Results and Discussion

4

RESULTS AND DISCUSSION

Laccase is a potentially important industrial enzyme that can be applied extensively in many fields which include waste detoxification and textile dye transformation, delignification of lignocellulosic material and cross linking of polysaccharides, upgrading of wine quality and removal of fermentation inhibitors to increase the yield of ethanol, improvement of drug analysis as well as construction of new energy-producing devices and enzyme sensors (Singh *et al.*, 2009). Due to these potentials, heterologous expression and protein engineering of laccase have been carried out for higher production and more excellent characters (Xu *et al.*, 2007).

The various microorganisms that produce the enzyme laccase have been studied intensively due to their potential applications in industrial and remediative processes. The production of laccase from microorganisms is dependent on number of factors which include the strains of microorganism, the composition of culture medium that is the compounds that provide nitrogen and carbon sources(Strong, 2011).

Laccase, one of the enzymes of phenol oxidase group, has high potential for industrial biotechnology. Many studies have been devoted to identify the most efficient laccase – producing source, to select the most suitable culture medium, to develop appropriate reproducible and inexpensive isolation procedures and mainly to optimize the enzyme production (Kumar *et al.*, 2010).

Hence the present study was aimed to identify the media components critical for laccase production and optimize their concentration for high

laccase activity from the isolated bacterial strain. The components were identified using "one time one factor approach " and further optimized using a statistical design namely Plackett- Burmann Design. The results obtained are discussed as follows.

4.1 Optimization of culture conditions for laccase production-One time One factor approach.

4.1.1 Effect of inoculum age on bacterial growth and enzyme production

4.1.2 Effect of temperature on bacterial growth and laccase production

4.1.3 Effect of carbon sources on bacterial growth and enzyme production

4.1.4 Effect of nitrogen sources on bacterial growth and enzyme production

4.1.5 Effect of Inducers on bacterial growth and enzyme production

4.1.6 Effect of inoculum volume on bacterial growth and enzyme production

4.1.7 Effect of metal ions on bacterial growth and enzyme production

4.1.8 Effect of surfactant on bacterial growth and enzyme production

4.2 Screening of the variables by Plackett-Burman Design

4.2.1 Effect of medium components on laccase production - Plackett-Burman Design

4.2.2 Effect of medium components on bacterial growth by - Plackett Burman Design

4.1 Optimization of culture conditions for laccase production-One time One factor approach.

4.1.1 Effect of inoculum age on bacterial growth and enzyme production

The optimization of age of inoculum of bacterial culture is important because low density gives insufficient biomass and high density produces excess biomass which results in depletion of nutrient necessary for enzyme production. The potential growth of bacteria in the starter culture with respect to the time duration was investigated by using the inoculum grown for 6, 12, 24 and 48 hr. The results obtained are depicted in Table 2 and Figure 4.

Table 2

Growth and Laccase Activity of the Isolated Bacterial Strain at selected inoculum age

S. No	Inoculum Age in hours	Growth (A_{660nm})	Laccase activity(U/L)
1	6	0.68 ±0.13	0.269 ±0.07
2	12	1.01 ±0.09	0.133 ± 0.04
3	24	1.23 ±0.03	0.338 ± 0.16
4	48	1.25 ±0.01	0.554 ± 0.06
	CD	0.151	0.193

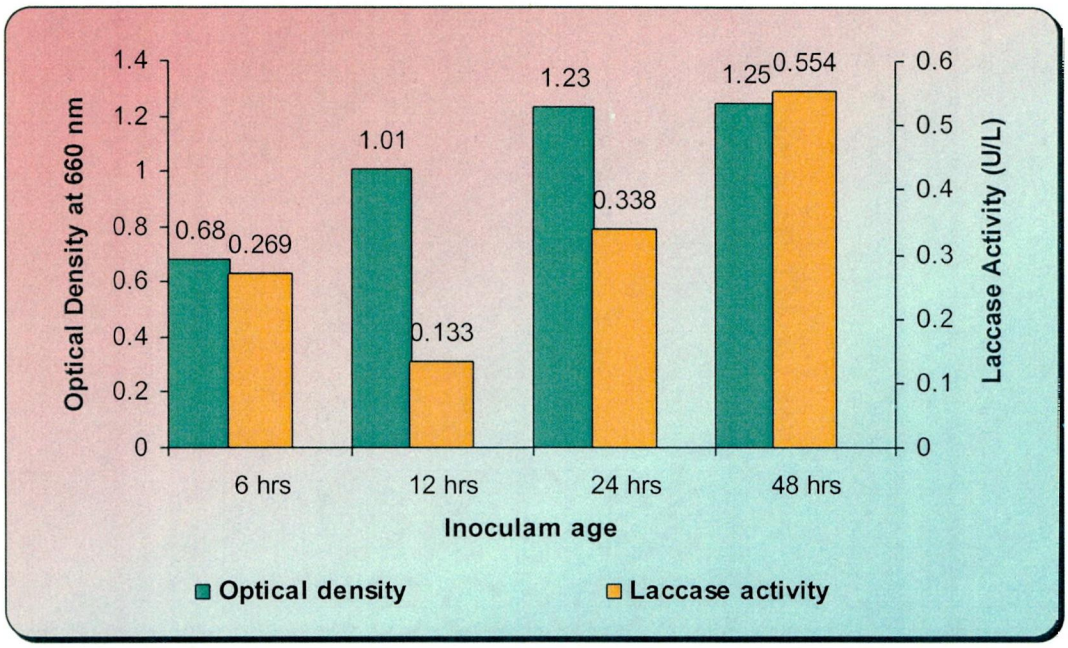
U = micromole of product formed per minute

Values are the mean of triplicates

A significant decrease in the activity of laccase was observed when the inoculum age was increased from 6 hours to 12 hours whereas the laccase activity was found to be significantly increased when the inoculum age was increased from 12 hours to 48 hours.

Figure 4

Growth and Laccase Activity of the Isolated Bacterial Strain at selected Inoculum age



The bacterial growth was found to be significantly increased with increasing inoculum age from 6hrs to 24hrs. However significant difference in bacterial growth was not noticed between 24hrs and 48hrs cultures.

Babu and Satyanarayana (1995) reported maximum amylase production by 48 hrs grown culture of *Bacillus coagulans.*

4.1.2 Effect of temperature on bacterial growth and laccase production

The growth and laccase production were studied at three different temperatures namely 27^0 C, 37^0 C and 47^0 C. The effect of temperature on bacterial growth and laccase production is presented in Table 3 and Figure 5.

An increase in temperature from 27^0 C to 37^0 C significantly increased the growth and Laccase activity and further increase in temperature from 37^0 C to 47^0 C significantly reduced both growth and Laccase activity.

