Freshwater Weeds as Low-cost Solid Substrate for the Production of Peroxidase by a Newly Isolated *Bacillus flexus* from Wastewater

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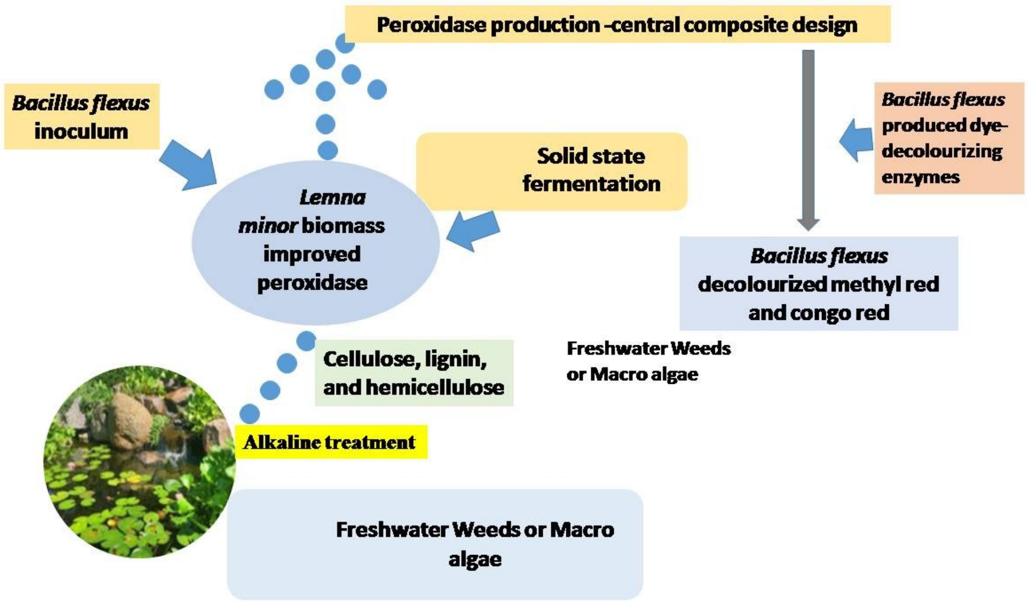
DOI: 10.15376/biores.20.4.10148-10169

GRAPHICAL ABSTRACT



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Freshwater weeds (*Eichhornia crassipes, Lemna minor, Azolla pinnata, Myriophyllum indicum* and *Nymphoides peltatum*) were used as fermentation substrates to increase production of peroxidase enzyme. The pretreated freshwater weeds released sugars which favoured bacterial growth and peroxidase yields. The cellulose content of macroalgae ranged from 15.9±0.42 to 26.4±0.18% and the maximum amount was detected in *L. minor* (26.4±0.18%). Lignin content was high (8.3±0.4%) in *A. pinnata* and hemicellulose content was highest (26.5±0.92%) in *E. crassipes*. Peroxidase production was high in the *L. minor* biomass (7.28 ±0.41 U/g), followed by *E. crassipes* (6.72±0.3 U/g). The isolated bacteria C18 produced dye degrading-enzymes such as tyrosinase (2.74±0.3 U/mL), lignin peroxidase (0.71±0.02 U/mL), NADH-DCIP reductase (1.49±0.03 U/mL), laccase (29.8±1.1 U/mL), and azoreductases (35.4±0.15 U/mL). Central composite design and response surface methodology were used to improve peroxidase production by *Bacillus flexus*. Peroxidase production improved with an increase in initial pH value, low levels of glucose, and ammonium sulphate. *B. flexus* decolourized methyl red (>75%) and Congo red (>60%) in the culture medium. *Lemna minor* is a cost-effective culture medium for peroxidase production. The enzyme-based bioremediation reduces toxic pollutants in water systems, thereby potentially reducing health risks due to environmental exposure.

DOI: 10.15376/biores.20.4.10148-10169

Keywords: Freshwater weeds; Biomass; Solid support; Peroxidase; Toxic pollutants; Disability prevention

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INTRODUCTION

Agricultural by-products are important feedstocks for the generation of biofuels, enzymes, and secondary metabolites. Lignin is one of the most prominent polymers in lignocellulosic wastes (Ghaffar and Fan 2013). Commercially, lignin is generated as a waste product or fuel from paper and ethanol production. The worldwide production of lignin is about 100 million tonnes/year, and the approximate value was 732.7 million UDD in 2015. It is expected to reach $913.1 million by 2025, and the annual growth rate is approximately 2.2%. Lignins are categorized into the organosolv category (2%), kraft lignins (9%), and ligno-sulphonate (88%). Over the decades, lignin has been burned as fuel for power and heat. And approximately 2% of the lignin is utilized as adhesives, dispersants, and surfactants. However, due to the development of the bio-refinery concept and the emerging bioenergy/biofuel technologies, lignins are being utilized as cost-effective raw material (Bajwa *et al*. 2019). The presence of lignin in the plant material is known to slow down the saccharification process. Therefore, it is common practice to remove the lignin from the hemicelluloses and cellulose by chemical pre-treatment. Nevertheless, these methods require higher energy, are costly, and require stringent conditions. Alternatively, biological processes mediated by catalytic processes have lower energy input requirements, increased specificity, and are considered an alternative for lignin utilization. Microorganism-mediated lignin degradation is used because of good adaptabilities to temperature, pH, and oxygen-limited conditions (Welker *et al*. 2015). Peroxidases are enzymes that can oxidize various recalcitrant compounds. These enzymes can be used in various applications, including cosmetics, water, chemical, pulp and paper, and textile industries (Draelos 2015). Moreover, the uses of peroxidases are severely affected by poor yield, high production cost, and poor stability due to high concentration of hydrogen peroxide (Pham *et al*.2022). Extracellular production of peroxidases has several advantages in comparison to intracellular production because of simple recovery and purification process and less time required (Musengi *et al.*2014). In recent years the interest in peroxidases from microbial origin has increased, as peroxidases from the microbial sources seem to be highly stable in comparison to enzymes of animal and plant origin (Anbu *et al.* 2017). Most of the ligninolytic bacteria have the ability to produce peroxidases that have potential to be used in various industrial applications. A lignin-degrading bacterial strain has been characterized (Falade *et al.*2017b). Some of the bacteria from the actinobacteria group produce peroxidase for industrial processes (Musengi *et al.* 2014). In addition, peroxidase-producing bacteria from the genus *Pseudomonas* has been reported previously (Linde *et al*. 2021). These previous reports revealed that bacteria produce types of ligninolytic enzymes. Peroxidases from bacteria have several advantages in comparison to fungal sources because in the case of bacteria, it is possible to enhance catalytic properties using protein engineering approach. Moreover, studies on bacterial peroxidases are limited due to lack of generally regarded as safe organisms for environmental and industrial applications. Peroxidases have been characterized from the fungi *Rodococcus jostii*, *Pseudomonas putida*, and *Streptomyces viridosporus*, and these sources were involved in lignin degradation (Kumar and Arora 2022; Ambatkar *et al*. 2022). Peroxidases are recently receiving much more attention because of their ability to oxidize various compounds, and they are well known for their resistance to enzymatic degradation due to high redox potentials (Falade *et al.*2017a). These properties have prompted researchers to use these enzymes for industrial processes. However, increased production cost of peroxidases has hampered the use of this enzyme in industrial applications. The commercially available peroxidases from *Streptomyces avidinii* (streptavidin), *Bjerkandera adusta* peroxidase, and horseradish peroxidase could not meet the growing industrial demand. Hence, a continuing search for a peroxidase-producing novel strain from various sources continues is needed to meet the increasing market demands. Bacteria can produce dye-decolourizing peroxidase, and these include *Bacillus subtilis* (Rao and Kavya 2014), *Bacillus aryabhattai* B8W22 (Elmetwalli *et al.* 2023), and *Enterobacter aerogenes* ES014 (Al-Ansari *et al*. 2022). These enzymes have been utilized for the bioremediation of azo dye from the effluent due to a broad range of substrate specificity. Peroxidases such as manganese peroxidase, lignin peroxidase, and versatile peroxidase have the potential to degrade xenobiotic compounds and azo dyes from the environment (Pinheiro *et al*. 2022; Ambatkar *et al.* 2022).

