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Production of Lignin Peroxidase from Aquatic Bacteria, *Alcaligenes aquatilis*

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ABSTRACT

The main objective of the present work was to decolorize dye-containing wastewaters of textile industries using dye decolorizing bacteria which isolated from different aquatic samples. The most potent bacterial isolate capable of decolorizing malachite green with decolorization rate of 97% within 48 h under aerobic condition was selected for phylogenetic identification. The 16S rRNA amplification revealed that the isolate DB 8 was highly related to *Alcaligenes aquatilis* by 100%. Activity of lignin peroxidase responsible for malachite green decolorization, was detected. The culture conditions for lignin peroxidase production by *Alcaligenes aquatilis* were optimized using response surface methodology. The optimal concentrations obtained were soluble starch, 10.2 (g/l); ammonium nitrate, 5.0 (g/l); KH_2PO_4, 2.0 (g/l); inoculums size, 10%; and time course, 36.6 (h) with optimized LiP activity of 5.6 U/ml.

INTRODUCTION

A wide variety of organic pollutants can introduce into the water environment and/or the wastewater treatment system through the discharge of effluents containing dyes from different sources (textile industries, tannery, pulp and paper industries, Kraft bleaching industries and pharmaceutical industries). Dye-containing wastewaters of textile industries give rise to worldwide severe pollution problems **(Carmen and Daniela, 2012)**. Those dyes can continue to exist in the environment for long periods without adequate treatments. The environmental pollution caused by the dyes remains a difficult problem because the effluents contain reactive dyes, synthetic dyes and other hazardous chemicals. These toxic materials disturb the environmental balance, cause water pollution, alter the pH of water, increase the biological oxygen demand (BOD) as well as the chemical oxygen demand (COD) of water and changing chemical content of the water body. In addition to oxygen consumption, acute toxic effects to the aquatic ecosystem can occur due to low light penetration into deeper layer of water bodies **(Pandey *et al*., 2007)**.

Physical and chemical processes such as ion exchange, reverse osmosis, activated carbon adsorption, flocculation, coagulation, advanced oxidation, photocatalysis, ozonation, filtration and electrochemical oxidation have been applied to degrade dye from colored wastewater **(Lade *et al.*, 2015)**. These methods are inefficient, costly and produce large bulk of sludge. Therefore, biological treatment of dyes has been receiving more attention as it is an efficient, ecofriend and cost effective alternative **(Chen *et al.*, 2009)**. Malachite green (MG) is one of the extensively used triphenylmethane dyes. MG is used as food additive, food coloring agent, industrial dye and medical disinfectant. Vast usage of MG is carcinogenic and mutagenic to many organisms including mammalian cells and aquatic life **(Srivastava *et al.*, 2004)**. Environmental bioremediation through enzymes has increased owing to the unusual properties of this class of enzymes. They operate in a wide range of contaminant concentrations, temperature, pH and salinity **(Rao *et al.*, 2014)**. The main enzymes involved in dye degradation are the ligninolytic enzymes including lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2). They have low substrate specificity, strong oxidative abilities and no steric selectivity **(Ashger *et al.*, 2014)**. LiP, a relatively nonspecific oxidant enzyme, has exceptional ability to mineralize various recalcitrant aromatic and halogenated phenolic compounds. Compared with the classical peroxidases LiP has stronger redox potential **(Wong, 2009)**. The Plackett and Burman design used to screening of nutrients to enhance LiP production with following optimization through central composite design. The response surface method is a statistical method applied for medium components optimization as well as other variables critical for production of enzymes **(Xiong *et al.*, 2004)**. One factor at a time approach (OFAT) methods for enzyme production optimization are good in assessing the independent effect of each variable but the interactive effect of variables on the response can never be assessed using OFAT. Statistical optimization has cost and time consumption advantages when compared with OFAT. In addition, number of experiments can be less and different variable interactions can be studied simultaneously **(Sondhi and Saini, 2019)**. The present study aimed to isolate efficient dye decolorizing bacteria, monitoring LiP activity during biodegradation process and enhanced production of LiP by *Alcaligenes aquatilis* using response surface methodology for screening nutrients as well as optimizing the significant nutrients.

