtilis* with an increase in pH from 3 - 8 was revealed. The Figure depict that *Aspergillus niger* produced more Laccase than *Bacillus subtilis* at different pH value. The optimal Laccase production (0.684 U/ml) was observed at pH of 6. It was also observed that Laccase are more produce at pH within 3 – 6. This implies to some extent *Aspergillus niger* and *Bacillus subtilis* can produce Laccase at control pH 3 -6.

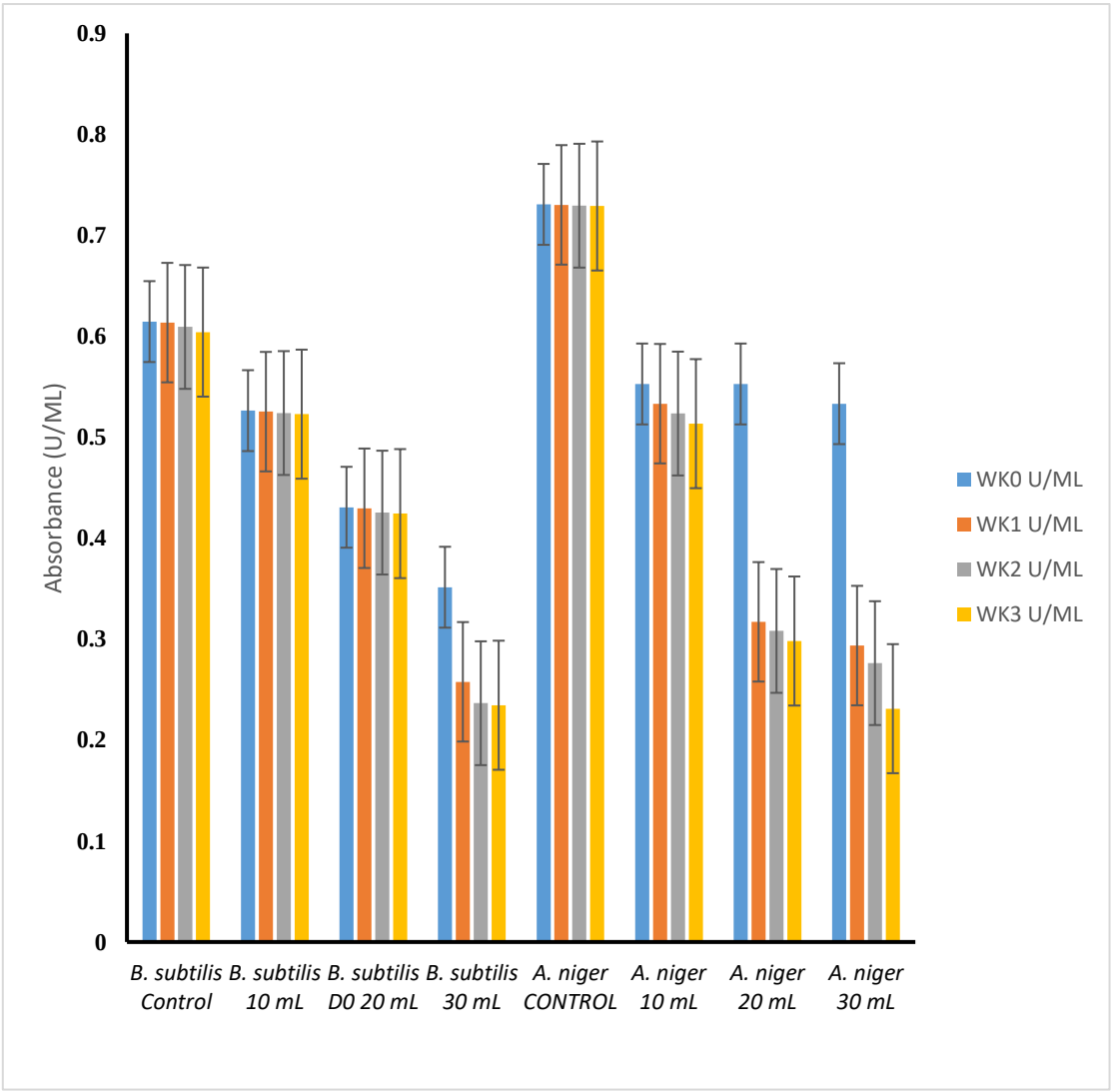

Figure 4: Treatment of Textile Effluent produces by *Aspergillus niger* and *Bacillus subtilis*

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Figure 4 shows that the absorbance Level of Laccase produced by *Aspergillus niger* and *Bacillus subtilis* from Control, 10ml, 20ml and 30ml. It was revealed that significance different at p> 0.05 exit between absorbance level of control and the addition of Laccase produced by Aspergillus *niger* and *Bacillus subtilis* at 10ml, 20ml, and 30ml. it was also observed *Bacillus subtilis* performs better in the treatment of effluent most especially at 30ml.