Azo dyes are synthetic organic compounds that are widely used in chemical industries. These inorganic waste substances polluted the environment (Balapure *et al.* 2014). Dye industries have discharged about 40% of unused dyes to the environment, and these cause serious threat to the environment and organisms (Sahasrabudhe *et al.*2014). The prolonged disposal of these dyestuffs into the soil and water bodies raises the organic load to natural ecosystem, which in turn leads to the negative impact on ecological functions. Hence, it is very important to eliminate these toxic substances from the environment before it gets discharged into the surrounding ecosystem (Das and Mishra 2017). Several traditional physico-chemical methods have been recommended for textile dye effluent treatment; however, these methods showed limited success. Moreover, microbial methods are eco-friendly and exhibit high substrate specificity (Govindwar *et al.* 2014). Bacteria from the stressed or contaminated environment generally modify and adapt themselves for the degradation of synthetic xenobiotic compounds, showing catabolic activity toward the surrounding polluted environment (Hsueh *et al.* 2017). The indigenous bacteria are more likely to be well suited for the removal of pollutants. In addition to bacteria, actinomycetes, fungi and algae also have been studied for degradation and decolouration of the azodyse, among which bacteria show promising results for the removal of textile azo dyes from the effluent (Kurade *et al.* 2016).

Aquatic plants, including emergent, submerged, and free-floating species, have attracted great attention due to their potential to generate a huge amount of biomass, being cheap and easy to harvest, and having high bioremediation rates. Among aquatic plant species, the free-floating type of aquatic weeds is widely used as a biomass due to a simple harvesting procedure. The widely studied aquatic weeds are water lettuce (*Pistia stratiotes*) (Mukherjee *et al*. 2015), water hyacinth (*Eichhornia crassipes*) (Ruan *et al*. 2018), Azolla (Brouwer *et al*. 2016), and duckweed (Verma and Suthar 2015). Water hyacinth has been considered an invasive aquatic weed, and due to its rapid growth rate and cellulose content, it has been applied in various industrial processes (Wu and Ding 2020). Duck weed (*Lemna minor*) is used in various ranges of applications in several research areas, including ecotoxicological testing, wastewater treatment, and production of bioethanol, protein, and starch (Ramírez-Morales *et al.* 2022). Aquatic weeds are considered the source of bioenergy feedstock, and can be utilized for biomolecules, bioenergy production, and to control the ecological damage (Kaur *et al*. 2018). In the present investigation, anazo dye degrading *Bacillus* strain was characterized from the dye effluent. To improve the efficacy of decolouration process, the physico-chemical and nutritional factors were optimized for peroxidase production. In addition, dye degradation was monitored by spectrophotometry analysis.

EXPERIMENTAL

Enrichment of Bacteria for Dye Degradation

Water sample was collected from the dye effluent discharged from the dye industry. It was diluted to 10% using sterile double distilled water. The diluted sample (5 mL) was inoculated into minimal medium and incubated at 37 °C for 3 days for enrichment. After 3 days, 5 mL of culture medium was transferred to 100 mL of fresh degradation medium to continue culturing and acclimatize it to completely decolorize under the same conditions.

When the decolorization became stable, the individual concentration of the dye (Congo red, or methylene blue) was increased to 200, 300, 400, 500, and 600 mg/L, respectively. The broth culture was serially diluted up to 10^{-8},and bacteria were isolated. These dye-resistant bacterial strains were cultured in mineral medium and incubated for 37°C for 24 h. The growth was monitored, and the rapidly growing 10 bacterial strains were selected for further studies. The selected bacterial strains (A1, A2, A3, A9, A16, C18, D10, D27, H1, and U10) were sub-cultured and finally maintained at 4 °C.

Veratryl Alcohol Oxidation Test

Veratryl alcohol (VA) oxidation test was performed to screen the oxidation performance of VA to veratryl aldehyde by the bacterial strains. The selected bacterial strains were cultured in Luria-Bertani (LB) broth medium for 48 h. The growth was monitored continuously and maintained to reach 1.5 to 2.0 optical densities at 600 nm. After 48 h, the culture was centrifuged at 10000 rpm 10 min, and the cell-free supernatant was used for analysis. For the analysis of VA oxidation, VA (2.0 mM) and H_2O_2 (0.4 mM) were mixed into cell-free supernatant (0.5 mL). The total volume of the reaction mixture was 1.0 mL volume. Finally, the generated veratryl aldehyde was detected by measuring the absorbance at 310 nm using a UV spectrophotometer. Based on VA oxidation test, 10 isolates were selected for peroxidase production.

Culture of Bacteria under Submerged Fermentation (SmF)

The isolated bacterial strains (A1, A2, A3, A9, A16, C18, D10, D27, H1, and U10) were cultured under submerged cultivation using alkali lignin medium containing, 0.1% (w/v) alkali lignin, yeast extract (0.1 g L^{-1}), NH_4NO_3 (5 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), KH_2PO_4 (0.53 g L^{-1}), and K_2HPO_4 (4.55 g L^{-1}). Briefly, the alkali lignin medium was sterilized and inoculated with 1% bacterial inoculum and incubated for 48 h, at 150 rpm at 30 ± 2 °C. The culture (2 mL) was withdrawn at regular intervals and centrifuged at 12000 rpm for 10 min. Then the cell free culture supernatant was used for the determination of peroxidase enzyme. Experiment was performed in triplicates, and an average value was considered for data analysis.

Peroxidase Activity

Peroxidase activity was determined from the sample, as suggested previously by Change and Maehly (1955). Briefly, 0.1 mL of crude sample (enzyme) was mixed with substrate (5% w/v pyrogallol), and 0.1 mL phosphate buffer (0.1 M, pH 7.0) was added. To this reaction mixture, 0.5% (v/v) H_2O_2 was added to initiate the reaction, and the absorbance of the sample was read at 420 nm. A 96 well microtiter plate was used for the analysis of enzyme activity. To the blank, double distilled water was added. One enzyme unit is defined as the amount of enzyme required for oxidation of one micro mole of pyrogallol to purpurogalin in 34 seconds.

Macro Algae Collection and Processing

The freshwater macro algae (*Eichhornia crassipes*, *Lemna minor*, *Azolla pinnata*, *Myriophyllum indicum*, and *Nymphoides peltatum*) were collected from the freshwater pond, Tamilnadu, India (Coordinates: 08°10'N 77°22'E). They were dried, crushed mechanically, powdered, and sieved with 60 meshes for further use.

