Enhancement of extracellular laccase production from Lenzites elegans KSG32: Taguchi orthogonal array experimental design methodology

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Abstract - Fungal extracellular laccases play a pre-eminent role in biotechnology applications due to their high redox potential and wide substrate specificity. For any industrial applications large quantity of enzyme is needed and must be produced economically. The present study focused on the enhancement of laccase production by Lenzites elegans KSG32 under solid-state fermentation method using Taguchi orthogonal array experimental design methodology. An L8 orthogonal array was designed using Qualitek-4 software to study the effect of seven factors selected on the basis of one factor at a time approach. The interactions and influences between the factors were studied. The predicted optimum conditions for the production were: incubation period (3 days), sugarcane bagasse size (0.02 m), pH (3), maltose concentration (2.5 %), beef extract concentration (0.1 %) and pyrogallol concentration (0.25 mM). Tween 20 was identified as unnecessary media component and excluded. After optimization of SSF conditions laccase production was increased by 39.74 %. The results suggest that both the design method and strain has promising potential in the production of laccases for biotechnological applications like synthetic dye decolourization.

Key words - Taguchi method; Design of experiments; Optimization; Laccase; Lenzites elegans KSG32; Solid state fermentation

I. INTRODUCTION

Laccases are lignin degrading enzymes that have cardinal industrial application in biobleaching and biopulping in paper industries [1] bioconversion of lignocellulosics [2], polycyclic aromatic hydrocarbon (PAH) contaminated soil, effluent bioremediation [3] and xenobiotic detoxification [4]. They non specifically catalyze monoelectronic oxidation of various organic and inorganic aromatic compounds with the concomitant four electron reduction of O_2 to water by multi copper enzyme centre [5]. Due to their unique catalytic properties, the practical applications of laccases have attracted research attention.

White rot fungi are known producers of extracellular laccases but the enzyme production is relatively low. The production is influenced by cultural conditions, development stage, nutrient levels and the presence of inducers in the culture medium [6]. The focus on increasing laccase production in the present study is to meet the industrial requirement for synthetic dye decolourization [7]. Increase in enzyme production may be achieved by either submerged fermentation or solid state fermentation (SSF) methods. In the case of laccase, higher yield was obtained by SSF rather than submerged fermentation [8]. Similarity to the natural habitat of fungi and better oxygen circulation are advantages of SSF [9]. Added advantages are the cheaper downstream processes and reduced energy and cost requirements of production [10].

The optimization of SSF parameters is a preliminary requirement for the industrial production of enzymes. However, optimization by classic factor at a time (OFAT) does not give a true optimum due to the interaction among variables. Thus, statistical design of experiments (DOE) is required that encompasses simultaneous study of different factors [11, 12]. Among different DOE, Taguchi's method gives a better understanding of the interaction between different factors involved in SSF. The fractional factorial approach of orthogonal array (OA) in this method helps to reduce the number and cost of experiments [13]. In this methodology, the complicated enzyme production optimization process got simplified by establishing a relationship between parameters and operational conditions hence the impact of individual factors can be easily identified. Based on the concept of robustness and S/N ratio, the optimal levels of process parameters can be determined. Analysis of experimental data by ANOVA gives a statistical significance to the software output data.

In the present study, the aim was to optimize the SSF factors to enhance intrinsic production of extracellular laccase from the white rot fungus, *Lenzites elegans* KSG32, isolated from decaying wood. The optimization entailed the study of factors by Taguchi DOE that successfully enhanced *L. elegans* laccase production by 39.74 %.

II. MATERIALS AND METHODS

A Microorganism

Lenzites elegans KSG32 [7] isolated from decaying wood, was used in the present study. The culture was maintained on potato dextrose agar plates at 4°C.

B Design of experiments using the Taguchi method

Taguchi experimental design was selected for the statistical optimization of fermentation factors. For designing the experiments, analysis of variance and optimization of process, Qualitek-4® software (Nutek Inc., MI, USA) was used. The factors- incubation period, sugarcane bagasse size, pH, maltose, beef extract, tween 20 and pyrogallol for designing the matrix experiments were selected based on the significant influence on laccase production during OFAT studies. The seven factors selected were studied in two levels using an L8 (2^7) orthogonal array (OA) (Table 1). The two levels were selected to find out the optimum production with the effect of interactions between the fermentation parameters. After the primary results, a validation test was performed to check the optimum conditions. Analysis of variance (ANOVA) for the obtained results was investigated.

C Solid state fermentation, enzyme extraction and assay

Laccase production was done by solid state fermentation method. Sugarcane bagasse was used as substrate and the moisture content was adjusted with 0.1M sodium citrate buffer of different pH (3, 5). The substrate was inoculated with *L. elegans* KSG 32 mycelial agar plugs of size 1 cm^2. Incubation was done at 26°C. After incubation 50 mL 0.1 M citrate buffer (3, 5) was added to each flask to extract the crude laccase. The enzyme supernatant was collected after centrifugation at 10,000 rpm for 10 min at 4°C and stored at -20°C for subsequent use.

Laccase activity was measured by the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) [14]. The absorbance was checked at 420 nm. Enzyme activity was expressed in international units (IU/mL). One IU of enzyme activity was defined as the amount of enzyme that oxidized 1µM of substrate per minute under standard conditions.

All the experiments were done in triplicates.

