ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of Dye Degradation Using *Streptomyces pactum* Strain JAAS1.

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ABSTRACT

The principle aim of this study was to isolate dye degrading actinomycetes from soil, mainly from agricultural farm lands and waste disposal areas, followed by characterization of the obtained isolates and to screen for dye degradation potential. Experiments included collection of soil samples; isolation of actinomycetes by serial dilution and spread plate technique on ISP2 medium, screening for blue, red, magenta and orange dye degradation and the biodegradation was monitored by FTIR and GCMS analysis. The role of enzymes like Laccase and peroxidases was studied to understand their activity in the degradation process and their activity was estimated. The aerobic degradation of dyes was carried out by seven different strains of actinomycetes isolated from two soil samples collected from farm lands at Vellore, Tamil Nadu. Among all the isolates, most potential strain (B2) was identified and sent for 16S rRNA gene sequencing and was used for dye degradation and optimization of degradation by response surface methodology (RSM). Their performance was evaluated *in vitro* with a view to understand their potential for treating effluents from textile industry especially considering the potential for toxicity reduction of the dye solution.

Keywords: Actinomycetes, dyes, FTIR, GC-MS, RSM

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ISSN: 0975-8585

INTRODUCTION

Bioremediation is the use of soil microbes to degrade pollutants to harmless substances [1]. Actinomycetes possess many properties that make them good candidates for application in bioremediation of soils contaminated with organic pollutants. They play an important role in the recycling of organic carbon and are able to degrade complex polymers [2]. Some reports have indicated that Streptomyces flora could play a very important role in degradation of hydrocarbons [3,4]. Many strains have the ability to solubilise lignin and degrade lignin-related compounds by producing cellulose- and hemicellulose-degrading enzymes and extracellular peroxidise [5]. In some contaminated sites actinomycetes represent the dominant group among the degraders [6]. Actinomycetes ecologically and metabolically are excellent candidates for research and commercialization of the metabolites generated in the process. Enzymes such as amylase, lipase, and cellulase produced from actinomycetes play an important role in food, fermentation, textile and paper industries.
The actinomycetes are a large group of aerobic, high G-C percentage gram-positive bacteria that form branching filaments or hyphae and asexual spores. These bacteria closely resemble fungi in overall morphology. They belong to the phylum Actinobacteria and are widely distributed in the terrestrial and aquatic ecosystems. Members of this group play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants, animals and fungal materials. They are also important in soil biodegradation and humus formation as they recycle the nutrients associated with recalcitrant polymers, such as chitin, keratin, and lignocelluloses [7,8], this produces several volatile substances like geosmin responsible of the characteristic wet earth odor [9] and exhibit diverse physiological and metabolic properties, for example the manufacture of extracellular enzymes [10]. Pharmaceutical companies all over the world depend on products of microbial origin, especially actinomycetes as one of the major source of novel drugs. Researchers are thus, exploring actinomycetes to discover novel molecules with potential therapeutic applications.

MATERIALS AND METHODS

Sample collection

In order to screen and isolate the actinomycete strains systematically, a total of 2 samples were collected from two different locations i.e agricultural farm and waste disposal site in Vellore, Tamil Nadu. 20 gm of soil was collected using sterile spatula from the top 10-15 cm soil depth where most of the microbial activity takes place, as most of the microbial population is concentrated. Soil samples were stored in clean, dry and sterile polythene bags. Care was taken that the points of collection had as widely varying characteristics as possible with regard to the organic matter, moisture content, particle size and color of soil.

Preparation of sample and isolation of strains

Numerous media have been used for the isolation of actinomycetes from soil and other natural materials. Glycerol Arginine medium [11], starch casein agar medium [12] and colloidal chitin agar medium [13] have been widely used for isolating soil actinomycetes. In the present work Yeast Malt Agar (yeast extract 0.4%, malt extract 1%, glucose 0.4%, pH 7.2) was used.

1 g of both the soil samples are added to 10 ml distilled water (10%w/v).The sample were serially diluted up to 10^{-3} and 10^{-4} concentrated 0.1 ml aliquot of both the dilutions were inoculated in duplicate on to the plates of the ISP2, starch casein nitrate agar and Actinomycetes isolation agar by the spread plate technique. Plating was done in triplicate and all the plates were incubated at 28°C for 7-10 days and the white powdery colonies were observed. The isolation plates of actinomycetes contaminated with bacteria and fungi were purified by streak-plate technique. Each actinomycete colony was isolated and pure cultured on Yeast Malt Agar. Subculturing continued till morphologically distinct actinomycete strains were obtained from both the samples.