Pretreatment of Macro Algae

The dried freshwater weeds (*E. crassipes*, *L. minor*, *A. pinnata*, *M. indicum*, and *N. peltatum*) were pretreated by soaking 50 g of weed in 1000 mL of 2% NaOH for 15 min at 28±1 °C. Then, the material was placed in a boiling water bath for 2 h with occasional mixing for every 10 min. Then it was washed with double distilled water to remove NaOH residues from the biomass. Washing of biomass was continued until the culture filtrate reached pH 7.0. Then it was dried at 60 °C for 24 h and stored at 4 °C.

Determination of Cellulose, Lignin, and Hemicellulose

The amount of cellulose content of the biomass was determined by Seifert method (Browning 1967). Lignin and hemicellulose content of macroalga was determined by the NREL laboratory analytical protocol (Sluiter 2008).

Characterization of Bacterial Strain

The strain C18 was subjected for characterization studies. Briefly, the strain C18 was cultivated in LB broth medium for 18 h at 130 rpm in a rotary shaker. After 18 h incubation, 4 mL culture was centrifuged at 10000 rpm for 10 min. The genomic DNA was isolated as described previously, using a DNA purification kit (Thermo Scientific, USA). Universal forward and reverse primers were used for the amplification of partial rRNA gene. The sequences were submitted to GenBank databases and accession number was assigned (PX257951).

Bacterial Growth and Peroxidase Production at Various Incubation Times

Enzyme production towards bacterial growth was determined by the method of Tuncer *et al*. (1999). The selected bacterial strain (C18) was cultured in LB medium and incubated for 48 h. The culture was further inoculated in newly prepared LB medium (pH 7.0) and incubated in a rotary shaker incubator at 150 rpm and at 30 ± 2°C. The culture was withdrawn for every 12 h for 96 h and enzyme activity was assayed. The bacterial growth was monitored continuously using a UV-visible spectrophotometer at 600 nm.

Production of Peroxidase in Solid State Culture by the Bacterial Strain C18

A total of 10 g algal biomass was weighed individually and added into the Erlenmeyer flasks. Then, 8 mL of sodium phosphate buffer (pH 7.2) was added and mixed evenly, and sterilized for 30 min at 121 °C. The bacterial strain C18 (10^6 CFU/mL) was inoculated (2%) and fermentation experiment was performed for 96 h. After 96 h incubation, peroxidase activity was assayed.

Evaluation of Process Parameters to Improve Peroxidase Production

To screen the variables to improve peroxidase production, four independent variables (pH, temperature, carbon source, and nitrogen source) were selected. The pH of the culture medium was optimized by adjusting the initial pH of the medium ranging from 5.5 to 9.0 using 0.1 M buffers. To determine optimum temperature for the production of enzymes, the culture was maintained between 25 and 50 °C at pH 7.5. To optimize the carbon sources, organic carbon sources such as glucose, fructose, sucrose, xylose, trehalose and maltose were supplemented at 0.5% level. To determine optimum nitrogen sources, the solid medium was supplemented with 0.1% peptone, yeast extract, beef extract, casein, and ammonium sulphate. All experiments were performed in triplicates and an average value was considered for analysis.

Analysis of Dye Decolourization Enzymes from the Strain C18

The bacterial strain was cultivated with azo dyes. The bacterial biomass was centrifuged after 48 h at 10000 rpm (10 min, and 4 °C) and biomass was harvested. The cell free extract was used for the determination of enzyme activity was performed as described previously by Balapure *et al.* (2014). Cell-free lysate was used for the determination of azo-reductase, tyrosinase, NADH-DCIP reductase, laccase, and lignin peroxidase. Laccase biosynthesis was performed as suggested previously by Hatvani and Mecs (2001). Reduction of Azo was carried out by analyzing the decreased absorbance of DR81 at 514 nm. Lignin peroxidase, tyrosinase, and NADPH-DCIP were analyzed, and the result was expressed as U/mL under standard conditions.

Optimization of Peroxidase Production by Response Surface Methodology (RSM)

Central composite design (CCD) and RSM was selected using software (Design Expert, version 8.0, Statease Inc., Minneapolis, USA) to optimize the selected individual variables. Peroxidase activity was expressed as response Y. The results (Y) were fed in software and analyzed to generate 3D response surface plots revealing the optimum physical variable (pH) and nutrient sources (glucose and ammonium sulphate). The generated 3D plots revealing optimum culture conditions and interaction among these selected variables. Analysis of variance was performed to determine the significance of the designed model. The collected data were subjected for regression analysis.

Biocatalytic Reduction of Azo Dyes by the Strain C18

The selected bacterial strain (C18) was cultured in minimal medium containing 1% solid substrate and maintained for 24 h at 35 °C. The bacterial strain was harvested by centrifugation (10000 rpm 10 min), and the biomass was resuspended in physiological saline (0.9% NaCl). Further, the bacterial cell was counted appropriately using plate count agar. The methyl red and Congo red concentrations were tested over incubation time, and the decolouration ability of strainC28 was determined against reagent blank. To the control (abiotic), bacterial strain was not inoculated. Microbial decolourization analysis was performed and the result was expressed as % decolourization. The culture was centrifuged and the cell free supernatant was used for the determination of azo dyes degradation. The decrease in λ max (absorption maxima) of methyl red (MR) was monitored at 525 nm and the decrease in λ max of Congo red at 490 nm.

RESULTS AND DISCUSSION

Screening of Peroxidase Producing Organisms

Peroxidase has many applications in various industrial processes. A total of 10 azo dye resistant bacterial strains were isolated and screened for peroxidase production. The yield of peroxidase ranged from 1.4±0.4 IU/mL to 6.83±0.09 IU/mL. The bacterial strain C18 showed the highest peroxidase yield (6.83±0.09 IU/mL) (Fig. 1). The use of peroxidases in industries is mainly based on the use of horseradish peroxidase. Hence, the search for efficient peroxidase producing bacteria is useful and can easily be genetically modified (Falade *et al*. 2017a). This study analyzed extracellular peroxidase production by bacteria in submerged fermentation. Bacteria isolated from the industrial effluent can be improved dye degrading ability (Bernal *et al*. 2021). Dawkar *et al*. (2009) isolated peroxidase producing *Bacillus* sp. VUS from the textile effluent and reported increased peroxidase producing potential. These peroxidase producing bacterial strains have application for textile dye decolouration (Santos *et al.* 2014; Linde *et al.* 2021).