Table 3

Growth and Laccase Activity of the Isolated Bacterial Strain at Selected Temperatures

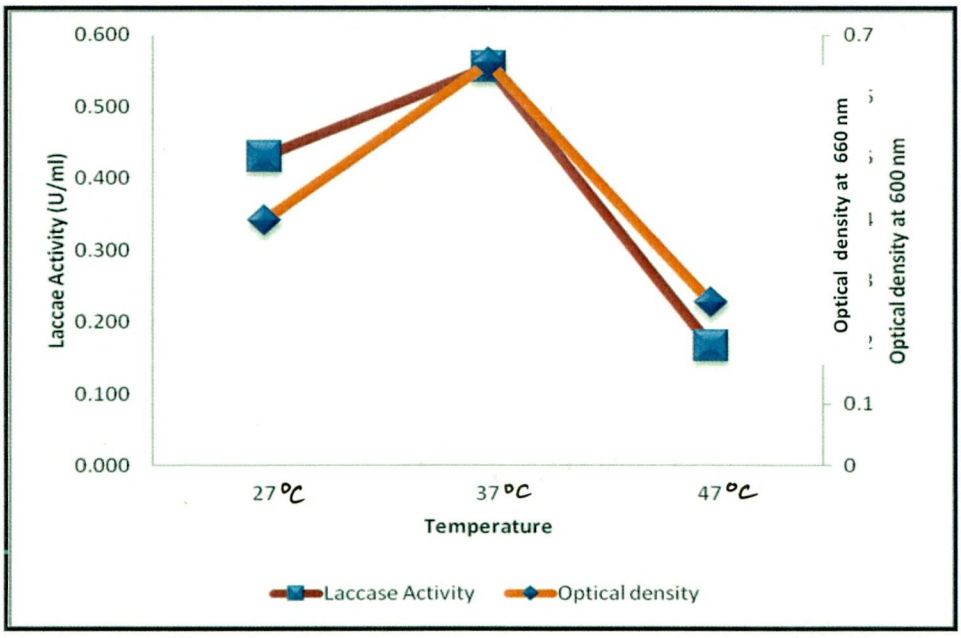
S. No	Temperature	Growth (A_{660nm})	Laccase activity(U/L)
1	27 ^{0}C	0.398±0.03	0.430±0.02
2	37 ^{0}C	0.655±0.05	0.556±0.40
3	47 ^{0}C	0.265±0.11	0.17±0.08
	CD	0.13	0.37

U = micromole of product formed per minute

Values are means of triplicates

Figure 5

Growth and Laccase Activity of the Isolated Bacterial Strain at the Selected Temperatures



Hence the results obtained in the present study indicated that the optimum temperature for the growth of the isolated bacterial strain and its laccase production as 37^0 C

Niladevi *et al.,* (2009) reported that optimum temperature for laccase production for *Streptomyces psammoticus* as 33^0 C.

4.1.3 Effect of carbon sources on bacterial growth and enzyme production

Laccase production is subjected to complex regulation by nutrients (C, N and inducers) in the culture medium during the growth of microbes (Dekker *et al.,* 2007). The effect of different carbon sources at 1% level on laccase production and growth of isolated bacterial strain was studied and depicted in Table 4 and Figure 6.

Table 4

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Carbon Sources

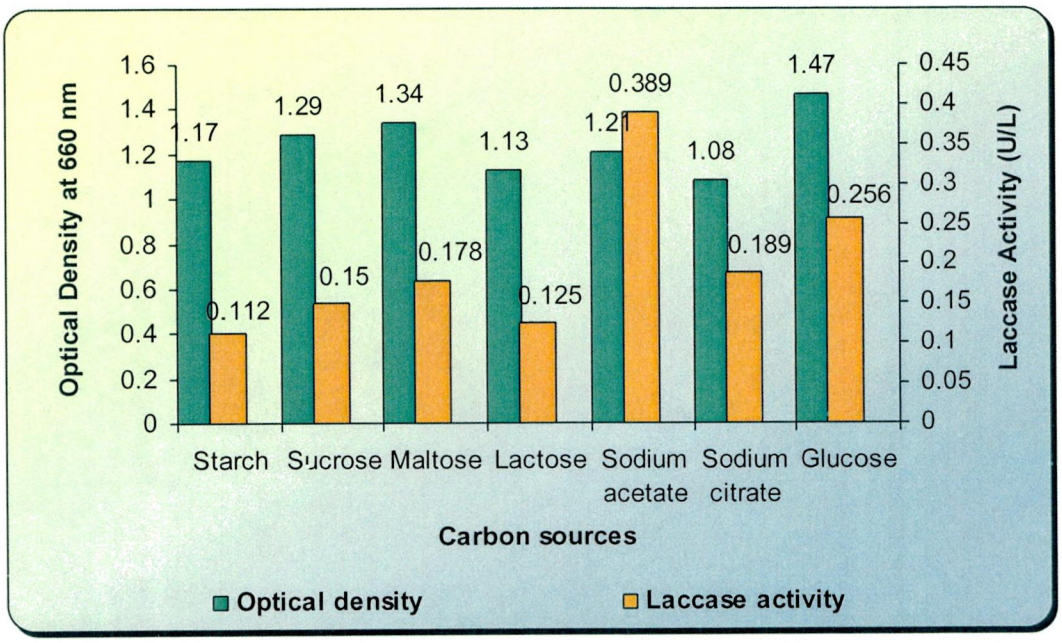
S. No	Carbon Sources	Growth (A_{660nm})	Laccase activity(U/L)
1	Starch	1.17±0.02	0.112 ± 0.01
2	Sucrose	1.29±0.19	0.150 ± 0.15
3	Maltose	1.34±0.13	0.178 ± 0.08
4	Lactose	1.13±0.02	0.125 ± 0.01
5	Sodium acetate	1.21±0.011	0.389 ± 0.04
6	Sodium citrate	1.08±0.04	0.189 ± 0.10
7	Glucose	1.47±0.01	**0.256** ± 0.04
	CD	0.153	0.12

U = micromole of product formed per minute

Values are means of triplicates

Figure 6

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Carbon Sources



Both inorganic and organic carbon sources used in the present study supported growth of the bacteria. The bacterial strain achieved maximum growth with glucose or maltose as the carbon sources when compared to the other sources. Among the inorganic carbon sources, sodium acetate significantly increased the growth of the bacteria when compared to sodium citrate.

The results of the present study suggested that glucose is the best organic carbon source for laccase production by the isolated bacterial strain when compared to the other organic carbon sources. Inorganic carbon source namely sodium acetate in the culture medium significantly increased laccase activity when compared to sodium citrate. The Laccase activity was found to be significantly increased with sodium acetate as carbon source in the medium than with glucose as carbon source in the medium. The carbon source of the growth medium appears to regulate Lac and Mnp

expression in microbes, and the activity of Lac and MnP can be increased by the choice of the carbon source (Vaithanomsat *et al.,* 2012).