MATERIALS AND METHODS

Sampling:

Industrial wastewater samples were collected in sterile glass bottles from two different locations at El- Khadrawia drain - Mubarak industrial zone - Menoufiya and Lake of Manzala (Old Bahr El-Bakar discharge point and Hadous discharge point). The samples were brought to the laboratory and processed within 48 h.

Isolation and screening of dye decolorizing bacteria:

1.0 ml of each water sample was serially diluted. 0.1 ml was withdrawn from the 10^{-5} dilutions and plated on nutrient agar containing 20 mg/l of MG. Plates were incubated at 37 °C for 3 days according to the method of **Shah *et al.* (2012)**. After incubation, morphologically distinct bacterial isolates showing higher clear zone around their colonies due to decolorization of dyes were selected for further studies.

Dye decolorization assay:

The isolates were screened to select the most potent dye decolorizing bacteria in nutrient broth by the procedure described by **Sinoy *et al.* (2011)**. Decolorization activity was performed in 100 ml nutrient broth containing 20 mg/l MG. The flasks were inoculated with 5 ml of 18 h old bacterial culture and incubated at 37 °C on a shaker for 48 h. After incubation, 10 ml medium was centrifuged at 5000 rpm for 10 min. and the supernatant decolorization degree was determined at 620 nm (the wavelength of maximum absorbance of the MG solution) using UV-Vis spectrophotometer (Jenway - 6800). The decolorization percentage was calculated from the following equation,

$$Decolorization\ (\%) = \frac{Initial\ O\ D - Final\ O\ D\ x\ 100}{Initial\ O\ D}$$

Identification of the most potent bacterial isolate by 16S rRNA sequencing:

Molecular analysis

Bacterial isolate with the most potent dye decolorization was characterized by 16S rRNA gene. Briefly, DNA was extracted performing protocol of Gene Jet genomic DNA purification kit (Thermo K0721), according to the manufacturer's protocol.

PCR amplifications

DNA extracts were amplified by PCR using primers 63f (5'-CAGGCCTAACACATGC AAGTC-3') and 1387r (5'-GGGCGGCGTGTACAAGGC-3') **(Marchesi *et al.*, 1998)**. The PCR reaction was conducted by mixing 12.5 µL of Master Mix (Thermo K1051), 1.25 µL of primer 63F , 1.25 µL of primer 1387R, 2 µL of DNA extract and 8 µL of ddH_2O. PCR condition was done as follows: predenaturation at a temperature of 94 °C for 2 min, followed by 30 cycles of denaturation stages at a temperature of 94 °C for 30 seconds, annealing at a temperature of 55 °C for 30 seconds, extension at 72 °C for 1 min, and finalizing extension at a temperature of 72 °C for 5 min. Then it was stored at 4 °C for use at proper time **(Marchesi *et al.*, 1998)**. PCR products were purified by GeneJet ™ PCR purification Kit (Thermo K0701) were sequenced by use ABI 3730xl DNA analyzer sequencer, in forward direction. The reading sequence data obtained in AP1 file, was analyzed using Codon Code Aligner program 3.5.6 (Codon Code Corp.) according to **Delbes *et al.* (2007)** to remove the noisy sequence of peaks.

Sequencing analysis

The obtained sequences were compared with those found in the FASTA network service of the EMBL-EBI database (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) to determine their similarity to known sequences in the DNA database. The recovered sequences were aligned using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) **(Elsaied *et al.*, 2016)**. Phylogenetic trees were constructed, the nucleotide sequences of the recovered rRNA gene phylotypes and their homologues sequences, from the DNA database, were aligned, using the online program "Clustal Omega" **(Elsaied *et al.*, 2016)**.