Preservation

For the short term preservation of isolated actinomycetes strains, YM agar slants were used. The strains were inoculated in slants under aseptic conditions using a sterile loop and incubated for 5-7 days at 28°C. Later on, the slants were stored in refrigerator for future use.

Identification

Physiological, morphological and biochemical characters are used for identifying actinomycete strains. For the routine identification of actinomycetes, was carried out according to Nonomura [14] and Bergey's Manual of Determinative Bacteriology [15].

Morphological characterization

Morphological characteristics contribute largely towards the identification and classification of actinomycetes. The following are the important morphological traits studied upon:

Aerial mass color

The color of the mature sporulating aerial mycelium was noted and the colors range from White, grey, blue, red, violet and green [16]. Once the aerial color falls between two colors series, both the colors were recorded. In the cases where aerial mass color of a strain showed intermediate tints, then in that case both the color series were noted [17,18].

Reverse side pigments

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive or none (-). A color with low chroma such as pale yellow, olive or yellowish brown occurs, it is included in the latter group (-).

Melanoid pigments

The grouping is made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colors) on the medium. The strains were grouped as melanoid pigment producer (+) and not producer (-). For the melanoid pigment observation the inoculated plates were kept under incubator for 4 to 5 days. The strains which showed cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color were recorded as positive (+) total absence of diffusible pigment, are recorded as negative (-) for melanoid pigment production [18].

Spore chain morphology

Spore surface features were observed under Zeiss EVO-LS scanning electron microscope (SEM). The cover slip culture technique was prepared for observation under the light microscope was used for SEM analysis. The electron grid was cleaned and adhesive tape placed on the surface of the grid. The spore structures in actinomycetes were reported to be any of the four types: smooth (sm), spiny (sp), warty (wa) and hairy (ha).

Enzymatic Activity

Actinomycetes are a dominant group of the microbial population ubiquitous in soil and can produce extracellular enzymes which can decompose various materials. Actinomycetes have been commercially exploited for the production of pharmaceuticals, neutraceuticals, enzymes, antitumor agents, enzyme inhibitors, and so on. Actinomycetes are regularly screened for the production of these enzymes due to their high stability and unusual specificity. A wide array of enzymes and their products applied in biotechnological industries and biomedical fields has been reported from various genera of actinomycetes such as proteases, cellulases, chitinases, amylases, xylanases, and others.

Carbon sources assimilation

The ability of two actinomycete strains to use various carbon compounds as source of energy was studied by following the method recommended in International *Streptomyces* Project using carbon utilization medium with slight modification [19]. Chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials were used. Stock solution of D-glucose, sucrose, D-fructose

and starch at 2% (w/v) were prepared separately in sterile water and sterilized by filtering through 0.22µm pore size membrane filters. In 150ml of basal mineral salt agar, 3g of sterilized carbon source was mixed to give a final concentration of 1% (w/v). Plates were streaked and incubated at 37°C for 7 to 10 days. Growth were observed by comparing them with positive (D-glucose, starch, sucrose and fructose) and negative control (no carbon source).

Nitrogen sources assimilation

The ability of different actinomycetes strains in utilizing various nitrogen compounds as source of energy was studied by following the method recommended in International *Streptomyces* Project (ISP). Stock solution of nitrogen sources namely ammonium sulphate, sodium nitrate, ammonium phosphate and potassium nitrate were prepared in autoclaved water and sterilized by filtering through 0.22 mm pore size membrane filters and stored at 4ºC. Medium was prepared by adding 2% nitrogen source in ISP2 media. Inoculated and incubated at 28ºC for 7 to 10 days. Growth was observed by comparing them with positive and negative control [19].

Dye degradation

The 7 different strains of actinomycete isolates were tested primarily for dye decolorisation by streak plate technique on Yeast Malt Agar plates containing 0.025% w/v of colored dyes Red, Orange, Blue and Magenta. The dyes used were Methyl red, Navy Blue HER, Reactive magenta HB and orange 3R in color. Basal minimal agar media is used in the process including $Na_2HPO_4.7H_2O$ 64 g/L, KH_2PO_4 15 g/L, NaCl 25 g/L, NH_4Cl 5 g/L, $MgSO_4$ 2ml (1 M) and $CaCl_2$ 0.1 ml (1M) and agar powder 20g/l. After inoculation the plates were incubated for 5-7 days at 28° C and the decolorization results were obtained.