Patel *et al.* (2009) obtained a maximum laccase activity with 1% glucose containing medium for *Pleurotus ostreatus*. Quarantino *et al.,* (2008) reported that medium containing glucose at a concentration of 12.5 g /L showed maximum laccase activity for *Panu tigrinus.*

4.1.4 Effect of nitrogen sources on bacterial growth and enzyme production

In microorganisms, nitrogen (both organic and inorganic forms) is metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components. The nature and concentration of nitrogen source are powerful nutritional factors regulating the laccase production in fungus (Galhaup *et al.,* 2002). There is much less report on the effect of nitrogen source on laccase production by bacteria. However several authors claim that nitrogen is an important factor for the economically important enzyme production by bacteria (Patel *et al.,* 2005).

The effect of different nitrogen sources at 0.5 % level on laccase production and growth of isolated bacterial strain was studied using 1% glucose as carbon source and at 37^0C. The results are highlighted in Table 5 and Figure 7.

It can be inferred from the results of the present study that the selected nitrogen sources had no inhibitory effect on the growth of the bacteria. The maximum growth of the bacteria was supported by potassium nitrate followed by urea, yeast extract, peptone, casein and gelatin whereas ammonium sulphate was found to be less favorable for bacterial growth.

Table 5

Growth and laccase activity of the isolated bacterial strain with various nitrogen sources

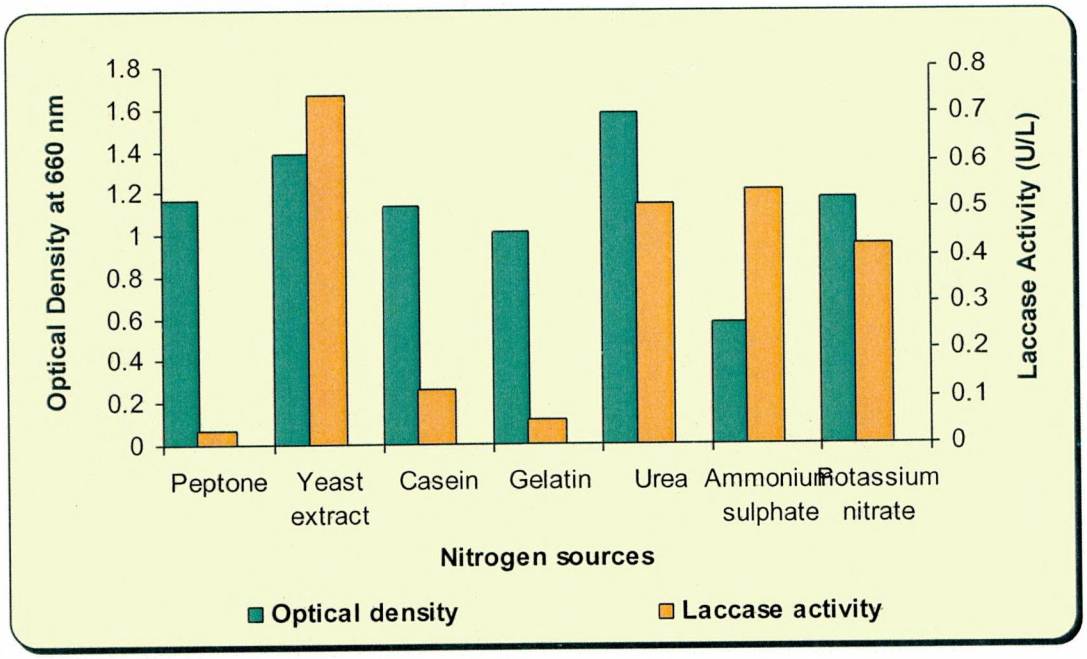
S.No	Nitrogen Sources	Growth (A_{660nm})	Laccase activity (U/L)
1	Peptone	1.16± 0.01	0.028 ± 0.01
2	Yeast extract	1.39±0.06	0.739 ± 0.24
3	Casein	1.14±0.01	0.117 ± 0.01
4	Gelatin	1.01±0.127	0.050 ± 0.01
5	Urea	1.58±0.02	0.511 ± 0.08
6	Ammonium sulphate	0.58±0.02	0.54 ± 0.03
7	Potassium nitrate	1.17±0.02	0.422 ± 0.23
	CD	0.08	0.19

U = micromole of product formed per minute

Values are means of triplicates

Figure 7

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Nitrogen Sources



The laccase production with different nitrogen sources ranged between 0.028 to 0.739 U/ml. The nitrogen source that showed the highest laccase production was yeast extract (0.739 U/ml) and the one that produced least laccase activity (0.028%) was peptone.

Potassium nitrate favoured bacterial growth to the greater extent as compared to other nitrogen sources however it did not provide utmost enzyme production. Ammonium sulphate provided the maximum laccase activity but it did not favou the growth of bacteria in terms of optical density. According to the results of the present study the yeast extract was found to be superior in terms of both bacterial growth and laccase production.

Hence yeast extract was selected as the nitrogen source in the medium for further optimization studies. The Combination of ammonium sulphate and yeast extract was selected for Plackett-Burman design.

Revenkar and Lee (2006) reported that fungus WR-1 showed maximum laccase production using yeast extract as nitrogen source.

Inorganic nitrogen sources proved to be less favorable towards both growth and alkaline protease secretion by bacillus species (Patel *et al.*, 2009).

4.1.5 Effect of Inducers on bacterial growth and enzyme production

The extracellular laccases are constitutively produced in small amounts however, their production can be considerably stimulated by the presence of inducers mainly aromatic or phenolic compounds related to lignin or lignin derivatives. The effect of twelve compounds which were previously reported as inducers in different organisms at 1mM concentration

on laccase production and bacterial growth was studied and the results are depicted in Table 6 and Figure 8.