Enzyme assays:

The culture was grown in minimal medium containing 20 mg/l of MG at 37 °C until the dye was completely decolorized. Cells were harvested by centrifugation and the supernatant was used for enzyme analysis. The activities of LiP, laccase, mangnase peroxidase (MnP), tyrosinase and MG reductase enzymes were assayed

spectrophotometrically in culture supernatant, in order to understand the role of each of these enzymes in the degradation of industrial dyes. LiP activity was determined at 420 nm through purpurogallin formation in a reaction mixture containing 2.4 ml of 100 mM potassium phosphate (pH 6.0), 0.3 ml of 5.33% pyrogallol, 100 µl enzyme extract and 200 µl of 10 mM H_2O_2 **(Ogola *et al*., 2009)**. Tyrosinase activity was determined by measuring liberated catechol quinone at 410 nm in 2 ml reaction mixture of 0.01% catechol in 0.1 M phosphate buffer pH 7.4 through the procedure described by **Zhang and Flurkey (1997)**.

The MG reductase activity was determined through monitoring the MG reduction at 620 nm in 50 mM potassium phosphate buffer, pH 7.4, according to the procedure described by **Du *et al.* (2011)**. The (4 ml) assay mixture composed of 20 mg/l MG, 50 µM NADH and 100 µL enzymes. Laccase activity was assayed using 0.4 ml 10 mM guaiacol containing 1.2 ml 100 mM acetate buffer (pH 5.0) and 0.4 ml enzyme source and monitored at 470 nm according to the method described by **Das *et al.* (1997)**. The MnP activity was determined through the oxidation of 1 mM $MnCl_2$ in 50 mM sodium malonate (pH 4.5) in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{+3}, formed a complex with malonate and the measurement was done at 270 nm **(Liu *et al*., 2009)**.

Optimization of lignin peroxidase production:

Response Surface Methodology

The response surface methodology is made up of two stages. Plackett- Burman design used in the first stage for significant nutrients identification to LiP production. The significant nutrients resulted from Plackett and Burman design was optimized in the second stage using the central composite design. Both the experimental design and statistical analysis of the data were done using Minitab 18 software package.

Plackett-Burman Design

Plackett-Burman design was applied for the most significant variables screening influencing the LiP production **(Plackett and Burman, 1946)**. Each variable was tested at a high level (+) and a low level (−) (Table 1). Eleven variables were screened through conducting 12 experiments. The significant variables at 5% level ($P < 0.05$) from the regression analysis were considered to have greater impact on LiP production and were further optimized by central composite design.

Table 1. Level of nutrients used for production of LiP using Plackett-Burman design.

Nutrient code	Nutrients	Low level (-)	High level (+)
A	Glucose	2 (g/l)	10 (g/l)
B	Sucrose	2 (g/l)	10 (g/l)
C	Cellulose	2 (g/l)	10 (g/l)
D	Soluble starch	2 (g/l)	10 (g/l)
E	Yeast extract	0.5 (g/l)	5 (g/l)
F	NH4NO3	0.2 (g/l)	2 (g/l)
G	MgSO4. 7H2O	0.2 (g/l)	1 (g/l)
H	KH2PO4	0.2 (g/l)	1 (g/l)
I	pH	6	8
J	Time course	24 h	48 h
K	Inoculums size	2%	10%

Central Composite Design

The most significant factors for LiP production were optimized using the central composite design. The effect of significant factors on LiP production was studied at 5

experimental levels (-*a*, -1, 0, +1, +*a* where $a=2^{n/4}$; here, *n* is the number of variables and 0 corresponds to the central point). LiP activity was analyzed using a second-order polynomial equation. The observations were further fitted to the equation by multiple regression procedure which represented in Eq. 1 as follows:

$$Y = \beta_o + \Sigma\beta_i X + \Sigma\beta_{ii} X_i 2 + \Sigma\beta_i X_i X_j \quad (1)$$

Y is the measured response, $X_i X_j$ are the independent variables and β_i is linear coefficients, β_{ii} is quadratic coefficients, and β_{ij} are interactive coefficient estimates with β_o is the intercept.In the present study, independent variables were coded as A, B, C, D, and E.
thus, Eq. (1) can be written as:

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{44} D^2\ \beta_{55} E^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} DE$$

Analysis of variance (ANOVA) and regression analysis were done and contour plots were drawn using Minitab 18 statistical software package.