Minimum Inhibitory Concentration

Secondary screening was done inorder to calculate the Minimum inhibitory concentration of dyes. All the four dyes were used in different concentration i.e 250 mg/L, 500 mg/L and 750 mg/L, were dissolved in sterilized minimal salt agar. The actinomycete strains were inoculated into the media and initial absorbance was recorded on the 0^{th} day followed by absorbance at every 24 h interval till 7^{th} day. Absorbance of all the dyes were taken at their respective wavelengths, blue dye was taken at a wavelength of 620 nm, red at 540 nm, orange dye absorbance was measured at 480 nm and magenta at 560 nm respectively. The conical flasks were incubated at 28ºC inn an orbital shaker for 5-7 days. The final absorbance was measured on the 7^{th} day and the percentage of decolorisation was calculated using the formula

$$\text{Percentage decolorisation} = \frac{(\text{Initial absorbance-Final absorbance}) \ *100}{\text{Initial absorbance}}$$

Lignin Peroxidase

To identify the role of lignin peroxidase in the dye degradation process, the following studies were conducted.

Crude Enzyme Production

For peroxidase production, actinomycetes isolates were inoculated directly into Yeast malt agar, which contained yeast extract (4 g/L), malt extract (10 g/L), D-glucose (4 g/L) and agar (15 g/L) at pH 7.2. Distilled-water suspensions of growth from agar-slant cultures were used as inoculum for all liquid cultures. For peroxidase production, isolates were inoculated directly into a sterile production medium (400 ml in a 500 m1 conical flask) as described by Ramachandra et al., [20]. This medium contained (g/L): oat spelt xylan, 6.0 g; yeast extract, 6.0g; $(NH_4)_2SO_4$, 0.1g; NaC1, 0.3g; $MgSO_4$. $7H_20$, 0.1; $CaCO_3$ 0.02g; trace-elements solution, 1 ml; pH 7.2. The trace-elements solution contained (g/L): $FeSO_4$. $7H_20$, 1.0; $ZnSO_4$, .$7H_2O$, 0.9; $MnSO_4.7H_20$, 0.2; pH 7.2. Cultures were incubated at 50°C in an orbital shaker at 150 rpm. for 50 h and harvested by centrifugation at 15000 rpm for 20 min. The culture supernatant was concentrated by filtration to produce a crude preparation for peroxidase purification and further analysis was done.

Determination of lignin peroxidase activity

Peroxidase activity was assayed using 2,4-dichlorophenol (2,4-DCP) as the substrate. The reaction mixture (total volume 1 mL) contained equal volumes (0.2 mL) of potassium phosphate buffer (100 mmol/L, pH 8.0) 2,4-DCP (25 mmol/L), 4-aminoantipyrine (16 mmol/L), enzyme solution and H_2O_2 (50 mmol/L). The reaction was initiated with the addition of H_2O_2 and the reaction was monitored at 30 ^oC for 1 min at a wavelength of 510 nm [20,21]. One unit (U) of peroxidase activity was defined as the amount of enzyme required for an increase in absorbance of 1 unit per minute. The specific activity of peroxidase was defined as the unit of enzyme activity per mg of filtrate (U/ml). The absorbance value obtained at the end of the one-min incubation was corrected by subtracting a control absorbance value obtained by replacing hydrogen peroxide with water in the reaction mixture.

Calculation of enzyme activity used an absorption coefficient of 2 l ,647 M^{-1} cm^{-1}.

FTIR

Infrared (IR) spectra of the dye degraded was recorded in the frequency range of 4,000-400 cm^{-1} with a Fourier transform infrared (FTIR) spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for Windows) with a helium-neon laser lamp as a source of IR radiation. Pressed pellets were prepared by grinding the extracted samples with potassium bromide in a mortar with 1:100 ratios and analyzed in the region of 4,000-400 cm^{-1} at a resolution of 4 cm^{-1}. The four degraded dye samples were collected and FTIR was done to analyze with the IR spectra of undegraded dyes to study about the functional groups present in them.

Response Surface Methodology

RSM is explained as a collection of statistical and mathematical techniques used for developing, improving and optimizing processes. Full Factorial Design is used to determine the variables that significantly affect the process. Two levels ten factor design with two blocks was adopted in the study. Data were analyzed using MINITAB-16^{TM} software to find the interaction between the variables and their responses. The experiment evaluates the influence of the independent variables and its possible interactions on the response. The influence of four factors namely pH, temperature, inoculums size and nitrogen source concentration, i.e ammonium sulphate, in the growth of actinomycetes was investigated in 12 runs using Plackett-Burman design. The percentage of dye degradation was calculated and its significance level is noted. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. All statistical analysis was performed using experimental results and expressed as mean of parallel duplicates ANOVA correlation were performed. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Sample collection and isolation of strains

Five Actinomycete strains were isolated from agricultural soil sample and two from sample collected from waste disposal area. The strains from agricultural land were named as A1, A2, A3, A4 and A5 whereas the isolated from other sample were named as B1 and B2.