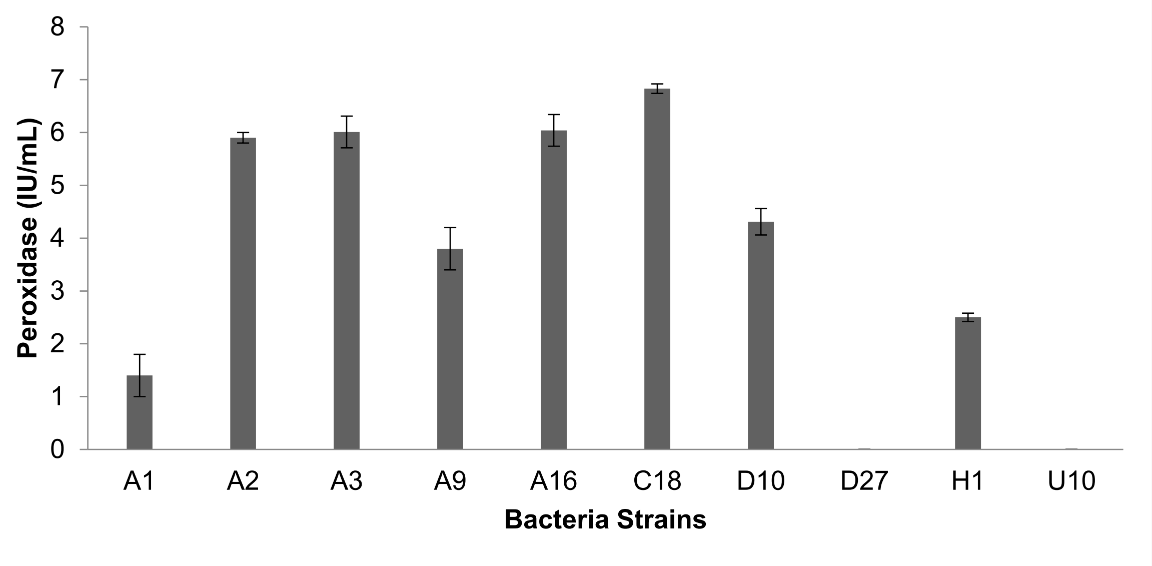

Fig. 1. Peroxidase production by bacteria isolated from the dye effluent in submerged fermentation. The bacterial strains were cultivated in minimal medium and peroxidase production was observed after 24 h incubation.

Cellulose, Hemicellulose, and Lignin Content of Macroalgae

The cellulose content of macroalgae ranged from 15.9±0.42 to 26.4±0.18% and the maximum amount was detected in *L. minor* (26.4±0.18%). Lignin content was high (8.3±0.4%) in *A. pinnata* and hemicelluloses content was maximum (26.5±0.92%) in *E. crassipes* (Table 1). In this study, alkali treated macroalgae showed these compositions. The biomass yield obtained after pretreatment method was similar with previous studies (Reales-Alfaro *et al*. 2013; Omondi *et al*. 2019). The cellulose, hemicelluloses, and lignin content of *Lemna minor* observed in this study were less than previously reported (Li *et al*. 2019). The cellulose content of *Azolla* was lower than water hyacinth and duckweed, which was consistent with Gupta *et al*. (2018).

Table 1. Lignocellulosic Biomass of Alkaline Pre-treated Freshwater Macro Algae

Macroalgae	Cellulose (%)	Lignin (%)	Hemicellulose (%)
E. crassipes (water hyacinth)	17.4±0.48	3.1±0.04	26.5±0.92
L. minor (duckweed)	26.4±0.18	7.8±0.18	20.4±0.16
A. pinnate (Mosquito fern)	18±0.11	8.3±0.4	9.11±0.22
M. indicum (Water milfoil)	20.4±0.92	4.3±0.17	4.7±0.28
N. peltatum (Floating heart)	15.9±0.42	5.4±0.26	6.9±0.47

Macroalgae Biomass: A Cheap Substrate for the Production of Peroxidases

Macroalgae are considered as an inexpensive source of bioactive compounds and enzymes. In the present study, peroxidase production was high in the *L. minor* biomass (6.72±0.3 U/g), followed by *E. crassipes* (6.72±0.3 U/g) (Fig. 2). Agroresidues are alternate carbon sources for microbial peroxidases production and reduce the production cost of enzymes. The present study revealed the use of freshwater macro algae for enzymes production. The utilized substrates are cheaper than inorganic carbon sources (Falade *et al*. 2020). Water hyacinth has been considered as the inducer for xylanase and cellulase production (Espinoza-Abundis *et al*. 2023). Agro-industrial wastes have been used for the production of lignocellulolytic enzymes (Rana *et al*. 2021), and this approach could be used to valorize agro-industrial residues for ecosystem management.

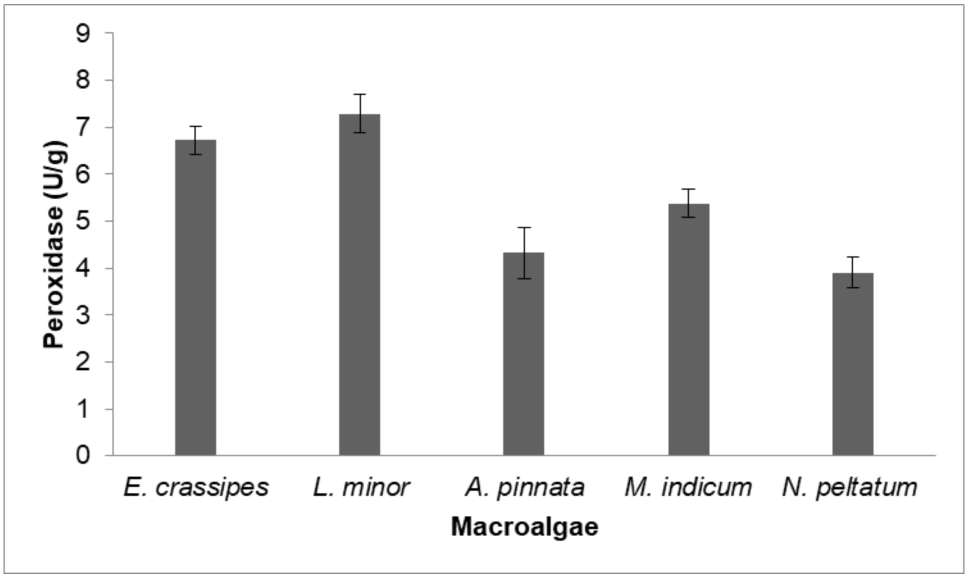

Fig. 2. Effect of macroalgae on peroxidase production in solid state fermentation

Effect of pH and Temperature on Peroxidases Production

In the present study, peroxidase producing ability of *B. flexus* was evaluated under optimized conditions, including pH and temperature. These are essential for optimal growth and product production in bacteria by amending the physical variables and nutrient factors. Bacterial growth is associated with extracellular enzymes production (Niladevi and Prema 2008). Hence, factors capable of affecting the growth of bacteria such as temperature, pH, as well as the nutrient composition will invariably influence in enzyme production by the bacteria. The pH of the culture medium is the important factors influenced on bacterial metabolism and microbial growth because nutrient absorption is mainly based on the surface charge (Saini *et al.* 2015). Hence, it is important to explore the pH of the culture medium that is highly favourable for various metabolic processes of microorganisms. The finding of culture pH for improved peroxidase production by the strain *B. flexus* is therefore important because pH of the culture medium influenced metabolic process. The results of culture medium pH on enzyme production are described in Fig. 3. The selected bacterial strain synthesized peroxidases over a range of pH values (pH 5.5 to 9.0), and the optimum pH was 7.5 (6.79 IU/mL). The selected bacterial strain showed its ability to produce enzyme at broad pH ranges; however, the variation in the enzyme yield was statistically significant ($p<0.05$). Rao and Kavya (2014) reported that acidic pH (6.0) was optimum to improve maximum peroxidases production in *Bacillus subtilis*. Moreover, maximum yield was reported when *Bacillus* sp. was cultured at alkaline pH value (8.0) (Rajkumar *et al.* 2013). These variations indicated the influence of pH on enzymes production in fermentation.