Table 6

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Inducers

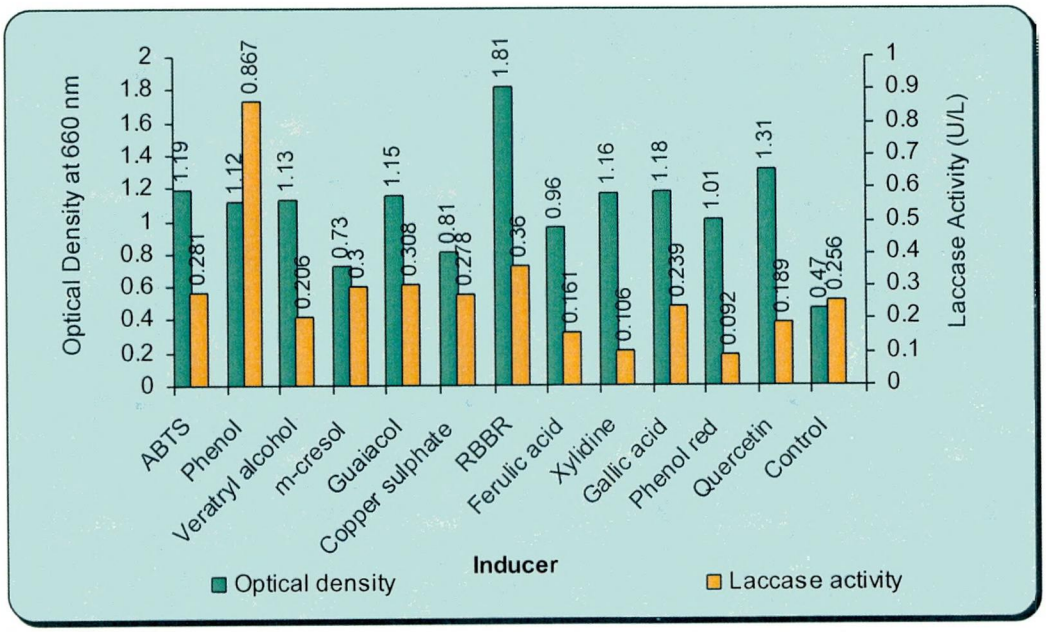
S. No	Inducers	Growth (A_{660nm})	Laccase activity(U/L)
1	ABTS	1.19±0.04	0.281 ± 0.26
2	Phenol	1.12±0.02	0.867 ± 0.66
3	Veratryl alcohol	1.13±0.02	0.206 ± 0.08
4	m-cresol	0.73±0.01	0.300 ± 0.21
5	Guaiacol	1.15±0.04	0.308 ± 0.21
6	Copper sulphate	0.81±0.03	0.278 ± 0.21
7	RBBR	1.81±0.02	0.367 ± 0.13
8	Ferulic acid	0.96±0.10	0.161 ± 0.13
9	Xylidine	1.16±0.03	0.106 ± 0.11
10	Gallic acid	1.18±0.01	0.239 ± 0.17
11	Phenol red	1.01±0.01	0.092 ± 0.03
12	Quercetin	1.31±0.02	0.189 ± 0.06
13	Control	.47±0.01	0.256 ± 0.04
	$CD_{0.05}$	0.06	0.234

U = micromole of product formed per minute

Values of the mean of triplicates

Figure 8

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Inducers



There is a significant decrease in the growth of bacteria in medium containing all the selected inducers except RBBR which significantly increased the growth of bacteria when compared to that of control.

No significant difference in laccase activity was noticed between control and in the presence of the selected inducers except phenol.

Among the twelve selected inducers only phenol was found to significantly increase laccase activity when compared to other inducers.

The results obtained might suggest that enhancement of laccase activity in response to various aromatic compounds depends on microbial strain and its physiological and genetic makeup. This finding is concordant with the report of Elisaashvili *et al.*, (2010) who showed that the structure of aromatic compound and concentration play an important role in the synthesis of laccase. They also suggested that enhanced laccase activity may function as defense mechanism against chemistry stress.

Mongkolthanaruk *et al.* (2012) reported that laccase activity of different strains could be triggered with different substrates.

4.1.6 Effect of inoculum volume on bacterial growth and enzyme production

Ten different inocula size of 0.1-1.0ml of bacterial culture were inoculated into each of 100ml minimal salt medium. The flasks were incubated at 37°C for 24 hours. The laccase activity and bacterial growth were determined.The results obtained are depicted in Table 7.

Table 7

Growth and Laccase Activity of the Isolated Bacterial Strain with Various inoculum

S. No	Inoculum (µl)	Growth (A_{660nm})	Laccase activity(U/L)
1	100	0.38	0.056
2	200	0.36	0.456
3	300	0.36	0.111
4	400	0.39	0.300
5	500	0.38	0.022
6	600	0.39	0.189
7	700	0.38	0.300
8	800	0.33	0.078
9	900	0.37	0.167
10	1000	0.39	0.322

U = micromole of product formed per minute

Values of the mean of triplicates

From the table it is clear that the growth was not altered much when the inoculums size was increased from 0.1ml to 1.0ml. The laccase activity was formed to be maximum with the inoculum size of 200µl.

4.1.7 Effect of metal ions on bacterial growth and enzyme production

The growth and laccase activity of the isolated bacterial strain were determined by supplementing the culture medium with metal ions namely barium, calcium, magnesium, iron, lithium and potassium. Table 8 and figure 9 represent the growth and laccase activity of the isolated bacterial strain in the presence of various metal ions.

Table 8

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Metal ions

S. No	Metal ions	Growth (A_{660nm})	Laccase activity(U/L)
1	Barium Chloride	1.87±0.04	0.017 ± 0.01
2	Calcium Chloride	1.45±0.04	0.042 ± 0.02
3	Magnesium Chloride	1.17±0.04	0.561 ± 0.27
4	Ferric Chloride	1.91±0.06	16.781 ± 2.49
5	Lithium Chloride	1.14±0.01	0.719 ± 0.72
6	Potassium Chloride	1.33±0.25	0.039 ± 0.01
	CD	0.150	1.344

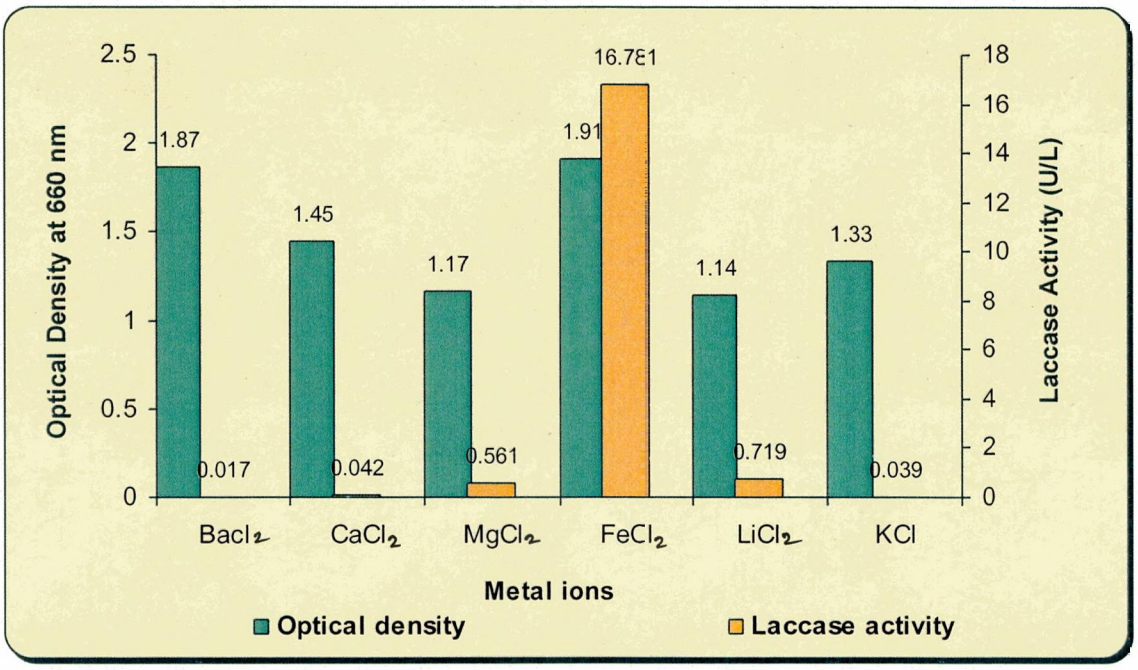
U = micromole of product formed per minute

Values of the means of the triplicates

The metal ions used in the present study supported growth, while the maximum growth was observed with Ferric chloride and Barium chloride and least growth was observed with Lithium chloride.