All purified isolates grew on yeast-extract-glucose-agar media showing morphology of typical *Streptomyces*; the colonies were slow growing, aerobic, glabrous or chalky, folded, and with aerial and substrate mycelia of different colors. In addition, all colonies possessed an earthy odor. All of the strains were acid fast negative and gram positive and fitted to the description of genus *Streptomyces* in Bergey's Manual of Systemic Bacteriology. The isolates were categorized into five color series according to their color of the aerial mycelium (Table 1). The gray series isolates were more predominant.

Minimum Inhibitory Concentration

All 7 isolates were inoculated with different concentrations (250 mg/L, 500 mg/L and 750 mg/L) and it was found that only strain B2 was able to show highest degree of decolorization at day 7 of incubation. Hence, it was further used for optimization, enzyme production and characterization studies.

ISSN: 0975-8585

Dye degradation studies

The B2 strain was tested for dye decolorisation by streak plate technique on basal minima agar plates containing 0.025% w/v of colored dyes Red, Orange, Blue and Magenta. The plates were incubated for 4-5 days at 37^{o}C and the following results were obtained.

Morphological Identification

Using a scanning electron microscope (SEM), the spore chain features of Actinomycetes were observed. It was found to be smooth with inward depression at the center as seen in figure 1. 16S rRNA gene sequence revealed the 99% similarity with *Streptomyces pactum* sp. After searching in BLAST it was revealed that the sequence alignment was in accordance with the *Streptomyces pactum* as shown in figure 2. The phylogenetic tree shows the similarities and differences with the closely related species of *Streptomyces*. The accession number obtained from NCBI is KT148627.

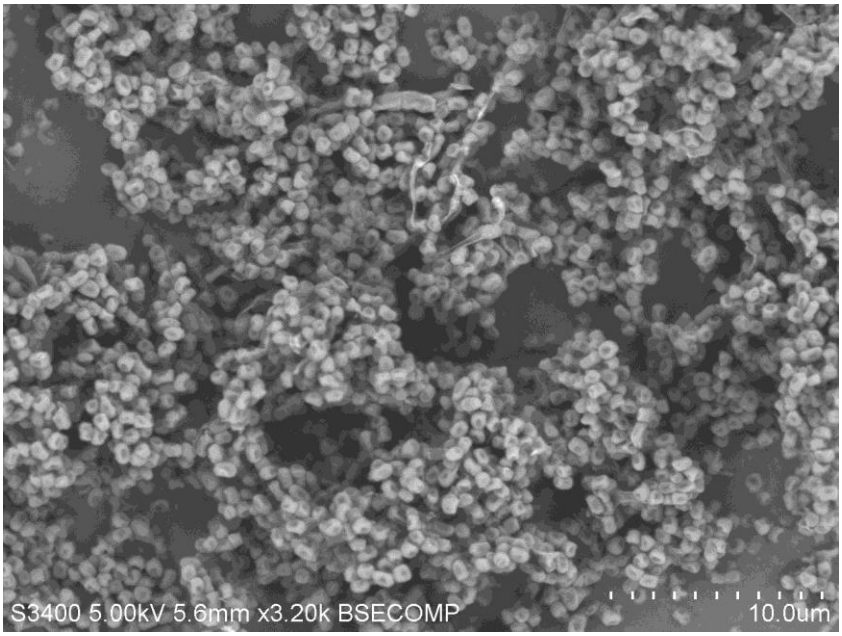

Figure 1. SEM image of spores of strain JAAS1.