Bacteria have the ability to produce enzymes within certain temperature ranges. Temperature is a major environmental factors influenced on enzymes production, influence intracellular metabolites, macromolecular composition, and cell growth. Hence it is important to analyze the optimum temperature that affects the growth and enzyme production. The effect of culture medium temperature on peroxidase production by the strain *B. flexus* is described in Fig. 4. Peroxidase production was found to be maximum at 35 °C. Moreover, the variation in peroxidase yield between temperature 30 and 40 was not statistically significant ($p>0.05$), and significant variation was observed between 25 °C and 50 °C ($p<0.05$).The reduced metabolic process at lower temperatures can explain lower enzyme biosynthesis (Ray *et al.* 2007; Tandon and Sharma 2014). The present finding was in accordance with the observations made previously with other bacterial species. In *Bacillus* sp, optimum peroxidase production has been achieved at 30 °C (Rajkumar *et al.* 2013), whereas 37 °C was optimum for *Bacillus subtilis* (Rao and Kavya 2014). The variation in incubation temperature for enzyme production may be species and strain dependent.

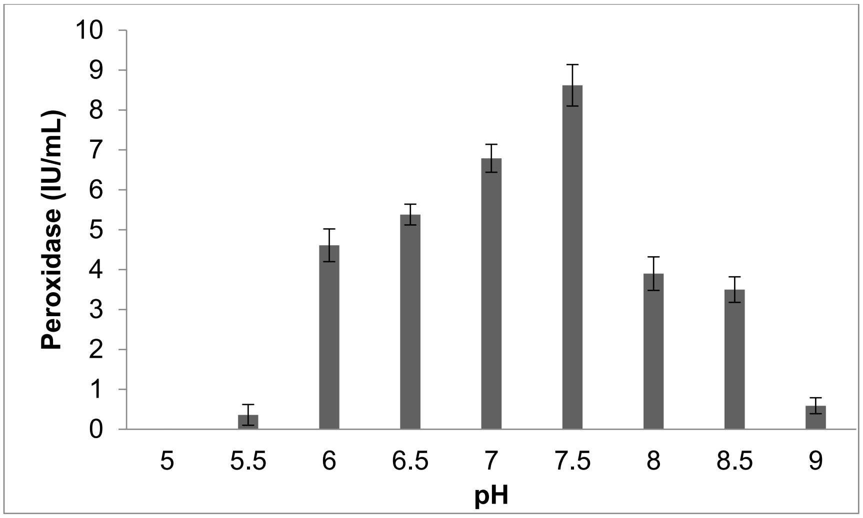

Fig. 3. Effect of medium pH on enzyme production by *B. flexus* in solid state fermentation

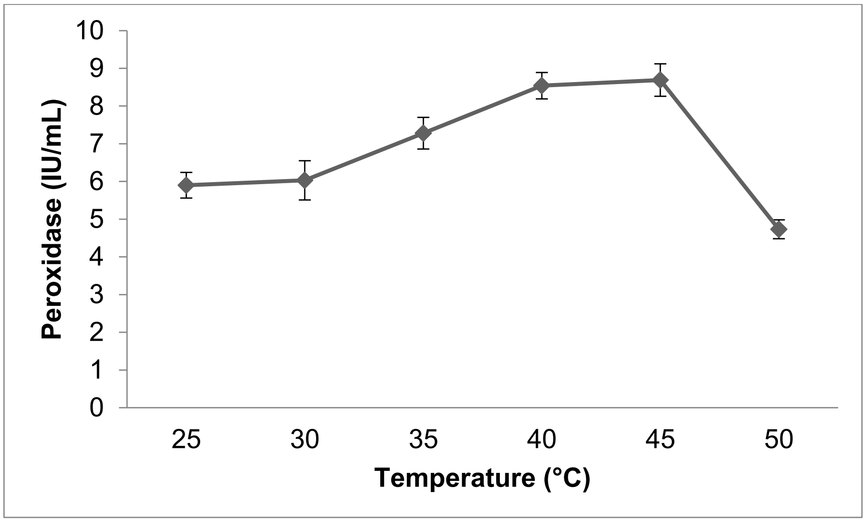

Fig. 4. Effect of incubation temperature on enzyme production by *B. flexus* in solid state fermentation

Optimization of Nutrient Factors on Peroxidase Production by *B. flexus*

The effect of carbon and nitrogen sources on peroxidase production was optimized. In this study, 0.5% carbon source was incorporated with solid substrate (glucose, fructose, sucrose, xylose, trehalose, and maltose). The present work revealed significant variation in peroxidase production when the organism was cultivated with different carbon sources ($p<0.05$) (Fig. 5). The effect of nitrogen sources on peroxidase production was carried out by incorporating 0.1% peptone, yeast extract, beef extract, casein, and ammonium sulphate with the solid substrate. Supplemented nitrogen sources affected peroxidases production (Fig. 6). The influences of various nitrogen sources on the production of ligninolytic enzymes were reported previously (Falade *et al.* 2019a). In this study peroxidase production was negatively regulated by the supplemented yeast extract, whereas, supplemented ammonium sulphate improved peroxidase production. Kaal *et al*. (1995) reported improved ligninolytic enzyme production; however, maximum lignin modifying enzyme production have been achieved in nitrogen-limited conditions (Galhaup *et al.* 2002). In this study, selected lower concentration of supplemented organic and inorganic nitrogen (0.1%) induced peroxidase production ($p<0.05$). Similarly, supplemented ammonium sulphate enhanced peroxidase production (9.67 IU/mL). The study conducted by Falade *et al.* (2019b) reported enhanced production of peroxidases in the medium containing ammonium sulphate in *E. adhaerens* NWODO-2. Moreover, ammonium chloride has been reported as the suitable nitrogen source for peroxidase production in *R. ornithinolytica* OKOH-1 (Falade *et al.*2019b). It has been reported that certain carbon sources in the medium affected the synthesis of bacterial enzymes (Abdel-Rhman *et al*. 2014). In the cited study, except for glucose, other carbon sources inhibited peroxidase synthesis. Similarly, the inhibitory effect was reported by Sadhu *et al*. (2012), who stated the production of enzyme using various carbon sources. Most of the tested nitrogen sources, repressed peroxidase activity and the increased production of observed with only ammonium sulphate. It has been previously reported that the nitrogen sources can either be inhibitory or stimulatory effect on lignocellulolytic enzymes (Pedri *et al.* 2015).