Figure 9

Growth and Laccase Activity of the Isolated Bacterial Strain with Various Metal ions



The metal ions influenced the laccase production and it was ranged between 0.017 to 16.781 U/ml. Among the metal ions the maximum laccase production was achieved with ferric chloride followed by lithium chloride, and magnesium chloride. The lowest laccase production was observed with calcium chloride, potassium chloride and barium chloride. Barium chloride supported the growth of isolated bacterial strain, but with lowest laccase production.

Since ferric chloride promotes both growth and laccase production, it was selected for further study.

4.1.8 Effect of surfactant on bacterial growth and enzyme production

There are number of reports in which the use of surfactant increased enzyme production.Although the exact nature of their action is not understood, it is assumed that surfactant increases the cell membrane

permeability thereby aiding the release of cell-membrane associated laccase. And also the surfactants increase the bioavailability of less soluble substrates for the growth of organism. In an attempt to increase the enzyme production by the isolated bacterium, various surfactants such as Tween 80, Triton X-100 and Sodium dodecyl sulfate, were incorporated in the medium.

Table 9

Growth and Laccase Activity of the Isolated Bacterial Strain with Various surfactants

S. No	Surfactants	Growth (A_{660nm})	Laccase activity(U/L)
1	Tween 80	1.19±0.05	0.186 ± 0.01
2	Triton X-100	0.69±0.08	0.339 ± 0.02
3	SDS	0.70±0.01	0.181 ± 0.06
4	Control	1.47±0.01	0.256 ± 0.04
	CD	0.0148	0.081

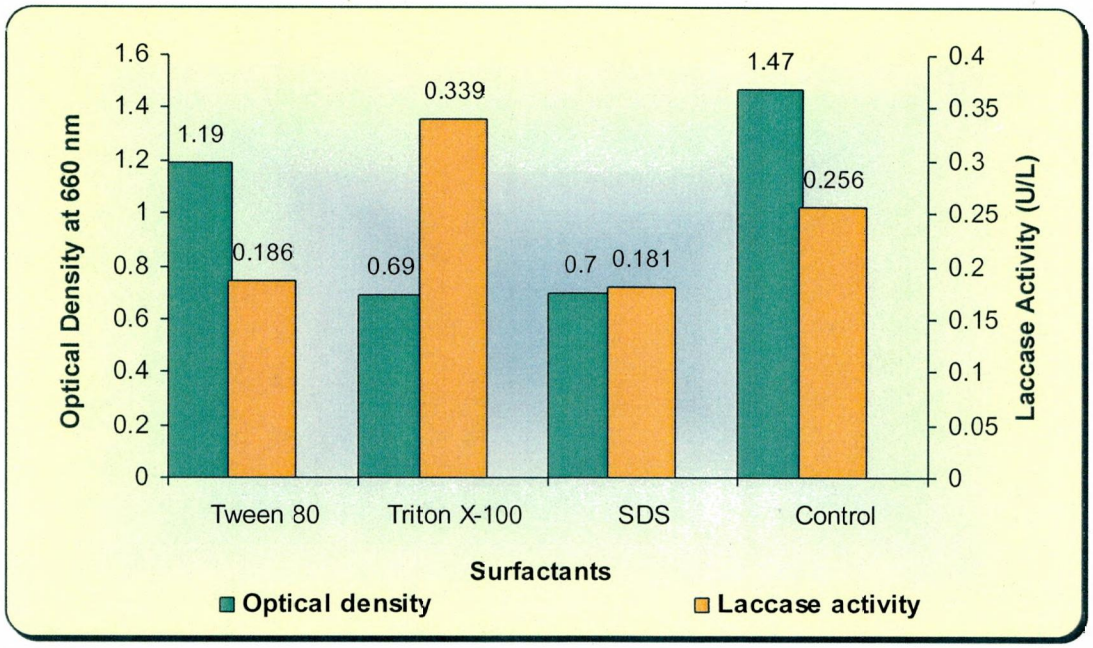
U = micromole of product formed per minute

Values of the means of the triplicates

From Table 9 and figure 10, it can be inferred that Tween 80 supported the maximum bacterial growth when compared to TritonX-100 and SDS. The results were found to be statistically significant at 5% level. All the surfactants studied were found to support the bacterial growth though there was a significant decrease in the growth of bacteria when these were present in this medium as compared with control.

Figure 10

Growth and Laccase Activity of the Isolated Bacterial Strain with Various surfactants



Among these surfactants studied Triton X-100 was found to yield the best laccase activity when compared to control. The presence of SDS and Tween 80 found to decrease significantly the laccase activity when compared to control. The Triton x concentration has to be further optimized for better laccase production and bacterial growth.

4.2 Screening of the variables by Plackett-Burman Design

4.2.1 Effect of medium components on laccase production - Plackett-Burman Design

Plackett-Burman design is a well established and widely used statistical design for the screening and selection of medium components. PB design offers a good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment. The model generated using PB design does not describe the interaction among the factors as such, it is only used to evaluate and select the important

factors that influence the response. A total of eleven medium components were analyzed with regard to their effect on laccase production using 12 runs of PB design. The experimental and predicted interval (95%) for laccase activity of new observation based on model for each run is shown in Table 10. Trail 3 showed greater enzyme production and trail 10 showed lowest enzyme production compared to others.

Table 10

Experimental and predicted laccase activity

Run / Trail	Experimental Value (U/ml)	Predicted Value (U/ml) at 95% prediction interval
1	0.074997	0.037045, 0.112949
2	0.11442	0.076490, 0.152394
3	0.18889	0.150940, 0.226844
4	0.036109	-0.001843, 0.074061
5	0.016667	-0.021285, 0.054619
6	0.016667	-0.021285, 0.054619
7	0.09932	0.061380, 0.137284
8	0.173232	0.135280, 0.211184
9	0.049997	0.012045, 0.087949
10	0.001	-0.037952, 0.037952
11	0.116667	0.078715, 0.154619
12	0.175002	0.137050, 0.212954

The data on enzyme production was subjected to multiple linear regression analysis using MINITAB 15.0 to estimate t-value, p-value, confidence level and it is presented in Table 11. The student's t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. The adequacy of the model was tested and

parameters with statistically significant effects were identified using the Fisher's test for analysis of variance (ANOVA).