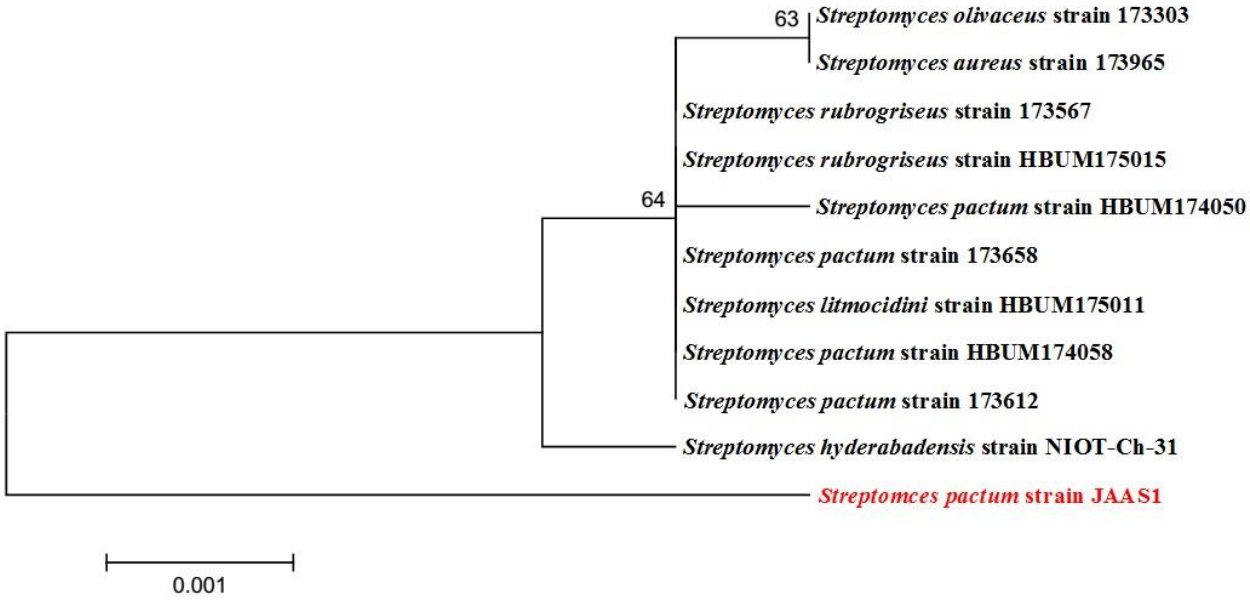

Figure 2. Phylogenetic tree of strain JAAS1 using neighbor joining method.

ISSN: 0975-8585

Lignin peroxidase assay

Actinomycete strain B2 was screened for the production of Lignin peroxidase and activity was calculated using the following formula and is shown in figure 3:

$$1 \text{ Unit} = \frac{\Delta E/\Delta t * V}{\Sigma * v * t}$$

Where AE/At = Increase in absorbance per minute
V = Volume of assay solution in the cuvette
Σ = Extinction coefficient
v = Volume of the sample
t = Time in minutes