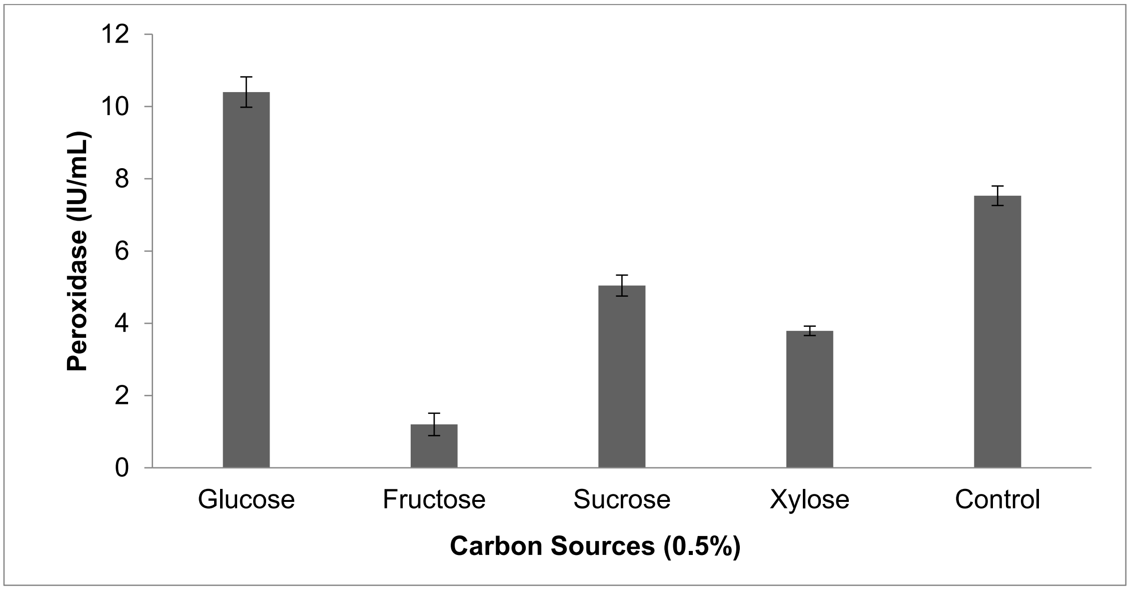

Fig. 5. Effect of carbon sources on enzyme production in solid state fermentation

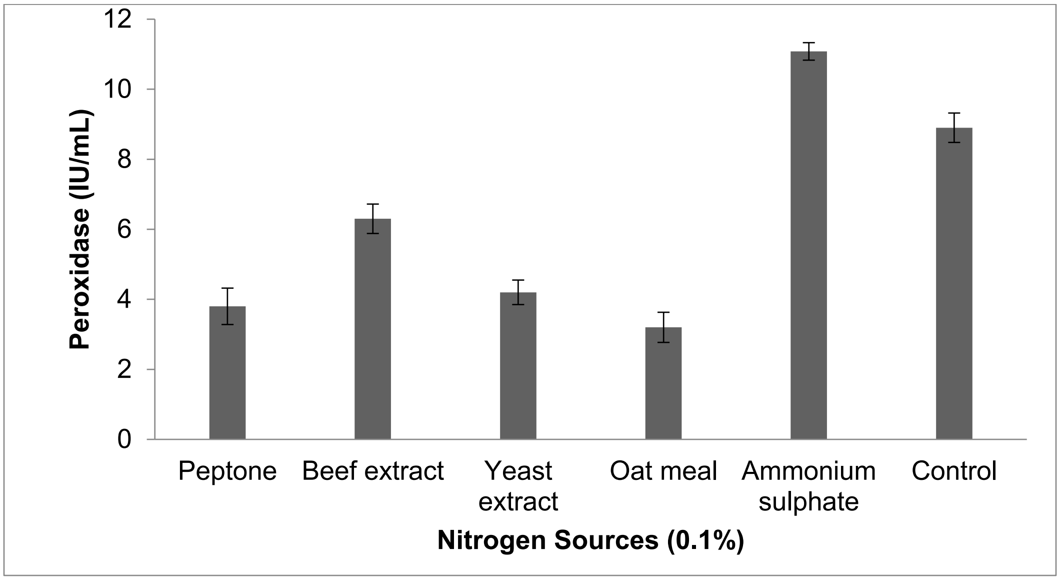

Fig. 6. Effect of nitrogen sources on enzyme production in solid state fermentation

Determination of Dye Degrading Enzymes

Bacteria degrade various synthetic dyes due to the activity of microbial enzymes. Microbial enzymes have been involved in dye degradation and decolourization. For degradation, these xenobiotic compounds required various enzymes from oxido-reductive classes (Saratale *et al.* 2013). In this study, enzyme activities such astyrosinase, lignin peroxidase, NADH-DCIP, laccases, and azoreductases were detected (Table 2). Bacteria synthesize azoreductases, which are able to break azo-bonds of the dyes (Zahran *et al.*2019). The selected bacterial strain produced azoreductases and lignin peroxidases and decolourized MR. These findings revealed synergistic activity of various oxido-reductive enzymes synthesized by the bacterial strain C18. Laccases have the ability to degrade various azo dyes. In *Bacillus stratosphericus* SCA1007 degradation of methyl orange was achieved by the activity of laccases (Akansha *et al.* 2019).

Table 2. Production of Dye-degrading Enzymes by Bacteria

Enzymes	Activity
Tyrosinase (U/mL)	2.74±0.3
Lignin peroxidase (U/mL)	0.71±0.02
NADH-DCIP reductase (U/mL)	1.49±0.03
Laccase (U/mL)	29.8±1.1
Azoreductases (U/mL)	35.4±0.15

Effect of Fermentation Period on Biomass and Peroxidase Production

The bacterial strain C18 was identified as *Bacillus flexus* and was used for further studies. As shown in Fig. 7, peroxidase production was high after 46 h incubation, and it declined after 48 h. Peroxidase production was 3.01±0 U/mL after 24 h, 7.92±0.027 U/mL after 48 h, and 5.76±0.33 U/mL after 72 h, respectively. During fermentation, bacteria utilize nutrients for their growth and enzyme production. The production of enzyme varied based on the availability of nutrient sources, and it declined after 48 h. Due to the decreased availability of nutrient sources, it reaches a declining phase in which growth and enzyme production decrease. This result is consistent with the previous studies about other bacterial species on enzymes production (Falade *et al*. 2020; Rambu *et al.* 2024). Peroxiase-producing *Bacillus* strains were isolated and characterized from fermented tea (Mohammad and Alireza 2007). Peroxidase characterized from *Bacillus* sp. VUS effectively decolourized textile dyes (Dawkar *et al*. 2009). A peroxidase enzyme isolated from *Bacillus subtilis* exhibited dye-degrading activity (Min *et al*. 2015). The microbial peroxidase shows unique abilities to degrade various environmental pollutants, including, industrial dye effluents. The efficacy of degrading capacity varied based on their source, and type of peroxidase (Twala *et al*. 2020).

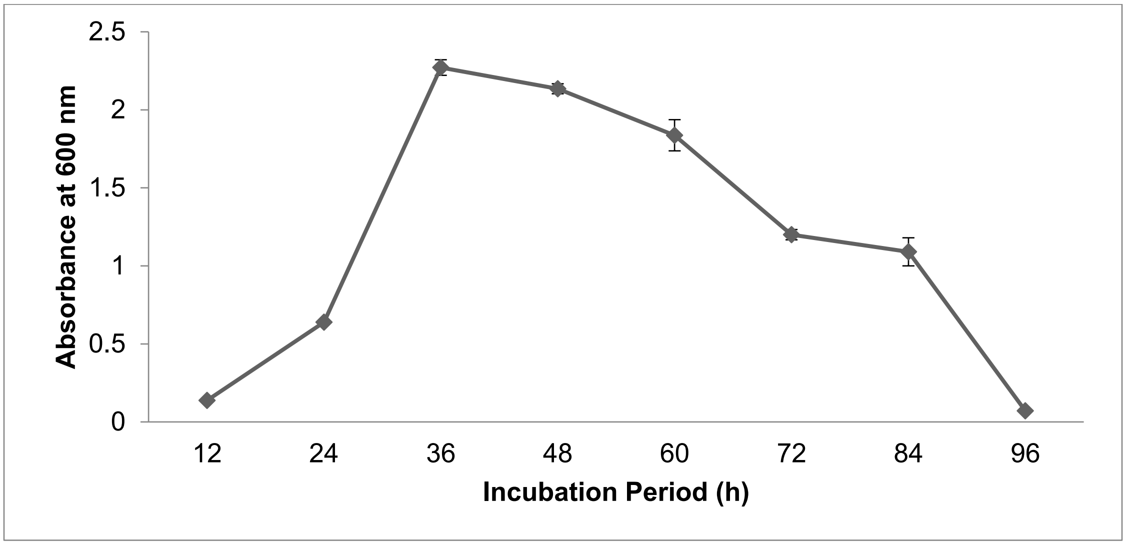

Fig. 7. Effect of incubation period on bacterial growth (a) and peroxidase production in submerged fermentation. Error bar represents standard deviation.