Table 11

Plackett-Burman Design showing estimated effect, coefficient values, t- and P-value for each variable for the laccase production

Variable	Main effect	Coefficients	t-value	p-value
Constant		0.08850	33.34	0.000
Inoculum (A)	0.02056	0.01028	3.87	0.001
pH (B)	-0.03256	-0.01628	-6.13	0.000
Magnesium sulphate (C)	-0.03615	-0.01807	-6.81	0.000
Incubation Period (D)	-0.03245	-0.01622	-6.11	0.000
Glucose (E)	-0.01241	-0.00620	-2.34	0.028
Yeast Extract (F)	0.5760	0.02880	10.85	0.000
Ammonium Sulphate (G)	-0.00500	-0.00250	-0.94	0.356
Sodium acetate (H)	0.00518	0.00259	0.98	0.339
Inducer (J)	-0.06589	-0.03295	-12.41	0.00
Agitation (K)	0.06571	0.03285	12.38	0.000
Trace elements (L)	-0.01648	-0.00824	-3.10	0.005

Table 12

Analysis of variance for laccase activity by Plackett-Burman Design

S.No	Sources	DF	Seq SS	Adj SS	Adj MS	F	P
1	Model	11	0.146659	0.146659	0.0133327	52.57	0.000
2	Inoculum	1	0.003803	0.003803	0.0038034	15	0.001
3	pH	1	0.009540	0.009540	0.0095396	37.62	0.000
4	Magnesium sulphate	1	0.011759	0.011759	0.0117586	46.37	0.000
5	Incubation	1	0.009474	0.009474	0.0094742	37.36	0.000
6	Glucose	1	0.001386	0.001386	0.0013860	5.47	0.028
7	Yeast extract	1	0.029856	0.029856	0.0298577	117.73	0.000
8	Ammonium sulphate	1	0.000225	0.000225	0.0002250	0.89	0.356
9	Sodium acetate	1	0.000242	0.000242	0.0002418	0.95	0.339
10	Inducer	1	0.039474	0.039474	0.0394745	154.08	0.000
11	Agitation	1	0.038857	0.038857	0.0388566	153.22	0.000
12	Trace elements	1	0.002444	0.002444	0.0024441	9.64	0.005
13	Residual error	24	0.006086	0.006086	0.0002536		
14	Pure error	24	0.006086	0.006086	0.0002536		
15	Total	35	0.152746				

R-Sq = 96.02% R-Sq (pred) = 91.03% R-Sq (adj) = 94.19%

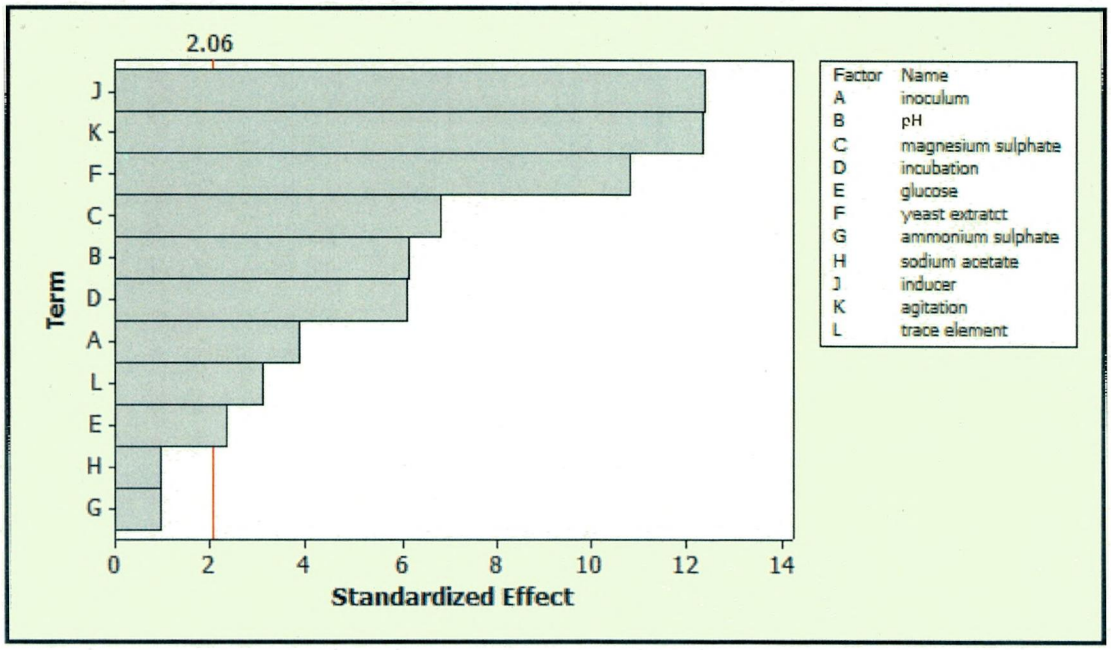
The analysis of variance for the selected factorial model (Table 12) showed that the model was significant with a Model F-value of 52.57. The factors having the values of $p < 0.05$ were considered to have significant effect on the laccase production. Except ammonium sulphate and sodium acetate, all other selected variables were found to have p value less than 0.05 indicating that they significantly affect the laccase production.

The correlation measures for testing the goodness of fit of the regression equation is the coefficient of determination (R^2). Predicted R-squared is to indicate how well the model predicts responses for new observations, whereas R-squared indicates how well the model fits the data. Predicted R-squared can prevent over fitting the model and can be more useful than adjusted R-squared for comparing models because it is calculated using observations not included in model estimation. For a good statistical model R^2 value should be close to 1.0 where a value > 0.75 indicates the aptness of the model.

The model generated using Plackett- Burman was found to have R^2 value of 0.9602 indicating good correlation between observed and predicted response. The adjusted determination coefficient (Adj R^2) corrects the R^2 value for the sample size and the number of terms in the model. Since the R^2 tend to overestimate the strength of association. The Adj R^2 of 0.9419 was slightly lower than the R^2 of 0.9602 which indicated that the model will have strong association even with the addition of extra variable. In addition, the predicted R^2 was found to be 0.9103 which was in reasonable aggrement with the Adj R^2 and model R^2. This indicated that there is good aggrement between the experimental and theoretical values predicted by the model and almost all the variation could be accounted by the model equation.