RESULTS AND DISCUSSION

Screening and identification of the most potent dye decolorizing bacteria:

Nine different morphologically distinct bacterial colonies having ability to decolorize MG were isolated. All nine bacterial isolates were screened for their efficacy in decolorizing MG from culture medium. Among them, the isolate DB 8 more potent in decolorizing the dye both in percentage (97%) and time (48 h) indicating its higher potential for dye decolorization compared to other isolates. While the isolate DB 6 showed decolorization of MG both in percentage (83%) and time (48 h) (Table 2). Based on these results, the isolate DB 8 was selected for further studies.

Molecular identification (16S rRNA sequencing analysis) of the bacterial isolate with the best decolorizing potentials revealed that DB 8 having 100% similarity to *Alcaligenes aquatilis* (KX345927.1). Some of the reported *Alcaligenes bacteria* with decolorizing activity include *Alcaligenes faecalis* **(Saini *et al.*, 2018)**, *Alcaligenes species* AP04 **(Pandey *et al.*, 2016)** and *Alcaligenes* Sp. AA09 **(Pandey and Dubey, 2012)**.The phylogenetic relationships between the current studied decolorizing bacteria and other relative species based on 16S rRNA sequences was presented in Fig. 1. The decolorizing bacteria in this study (indicated with red tips) are perhaps more closely related.

Table 2. Decolorization rate of MG by different bacterial isolates.

Isolate	Locations	Decolorization time (h)	Decolorization (%)
DB 1	Hadous drain	72	77
DB 2	Hadous drain	72	71
DB 3	Bahr El-Baker drain	72	68
DB 4	El-Khadrawia drain	72	78
DB 5	El-Khadrawia drain	72	71
DB 6	Industrial wastewater	48	83
DB 7	Industrial wastewater	72	80
DB 8	Industrial wastewater	48	97
DB 9	Industrial wastewater	48	81

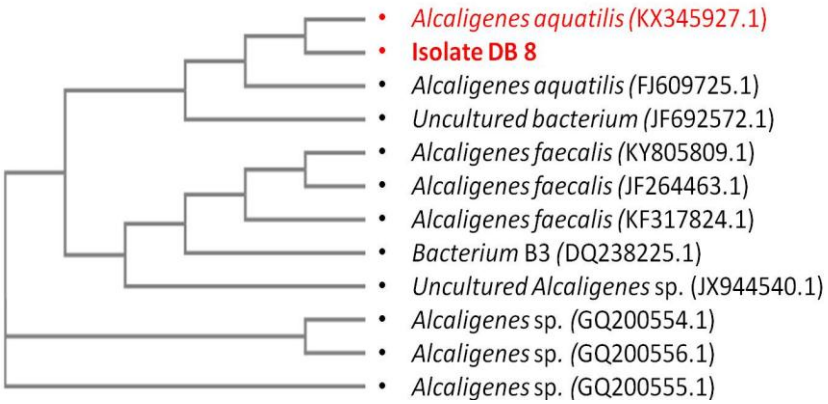

Fig.1. Neighbor-joining phylogenetic tree of isolate DB 8 and other relative species based on 16S rRNA sequences.