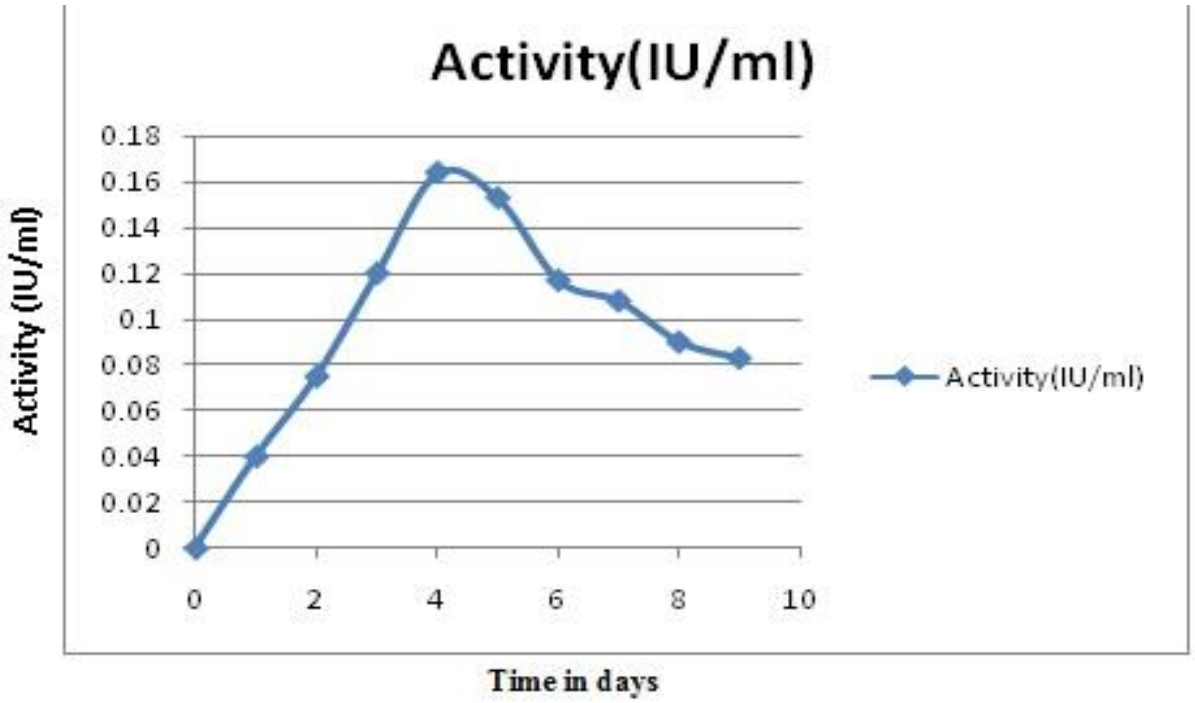

Figure 3. Lignin peroxidase activity produced by JAAS1.

FTIR

Fourier Transform Infrared (FTIR) spectroscopy was performed for testing the degradation products present in the degraded dye samples. For FTIR analysis, biological treatment process was performed with 250 mL solution containing 250 mg/l of dyes and 1% of B2 strain. The FTIR spectra of degraded dye and undegraded dye are shown in figure 4. FTIR spectra of undegraded dye showed the specific peaks in a range between 1500 and 500 cm for the mono and para-di-substituted benzene rings.

In the FTIR analysis of degraded blue dye the peaks at 3248.13 and 3267.13 cm show the presence of alcohol group, (-OH) stretching vibrations and strong, broad intensity. Peaks between 400 and 550 cm represented the presence of sulphide groups and bromine groups. Also the peak at 1631.78 cm for the C=C stretching vibrations and peak at 1076 cm for CH stretching. The degraded dye revealed an overall reduction in the spectra and, sulphates and CH, OH, NH and C ≡ N groups. The peak reduction was attributed to the cleavage of synthetic dye bonds.