Figure 11

Pareto Chart of the Standardized Effects

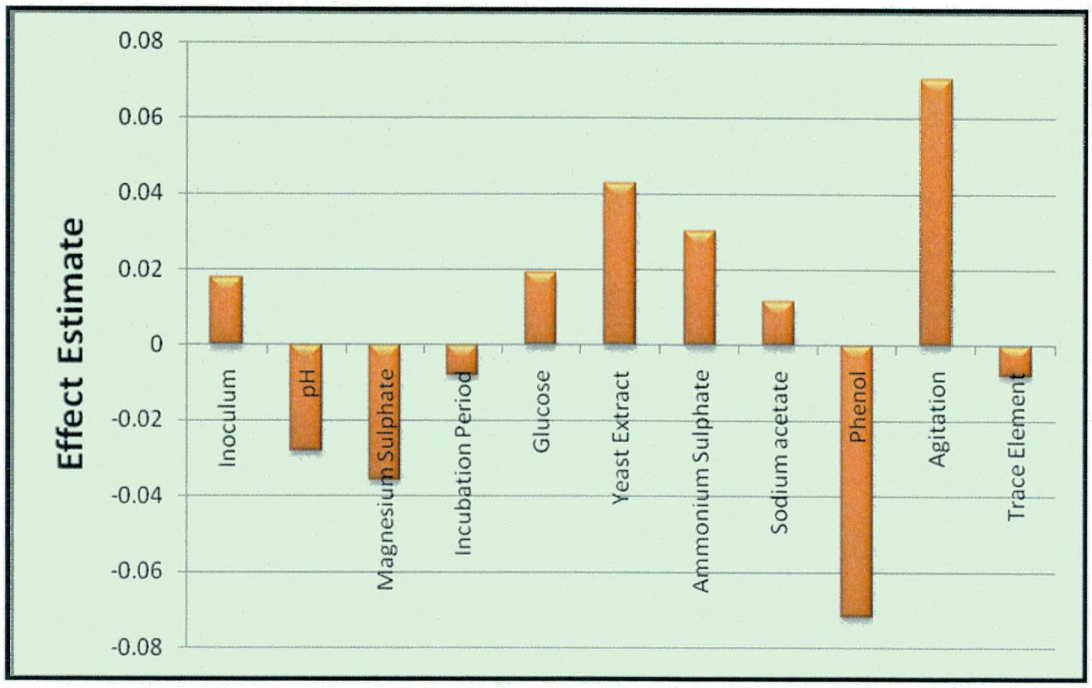


Pareto plot shows the main effect of all medium components and operating conditions. It gives essential information required for setting priorities on media components and process variables. Hence, the main effect of media components and process parameters on laccase production was also studied graphically using Pareto chart as shown in figure 11.

The pareto chart depicts that inducer and agitation were found to be the most influenzing factors followed by the concentration of yeast extract, magnesium sulphate, pH, Incubation period, inoculum size, trace element and glucose concentration. Out of these nine significant variables identified inoculum size, yeast extract, glucose and agitation were found to exert positive effect on laccase production while other factors such as magnesium sulphate, incubation period, trace element, pH and phenol were found to exert negative effect on laccase production as given by effect of estimate figure 12.

Figure 12

Effect of different Operational variables on laccase production



In Plackett-Burman design the effect of independent variables on laccase production is given by the first order linear model and is given by the following equation:

Y(laccase activity U/ml) =0.08850+0.01028 A - 0.01628 B -0.01807 C -0.01622 D-0.00620 E +0.2880 F -0.00250 G +0.00259 H -0.03295 J + 0.3285 K -0.00824 L

Where, A, B, C, D, E, F, G, H, I, J, K and L represent the inoculum size, pH, Magnesium sulphate, Incubation period, glucose, yeast extract, ammonium sulphate, sodium sulphate, inducer (phenol), agitation and trace elements respectively.

4.2.2 Effect of medium components on bacterial growth by Plackett Burmann design

The significant medium components for the bacterial growth were screened using Plackett Burman design. The experimental biomass and 95 % prediction intervel calculated based on model for each trail are presented in Table 13. The maximum biomass was observed in Trial 3 and lowest biomass was observed in Trial 7.

Table 13

Bacterial Biomass for each trail

Run/ Trial	Experimental Value (mg/ml)	95% Predicted Value (mg/ml)
1	2.10	1.85447 - 2.34553
2	0.84	0.59797- 1.08903
3	2.52	2.27947- 2.77053
4	0.975	0.72947 - 1.22053
5	0.910	0.66447- 1.15553
6	0.767	0.52147-1.01253
7	0.415	0.16947- 0.66053
8	0.819	0.57347-1.06453
9	0.663	0.41797-0.90903
10	1.060	0.81447-1.30553
11	1.85	1.60447-2.09553
12	1.685	1.43947-1.93053

Table 14

Statistical analysis of Plackett-Burman Design showing estimated effect, coefficient values, t- and P-value for each variable for the bacterial growth

Variable	Main effect	Coefficients	t-value	p-value
Constant		0.01563	77.92	0.000
Inoculum (A)	0.3442	0.1721	11.01	0.000
pH (B)	0.6568	0.3284	21.01	0.000
Magnesium sulphate (C)	-0.4247	-0.2123	-15.84	0.000
Incubation Period (D)	-0.4247	-0.2123	-13.59	0.000
Glucose (E)	-0.3170	-0.1585	-10.14	0.000
Yeast Extract (F)	0.4320	0.2160	13.82	0.000
Ammonium Sulphate (G)	0.0878	0.0439	2.81	0.008
Sodium acetate (H)	0.3857	0.1928	12.34	0.000
Inducer (J)	-0.0403	-0.0202	-1.29	0.205
Agitation (K)	0.3348	0.1674	10.71	0.000
Trace elements (L)	-0.2157	-0.1078	-6.90	0.000

The factors having the values of $p < 0.05$ were considered to have significant effect on the bacterial biomass. Except phenol, all other selected variables were found to have p value less than 0.05 indicating that they are significantly affect the bacterial growth.