Central Composite Design and Response Surface Methodology

The levels of the selected three process variables which have positive impact on peroxidase production were selected to design central composite design model. The peroxidase yield is presented in Table 3 along with experimental matrix. In the response surface experiment, maximum yield was obtained in run 8 and peroxidase activity was 30.4 U/g. Response surface methodology has been widely used for the production of peroxidises from *Bacillus mycoides* (Rath *et al*. 2022), *Enterobacter wuhouensis* (Thatoi *et al.* 2023), and *Bacillus subtilis* strain KSK02 (Selvam *et al*. 2024). The model F-value of 74.99 implies the model was significant. In this designed model, the variable C and the model terms, AB, A^2, B^2, C^2 were statisticallly significant. The p-value for lack of fit was 0.50, revealing that the fitted quadratic model was not significant (Table 4). The determination coefficient of the model R^2 was >0.90, which showed that the experimental and predicted values had coherence with each other. The predicted R^2 value was 0.948 and the Adj R^2 value was 0.972. The adequate precision of the model value was 22.32, which was judged to be adequate signal. The model F-value, p-value, and lack of fit value obtained in this study showed significance of the designed model as described previously in the literature (Arokiyaraj *et al.* 2024; Alarjani *et al*. 2024; Alfarhan *et al*. 2024).

The interaction between pH, glucose, and ammonium sulphate was presented in 3D response surface plots. In a 3D plot, the interactive effect of two variables was analyzed at a time, and a third variable was kept at zero level (middle level). The 3D surface plot showing the interactions between the selected variables and used to identify the significant factors (Mariyam *et al*. 2024; Zouhair *et al*. 2024). Peroxidase production improved with an increase in initial pH value and the low level of supplemented glucose improved enzyme production (Fig. 8a). The optimum pH obtained in this study was similar with *Pseudomonas stutzeri* laccase, and optimum enzyme production was achieved at pH 6.8 using wheat straw and cotton stalk substrate (Minhas *et al.* 2024).Maximum enzyme peroxidase yield was obtained at increasing concentration of ammonium sulphate in the culture medium, in comparison to pH and glucose levels (Fig. 8b). Ammonium sulphate has been identified previously as an efficient inducer for peroxidase production (Falade *et al*. 2019b) and the result obtained in our study was similar with previous report. The pH and ammonium sulphate content in the culture medium were increased enzyme yield at central level (Fig. 8c).The pH is a major abiotic factor influencing on enzymes production, whereas, ammonium sulphate is a major nitrogen source for microbes for laccase and lignin peroxidase production. The optimum level of ammonium sulphate stimulated enzymes production and the amount of enzyme production varied based on types of organism and culture conditions (Jing 2010).

Table 3. Central Composite Design Matrix with Experimental Results of Peroxidase Production from *B. flexus* in Solid State Fermentation

Run	pH (A)	Glucose (%)	Ammonium sulphate (%)	Peroxidase (U/g)
1	6	0.01	0.01	5.88
2	8	0.01	0.01	3.33
3	6	0.1	0.01	0
4	8	0.1	0.01	4.96
5	6	0.01	0.05	15.1
6	8	0.01	0.05	13.15
7	6	0.1	0.05	11.06
8	8	0.1	0.05	18.94
9	5.31	0.055	0.03	0.25
10	8.68	0.055	0.03	0.58
11	7	-0.020	0.03	16
12	7	0.130	0.03	11.1
13	7	0.055	-0.003	7.05
14	7	0.055	0.063	28.7
15	7	0.055	0.03	24.3
16	7	0.055	0.03	28.8
17	7	0.055	0.03	29.8
18	7	0.055	0.03	30.42
19	7	0.055	0.03	28.8
20	7	0.055	0.03	28.6

Table 4. Analysis of Variance for Central Composite Design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	2344.3	9	260.4	74.9	< 0.0001
A-pH	5.79	1	5.79	1.66	0.22
B-Glucose	8.44	1	8.44	2.43	0.15
C-Ammonium sulphate	474.39	1	474.4	136.57	< 0.0001
AB	37.58	1	37.58	10.82	0.0082
AC	1.54	1	1.548	0.445	0.5194
BC	4.5	1	4.5	1.295	0.2816
A^2	1445.18	1	1445.2	416.0	< 0.0001
B^2	415.59	1	415.6	119.6	< 0.0001
C^2	212.61	1	212.62	61.20	< 0.0001
Residual	34.73	10	3.4737		
Lack of Fit	11.54	5	2.3088	0.4977	0.7689
Pure Error	23.19	5	4.6387		
Cor Total	2379.06	19			

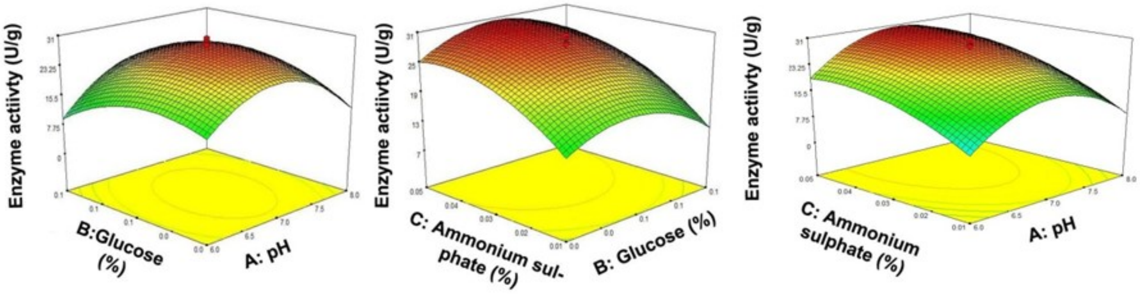

Fig. 8. 3D-response surface graphs showing the effect of interaction of (a) pH and glucose (b) ammonium sulphate and glucose (c) ammonium sulphate and pH on peroxidase production from *B. flexus* in solid state fermentation

Validation of the Model

Triplicate experiment was performed to validate the predicted response. The predicted response of the designed model was 32.0 U/g and the experimental value was 30.2 U/g. The optimization experiment increased 3.0-fold enzyme yield than unoptimized medium. Thus, the present results of optimization studies helped in significant increase in enzyme yield from *B. flexus* for industrial processing.

Azo Dye Degradation

In this study, the decolouration ability of *B. flexus* strains on MR and CR dyes in submerged fermentation was detected using 1% solid substrate. After 24 h incubation, 45 and 75.2% MR dye decolouration was achieved in *B. flexus* culture and was monitored at 525 nm. The dye decolouration efficacy increased after 48 h (75.2%) of incubation. The dye decolouration potential was 25.7% after 24 in the culture medium containing 50 ppm CR and it improved after 48 h (60.4%) and it was monitored at 490 nm.