ISSN: 0975-8585

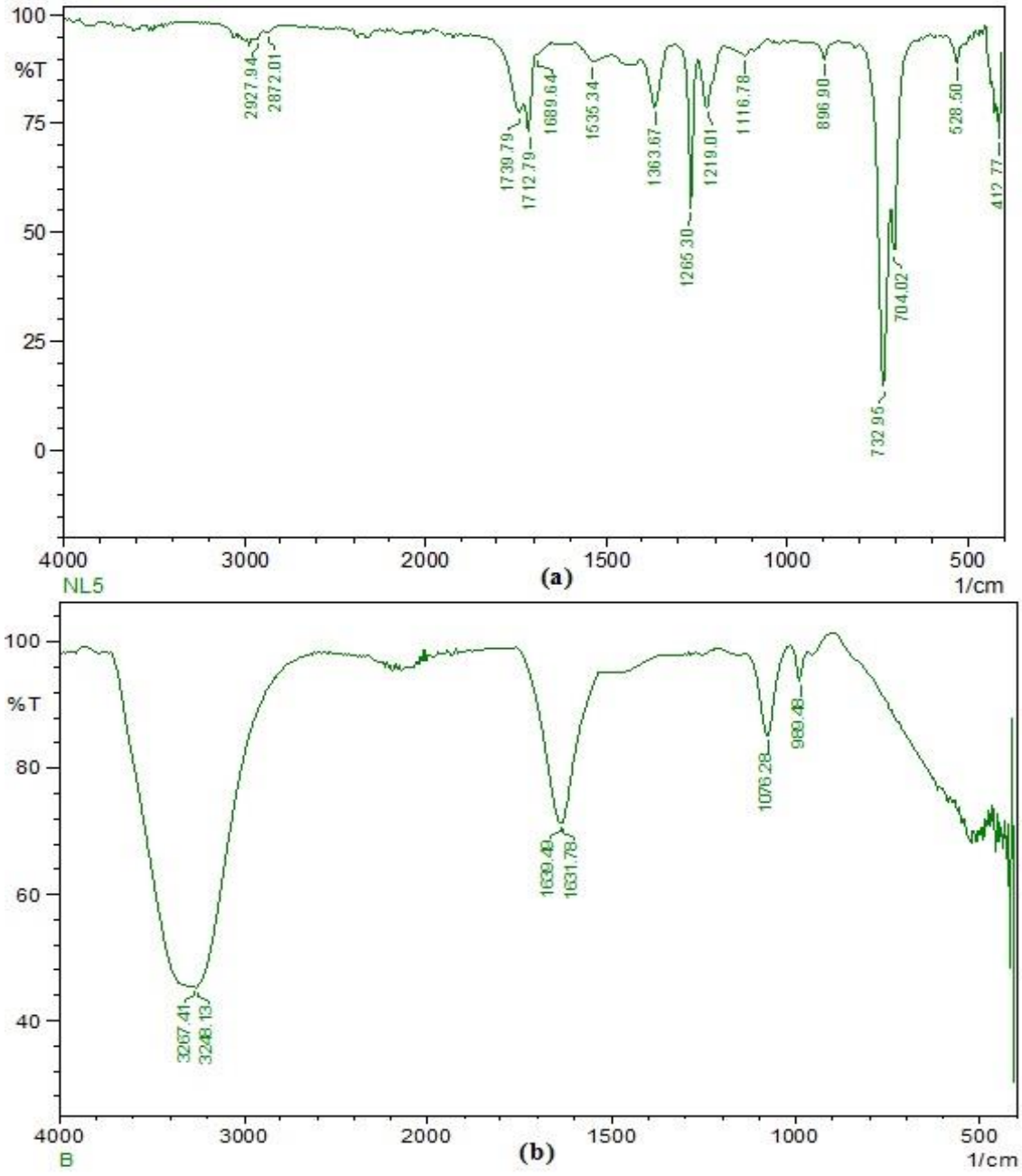

Figure 4. FTIR analysis of (a) standard against (b) sample degraded using strain JAAS1.

RSM

Based on Plackett-Burman design, pH, inoculum size, temperature and ammonium sulphate were selected for optimization using response surface methodology. The first set of experiments followed the Fractional Factorial Design which contributes to the selection of explanatory variables that have an impact on the dye degradation capacity of actinomycetes. Based on this, the significant explanatory variables such as pH, inoculum size and ammonium Sulphate concentration are employed in a more complicated design, such as a central composite design can be implemented to estimate a second-degree polynomial model.

To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of –2 to +2 in relation to degradation of blue dye by actinomycetes. The dye degradation percentage was calculated and the medium is optimized from the output of the central composite design. Contour plots and surface plots are obtained, analyzed and plotted.

ISSN: 0975-8585

From figure 5, it is seen that more than 80% degradation of blue dye takes place when ammonium sulphate concentration is between 0.5% -2.5% and inoculums size between 2%-5.5% at a constant pH of 7. Less than 50% decolorisation takes place at low ammonium sulphate concentration and inoculums size. It shows maximum degradation around 80% when inoculums size is between 2-6% at a pH of around 6.5-8.0 while ammonium sulphate concentration is kept constant. It also shows that more than 80% degradation of blue dye takes place when inoculums size is kept constant and ammonium sulphate concentration is between 0.5% -2.5% and pH between 6.5 -8.5.. The surface plots give 3D view of the optimization conditions in the form of overlaying graphs. Also contour plots show the optimum values for each variable for maximum enzyme activity (Figure 6).