Enzymes analysis:

The mechanism of dyes degradation is largely related to enzymes activities such as tyrosinase, laccase, Mn-peroxidase (MnP) and LiP. However, the contributions of these enzymes may be dissimilar **(Shedbalkar *et al.*, 2008)**. The results showed that the maximum activities of LiP, laccase and MG reductase in MG-containing medium were 2.3, 0.4, and 0.06 U/ml, respectively. While, MnP and tyrosinase, were not detected, suggesting that MnP and tyrosinase had no part of the MG decolorization. Since the LiP activity level produced was much higher than the laccase activity, LiP seemed to play the most important role in the decolorization. Therefore, LiP act as high-efficient biocatalysts in MG decolorization by *Alcaligenes aquatilis.* Several studies have shown that ligninolytic enzymes are efficient for dye decolorization **(Singh *et al.*, 2017; Sudiana *et al.*, 2018). Zhao *et al.* (2014)** have shown the presence of NADH-DCIP reductase, laccase, and azoreductase produced by *Bacillus* sp. strain UN2 during methyl red decolorization.

Screening of important variables for LiP production using Plackett-Burman design:

The Plackett-Burman design was carried out and the influence of the 11 variables on LiP production by *Alcaligenes aquatilis* DB 8 was investigated (Table 3). According to the regression analysis, factors with $p < 0.05$ were considered to have significant effects on the LiP production and selected for further optimization studies.

Table 3. LiP activity by *Alcaligenes aquatilis* for Plackett-Burman design.

Run	A	B	C	D	E	F	G	H	I	J	K	LiP (U/ml)
1	+	-	+	-	-	-	+	+	+	-	+	1.17
2	+	+	-	+	-	-	-	+	+	+	-	2.38
3	-	+	+	-	+	-	-	-	+	+	+	1.30
4	+	-	+	+	-	+	-	-	-	+	+	2.69
5	+	+	-	+	+	-	+	-	-	-	+	1.17
6	+	+	+	-	+	+	-	+	-	-	-	3.35
7	-	+	+	+	-	+	+	-	+	-	-	2.79
8	-	-	+	+	+	-	+	+	-	+	-	3.02
9	-	-	-	+	+	+	-	+	+	-	+	3.26
10	+	-	-	-	+	+	+	-	+	+	-	2.81
11	-	+	-	-	-	+	+	+	-	+	+	3.12
12	-	-	-	-	-	-	-	-	-	-	-	1.20

Ammonium nitrate with a p value of 0.000, was considered as the most significant factor, followed by KH_2PO_4 (0.003), inoculums size (0.018), time course (0.035), and soluble starch (0.036), respectively. Among the carbon sources, soluble starch showed significance at high level and others had no significance on LiP production. Ammonium nitrate showed significant effect on LiP production than other nitrogen sources. Also, KH_2PO_4, inoculums size and time course were found to have significant influence on LiP production. The determination coefficient (R^2) of the model was 0.955 indicating that the model can clarify data variation of 95.5%. The Plackett-Burman design experiments on LiP production from *Alcaligenes aquatilis* indicated that the most significant parameters were (soluble starch, Ammonium nitrate, KH_2PO_4, inoculums size and time course). Hence, those five factors were considered as the independent variables and their effects on LiP production were further studied using a central composite design. **Padma and Sudha (2013)** reported that incubation period, agitation, tween-80, pineapple leaf length and beef extract were the most significant variables affecting LiP productivity from *Ganoderma lucidum.* **Zanirun *et al*. (2009)** showed that nitrogen concentration, agitation speed, pH, inoculum concentration and the addition of inducer (veratryl alcohol) had an enhancing effect on LiP production by *Pycnoporus* sp.

Optimization of significant variables using central composite design:

The central composite design was applied for optimizing the concentration of significant nutrients resulted from the Plackett-Burman design experiments. Thirty-three experiments were carried out from the design and the experimental values are given in Table 4.

Table 4. LiP production by *Alcaligenes aquatilis* using significant nutrients by central composite design.