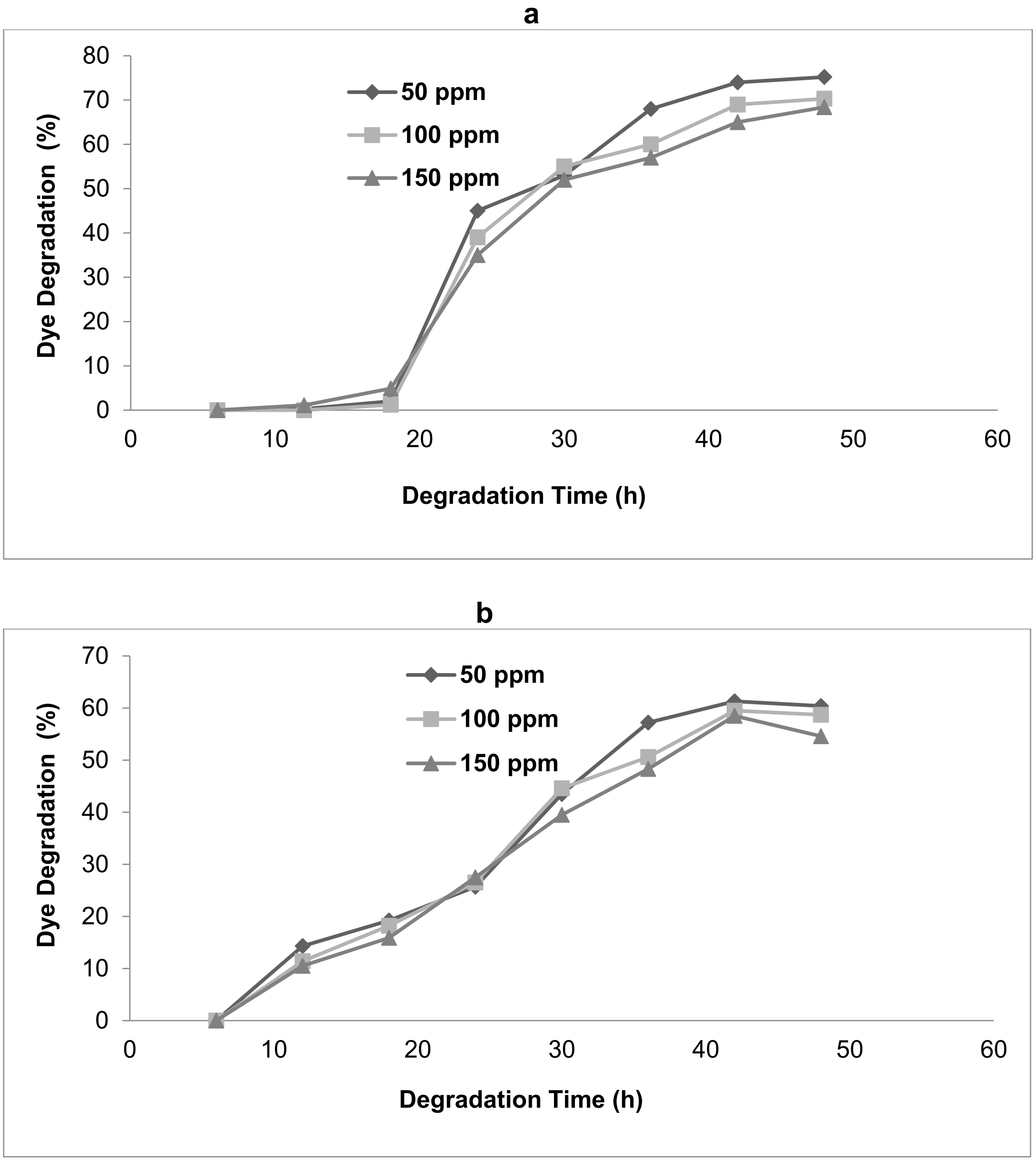

Fig. 9. Azo dye Degradation by *B. flexus* cultured in Minimal Medium containing 1% solid substrate in Submerged Fermentation. To the culture flask, 50, 100, and 150 ppm methyl red (a) or Congo red (b) was added individually and treated for 48 h. The culture was withdrawn for every 6 h and azo dye degradation (%) was assayed.

At higher dye concentration (>50 ppm) in the culture medium, less decolouration was achieved (Fig. 9). The variation in dye decolouration can be mainly attributed to the difference in the structure of inorganic dyes and chemical nature (Pramanik and Chaudhuri 2018). Recently the degradation of azo dye by *Klebsiella* sp. and *Acinetobacter* sp. has been demonstrated in the case of bacteria isolated previously from dye-contaminated wastewater. Generally azo dyes contain the–SO_3 functional group, and this functional group has a low susceptibility to bacterial degradation (Meerbergen *et al.*2018). To improve the decolourization process, various carbon and nitrogen sources were supplemented in the culture medium (Balapure *et al.* 2014). In this study, dye decolourization ability was validated at various concentrations of MR and CR in the medium. In certain dyes, decreased efficacy of bacteria has been reported due to high toxicity of dye at higher dye concentrations and also the toxicity of degradation products (Tan *et al.* 2016). In most cases, the decolourization process increased at lower concentrations and was affected at higher concentrations due to toxic effects (Guo *et al.* 2020).

Agricultural residues such as freshwater weeds, including *Lemna minor* have a highly complex structure, are environmentally friendly, and have global availability. These freshwater weeds are suitable for the production of microbial enzymes and biofuels. Freshwater weeds are considered a cheap and low-cost medium for enzyme production. The renewable wastes are low-cost materials for the cultivation of bacteria for the production of enzymes and in the breakdown of highly toxic azo dyes. These azo dyes are carcinogenic, causing environmental pollution and pose a danger to public health. Bacteria from the genus *Bacillus* produce several enzymes, including peroxidases, which break down azo dyes. Bioremediation of azo dye using *Bacillus* species is economically viable, chemical-free, and less energy is required.

CONCLUSIONS

1. Freshwater macro algae are the cost-effective substrate for solid-state fermentation. These substrates were induced for the production of peroxidase. The alkaline treated algal biomass increased the availability of glucose which improved bacterial biomass growth and enzyme production.
2. *Bacillus flexus* C18 isolated from the wastewater environment showed promising peroxidase producing potential under solid-state fermentation. The isolated bacteria produced dye degrading enzymes such as tyrosinase (2.74±0.3 U/mL), lignin peroxidase (0.71±0.02 U/mL), NADH-DCIP reductase (1.49±0.03 U/mL), laccase (29.8±1.1 U/mL), and azoreductases (35.4±0.15 U/mL).
3. Environmental factors (pH and temperature), and nutrient sources (carbon and nitrogen) influenced peroxidase production. Response surface methodology improved peroxidase production in solid state fermentation. The enzyme-based bioremediation reduces toxic pollutants in water systems, thereby helping to prevent neurological and developmental impairments linked to environmental exposure.

ACKNOWLEDGMENTS

The authors acknowledge Research Institute Supporting Program (RICSP-25-1), King Saud University, Riyadh, Saudi Arabia.

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Article submitted: January 10, 2025; Peer review completed: March 15, 2025; Revised version received September 3, 2025; Accepted: September 8, 2025; Published: October 9, 2025.
DOI: 10.15376/biores.20.4.10148-10169