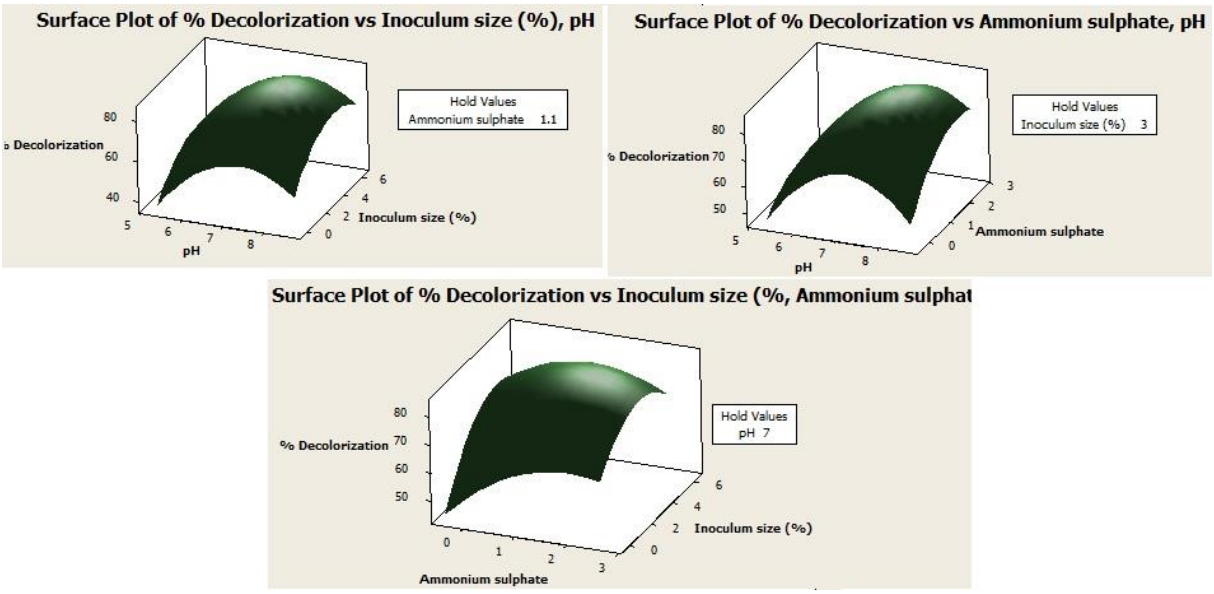

Figure 5. Surface plots of various parameters used in RSM.

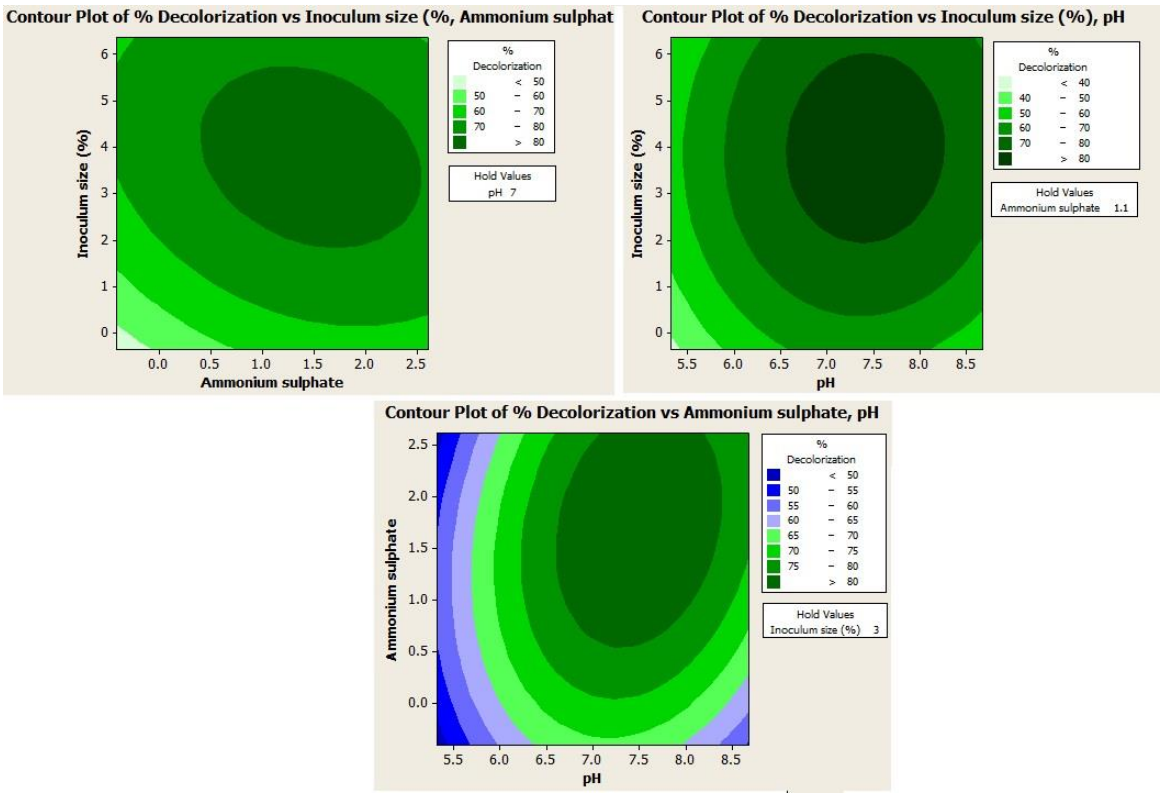

Figure 6. Contour plots showing effects of each parameter with hold values.

CONCLUSION

This study deals with the evaluation of actinomycetes strains in bioremediation of textile dyes at optimum conditions. The experiments revealed that oxidative biodegradation takes place upon action of enzymes such as lignin peroxidase. Response Surface Methodology was employed to optimize the dye degrading conditions of actinomycetes. It can be concluded that the actinomycetes isolate can effectively be used in the aerobic treatment of dyes before discharging textile effluents into the environment. However, further research is required to better understanding and effective employment of these isolates.

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