Table 15

Analysis of variance for Bacterial Biomass by Plackett-Burman Design

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	11	18.9508	18.9508	1.72280	146.93	0.000
Inoculum	1	1.4214	1.4214	1.42141	121.23	0.000
pH	1	5.1772	5.1772	5.17716	441.55	0.000
Magnesium sulphate	1	2.9423	2.9423	2.94228	250.94	0.000
Incubation	1	2.1641	2.1641	2.16410	184.57	0.000
Glucose	1	1.2059	1.2059	1.20587	102.85	0.000
Yeast extract	1	2.2395	2.2395	2.23949	191.00	0.000
Ammonium sulphate	1	0.0926	0.0926	0.09258	7.90	0.008
Sodium acetate	1	1.7849	1.7849	1.78487	152.23	0.000
Phenol	1	0.0195	0.0195	0.01952	1.66	0.205
Agitation	1	1.3454	1.3454	1.34536	114.74	0.000
Trace element	1	0.5581	0.5581	0.55815	47.60	0.000
Residual Error	36	0.4221	0.4221	0.01173		
Pure Error	36	0.4221	0.4221	0.01173		
Total	47	19.3729				

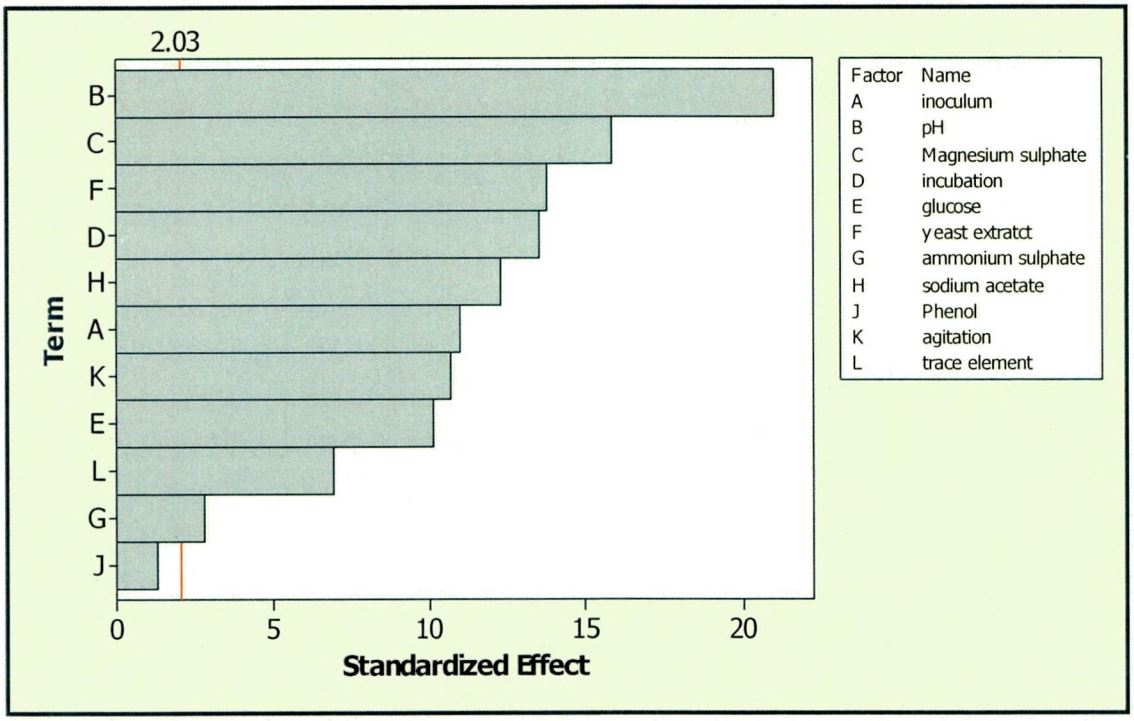
Model Summary S = 0.108282 PRESS = 0.750404

R-Sq = 97.82% R-Sq(pred) = 96.13% R-Sq(adj) = 97.16%

The analysis of variance for the selected factorial model showed that the model was significant with a Model F-value of 146. 93. The coefficient of the model (R^2 = 97.82; R^2 adj =97.16) validates that the model is well suited to experimental results.

Figure 13

Pareto Chart of the Standardized Effects



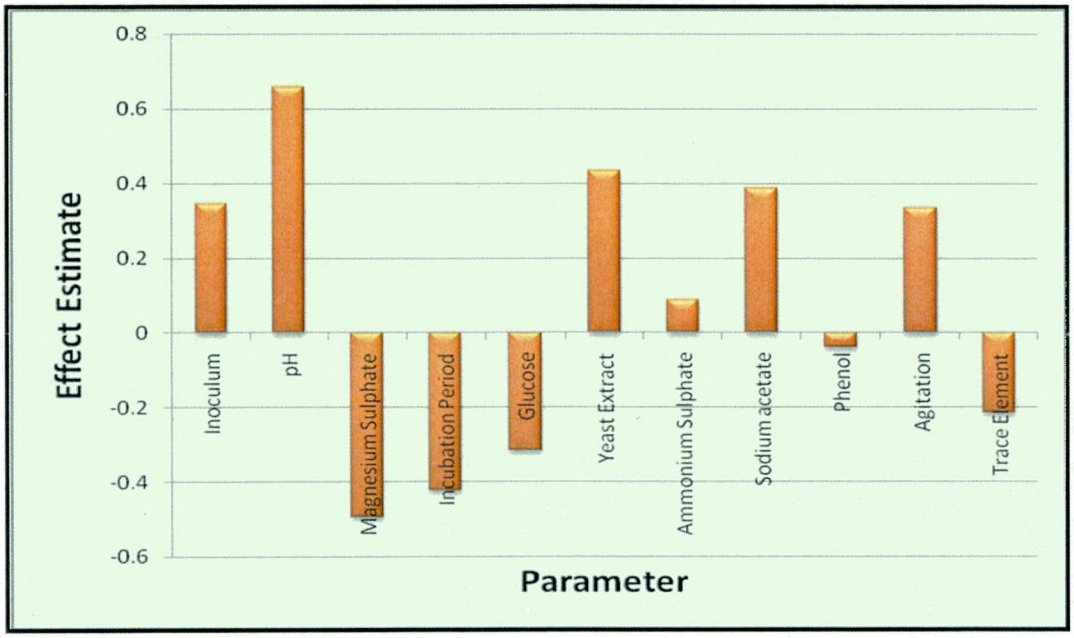
The Pareto chart depict that pH is most influenzing factor for bacterial growth followed by magnesium sulphate, yeast extract, incubation period, sodium acetate, inoculum size, agitation, glucose and trace element solution. The phenol and ammonium sulphate were found to have less effect on bacterial growth.

Out of the nine significant variable identified from 11 variables tested, inoculum, pH, yeast extract, sodium acetate, ammonium sulphate and agitation were found to have positive effect whereas incubation period,

glucose, magnesium sulphate, phenol and trace elements were found to have negative impact on biomass production.

Figure 14

Effect of different Operational variables on Bacterial growth



In Plackett-Burman design the effect of independent variables on bacterial biomass production is given by the first order linear model and is given by the following equation:

Y(biomass mg/ml) = 1.2178+0.1721 A - 0.3284 B -0.2476 C -0.2213 D-0.1585 E +0.2160 F +0.0439 G +0.1928 H -0.0202 J +0.1674K -0.1078 L

Where, A, B, C, D, E, F, G, H, J, K and L represent the inoculum size, pH, Magnesium sulphate, Incubation period, glucose, yeast extract, ammonium sulphate, sodium acetate, inducer (phenol), agitation and trace elements respectively.