Run order	S. starch (g/l)	Am. nitrate (g/l)	KH_2PO_4 (g/l)	Inoculum (%)	Time (h)	Lip (U/ml)
1	3	0.5	0.2	2	48	2.06
2	15	0.5	0.2	2	24	2.43
3	3	5	0.2	2	24	2.33
4	15	5	0.2	2	48	2.85
5	3	0.5	2	2	24	2.24
6	15	0.5	2	2	48	2.77
7	3	5	2	2	48	2.68
8	15	5	2	2	24	3.04
9	3	0.5	0.2	10	24	2.00
10	15	0.5	0.2	10	48	2.90
11	3	5	0.2	10	48	2.84
12	15	5	0.2	10	24	3.00
13	3	0.5	2	10	48	2.89
14	15	0.5	2	10	24	3.21
15	3	5	2	10	24	3.13
16	15	5	2	10	48	3.40
17	9	2.75	1.1	6	36	4.82
18	9	2.75	1.1	6	36	4.63
19	9	2.75	1.1	6	36	4.88
20	9	2.75	1.1	6	36	4.87
21	9	2.75	1.1	6	36	4.64
22	9	2.75	1.1	6	36	4.66
23	3	2.75	1.1	6	36	3.86
24	15	2.75	1.1	6	36	5.03
25	9	0.5	1.1	6	36	4.39
26	9	5	1.1	6	36	5.10
27	9	2.75	0.2	6	36	4.56
28	9	2.75	2	6	36	4.94
29	9	2.75	1.1	2	36	4.43
30	9	2.75	1.1	10	36	4.93
31	9	2.75	1.1	6	24	2.91
32	9	2.75	1.1	6	48	4.47
33	9	2.75	1.1	6	36	4.60

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain LiP production by *Alcaligenes aquatilis*.

Lip (U/ml) = -10.16 + 0.3340 A (g/l) + 0.0859 B (g/l) + 0.2056 C (g/l) + 0.0482 D (%)
+ 0.6809 E (h)- 0.01619 A (g/l)*A (g/l) - 0.00929 E (h)*E (h)

Variance analysis obtained from this design for the production of LiP is given in Table 5. ANOVA gives the model value and can explain this model adequately fits the variation observed in LiP production with the designed nutrients level. If the F test for the model is significant at the 5% level ($P< 0.05$), then the model is fit and can adequately explain the variation observed. The equation of regression obtained from ANOVA with the R^2 value (multiple correlation coefficients) of 0.9480 revealed that the model could explain 94.8% variation in the response. In the present study, for LiP production, the adjusted R^2 value (0.9306) was less than the R^2 value (0.9480). The adjusted R^2 may be distinctly smaller than the R^2 with a low value of the variation coefficient which indicates good precision and reliability of the study. In order to gain a better understanding of the interaction effects of variables on LiP production, contour plot was plotted against any two independent variables, while keeping another variable at its central (0) level.

Table 5. Analysis of variance for LiP production by *Alcaligenes aquatilis* using central composite design.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	32.8948	4.11185	54.66	0.000
Blocks	1	0.1027	0.10270	1.37	0.254
Linear	5	3.5003	0.70006	9.31	0.000
Square	2	19.0177	9.50887	126.41	0.000
Lack-of-Fit	19	1.7346	0.09129	6.45	0.024
Pure Error	5	0.0708	0.01416		
Total	32	34.7002			

The contour plot of calculated responses (LiP production) for the interactions between the variables are shown in Figs. 2, 3, 4 and 5, and the contour plots of LiP production for each pair of nutrient concentration by keeping the other three nutrients constant at its middle level. Maximum LiP was produced at middle level of each pair of nutrients at a constant middle level of the other three nutrients.