Physicochemical Parameters of Effluent Before and After the Treatment

The analysis of physicochemical parameters of effluent before and after the treatment with *Aspergillus niger* and *Bacillus subtilis*

Table 2: Physicochemical Parameters of Effluent Before and After the Treatment

	^{0}C		Mg\l	Mg\l	Ps\cm	Mg\l	Mg\l	Mg\l	Mg\l
Sample	**Temp**	**pH**	**TDS**	**TSS**	**Elect Cond**	**D.O**	**BOD**	**COD**	**Nitrate**
Before Treatment	34±0.00^{a}	13.10±0.00a	39.53±0.00a	3.00±0.00^{a}	5.90±0.00^{a}	7.0±0.00^{a}	5.20±0.00^{a}	6.10±0.00^{a}	18.00±0.00^{a}
Control After Treatment	31.3±31.11^{b}	5.45±2.79^{b}	28.96±9.54^{b}	3.72±0.50^{a}	4.66±1.87^{a}	6.47±0.17^{a}	3.75±0.35^{b}	5.42±0.20^{a}	14.63±0.31^{b}
Aspergillus niger	31.89±1.20^{b}	7.60±0.36^{b}	22.71±6.46^{c}	7.52±0.57^{b}	3.39±0.96^{b}	6.51±0.21^{a}	3.70±0.34^{b}	5.55±0.18^{a}	14.84±0.16^{b}

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After Treatment									
Bacillus subtilis	31.78± 0.60 [b]	2.91±0.25 [c]	13.08± 2.65 [d]	5.23± 0.25 [b]	1.95±0.39 [b]	6.40± 0.09 [a]	3.61±0.38 [b]	5.50±0.09 [a]	14.90± 0.15 [b]

Table 2 revealed that temperature (^oC) before treatment (34 ± 0.00) was significantly different at $p < 0.05$, to the control (31.3 ± 31.11), *Aspergillus niger* (31.89 ± 1.20) and *Bacillus subtilis* (31.78 ± 0.60). From the result it was also observed that there was no significant different at $p > 0.05$ between the control, effluent treated with *Aspergillus niger* and *Bacillus subtilis*

Discussion of Results

This study focusses on production of laccase by *Bacillus subtilis* and *Aspergillus niger* isolated from soil for the treatment of textile effluent. From the outcome of the study the *Bacillus subtilis* and *Aspergillus niger* production of laccase was successful with result of molecular characteristic and biochemical identification of isolate.

From the findings of the study it was revealed that the optimal laccase of 0.642 U/ml by produce by *Aspergillus niger* at temperature of 35^oC. to *Bacillus subtilis* produced maximum Laccase of 0.581 U/ml at increase temperature of 50^oC. This implies that to high extent *Aspergillus niger* produce more Laccase at temperature as low as 35^oC compared to *Bacillus subtilis* that may required high temperature to produce large quantity of laccase (Figure 2)

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Finding also revealed that the optimal production of Laccases by *Aspergillus niger* and *Bacillus subtilis* with an increase in pH from 3 - 8 was revealed. More so *Aspergillus niger* produced more Laccase than *Bacillus subtilis* at different pH value. The optimal Laccase production (0.684 U/ml) was observed at pH of 6, and laccase are more produce at pH within 3 – 6 (Figure 3)

The study the absorbance Level of Laccase produced by *Aspergillus niger* and *Bacillus subtilis* from Control, 10ml, 20ml and 30ml. It was revealed that significance different at p> 0.05 exit between absorbance level of control and the addition of Laccase produced by *Aspergillus niger* and *Bacillus subtili* at 10ml, 20ml, and 30ml. Hence*, Bacillus subtilis* performs better in the treatment of effluent most especially at 30ml. This is found similar to the finding of Ikram *et al*. (2022) also investigated the Bacillus subtilis: as an efficient bacterial strain for the reclamation of water loaded with textile azo dye, orange II. The azo dye orange II is used extensively in the textile sector for coloring fabrics.

The findings of the study on physicochemical parameter (Table 2) also depict that temperature (^{o}C) varies significantly before treatment and after treatment with *Aspergillus niger* and *Bacillus subtilis* although there was no significant different in temperature between effluent treated with *Aspergillus niger* and *Bacillus subtilis*. The pH was also observed to be significantly different before treatment and after treatment. more so the pH of *Aspergillus niger* was signifncant different from *Bacillus subtilis*, with *Bacillus subtilis* having the least pH. The TDS also revealed to varies significantly, before and after treatment with *Bacillus subtilis* having the least TDS. Sen and Nigam (2022) findings on Bioengineering for Decolorization of Synthetic Dyes in Textile Effluents using Microbial Enzymes affirmed this finding.

The TSS was revealed to also varies significantly before and after treatment with *Aspergillus niger* and Bacillus subtilis, same with electrical conductivity and BOD, while the there was no significant different at $p > 0.05$ for the dissolve oxygen DO and Nitrate before treatment and after treatment with *Aspergillus niger* and *Bacillus subtilis*.

Conclusion

Based on the findings of the study on the production of laccase by *Bacillus subtilis* and *Aspergillus niger* for treatment of textile effluent. The *Bacillus subtilis* and *aspergillus niger* were identified, and it is possible to concluded that the laccase produced by *Bacillus subtilis* and *Aspergillus niger* are essential, high-potential, and unavoidable components of an effective for treatment of textile effluent. Laccase by *Bacillus subtilis* and *Aspergillus niger* were identified to have absorbance properties that can aid the treatment of effluent. It is paramount to emphasize that laccase produced by *Bacillus subtilis* does better than *Aspergillus niger*

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