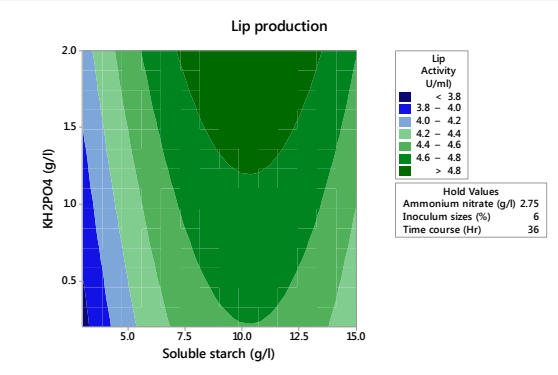

Fig. 2. Contour plot for LiP production at varying concentration of starch and KH_2PO_4

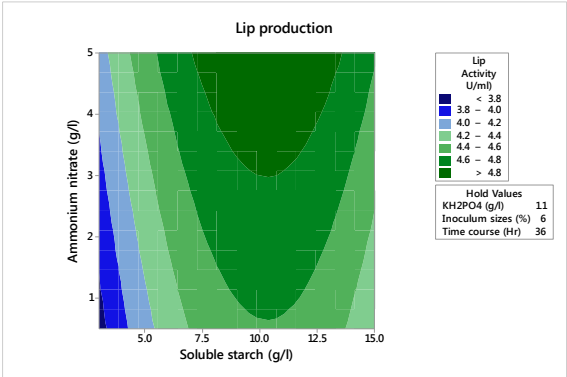

Fig. 3. Contour plot for LiP production at varying concentration of starch and Am. nitrate

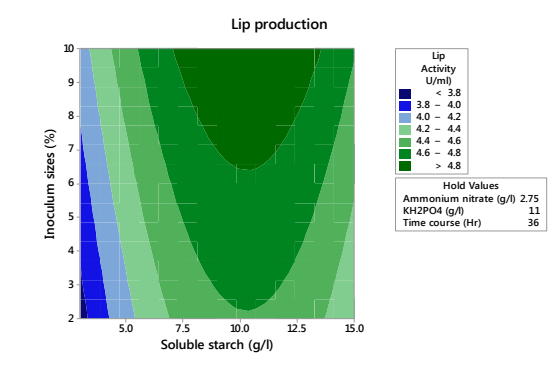

Fig. 4. Contour plot for LiP production at varying concentration of starch and inoculums size

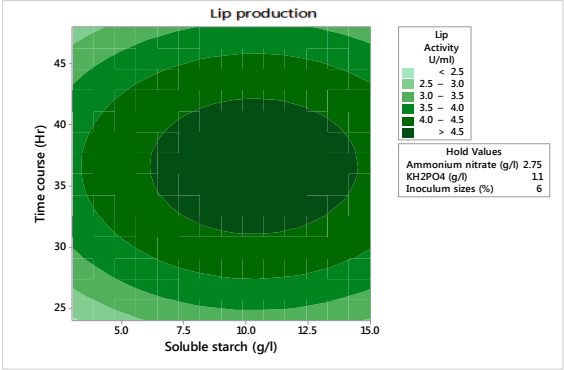

Fig. 5. Contour plot for LiP production at varying concentration of starch and time course

The optimized concentration obtained from response optimizer was: soluble starch, 10.2 (g/l); ammonium nitrate, 5.0 (g/l); KH_2PO_4, 2.0 (g/l); inoculums size, 10%; and time course, 36.6 (h). LiP activity in the optimized concentration of the nutrients was 5.6 U/ml, which was significantly greater than the reported values for this strain and was close to the predicted value 5.35 U/ml. The measured LiP activity was 5.6 U/ml higher than many microorganisms that synthesize LiP including *Phanerochaete chrysosporium* **(Alam *et al*., 2009)** and *Pycnoporus* sp. **(Zanirun *et al*., 2009)**.

CONCLUSION

Dye decolorizing bacteria were isolated from different aquatic samples. The more potent bacterial isolate *Alcaligenes aquatilis* DB 8 was selected and identified using 16S rRNA. LiP was responsible for malachite green decolorization and the enhanced production of LiP was optimized using response surface methodology.

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