III. RESULTS AND DISCUSSION

A Taguchi experimental design

As a preliminary approach to industrial scale production, statistical optimization is a must for any fermentation experiment. Taguchi method of DOE is suitable for the SSF experiments [11]. Both environmental factors and culture medium components have influence on SSF [15]. In classical one factor at a time approach, interaction between these factors was not considered even though the interactions are known to influence solid state fermentation. With Taguchi method interactions of both environmental factors and medium components can be analyzed [11]. These factors and their assigned levels used to design OA are shown in Table 1. All the selected factors have an influential role in the production of laccase as it increases the activity from 1147.1±21.21 IU/mL to 1603±12.6 IU/mL after optimization.

Table 1: Assigned levels of selected factors and their main effects

Factors	Assigned levels		L2-L1
	Level 1	*Level 2*	
Incubation period (days)	3	5	-5.06
Sugarcane bagasse size (m)	0.02	0.04	-4.553
pH	3	5	-4.242
Maltose concentration (%)	1	2.5	2.115
Beef extract concentration (%)	0.1	0.2	-.462
Tween 20 concentration (%)	0.1	0.3	-.135
Pyrogallol concentration (mM)	0.25	0.5	-1.435

B Relative influence of individual factors

The relative influence of the effect is indicated as the difference between the average values of factors at each level (L2-L1) (Table 1). The + or – shows the increase or decrease in the production from level 1 to level 2. Except maltose concentration, increase in the concentration of all other factors decreased the laccase activity. SSF trials as per OA design was conducted with conditions given in Table 2. OA trial 2 with experimental conditions of incubation period (3 days), sugarcane bagasse size (2cm), pH (3), maltose (2.5%;w/v), beef extract (0.2%;w/v), tween 20 (0.3%;w/v), and pyrogallol (0.5mM) gave maximum laccase activity of 1213.2±16.29 IU/ml.

Table 2: L_8 orthogonal array design

Trials	Columns							Laccase activity (IU/mL)
	1	*2*	*3*	*4*	*5*	*6*	*7*	
Trial 1	1	1	1	1	1	1	1	1201.16±18.10
Trial 2	1	1	1	2	2	2	2	1213.2±16.29
Trial 3	1	2	2	1	1	2	2	364.5±9.12
Trial 4	1	2	2	2	2	1	1	524.86±6.78
Trial 5	2	1	2	1	2	1	2	332.16±20.35
Trial 6	2	1	2	2	1	2	1	517.86±19.35
Trial 7	2	2	1	1	2	2	1	370.9±6.97
Trial 8	2	2	1	2	1	1	2	429.76±8.96

C Interactions of fermentation factors on laccase production

The interaction between the factors involved in fermentation gives a better understanding of the overall process of laccase production in a fermentor. Thus influence of all the factors accruing to laccase production was studied and the main effects of

all the seven factors were shown in Fig 1. The results were represented as signal to noise (S/N) ratio. A 100% interaction was represented by perpendicular line and null effect by parallel lines. Our result shows tween 20 concentrations has the least influence on laccase production.

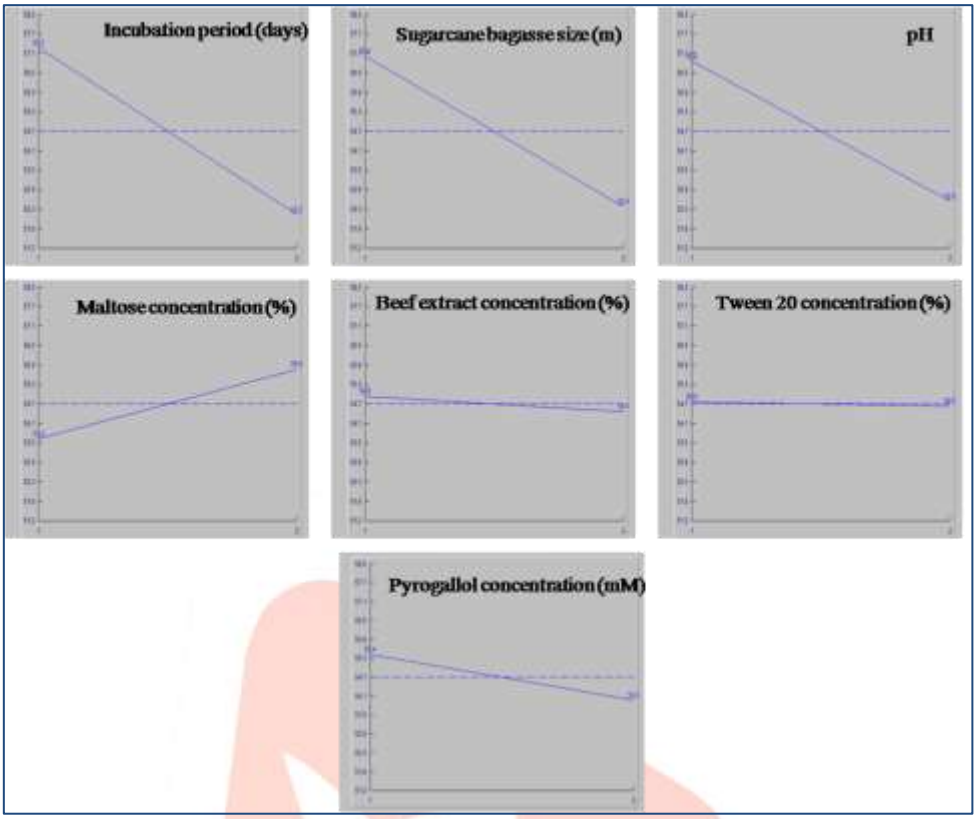

Fig. 1: individual influence of selected factors on laccase production by *Lenzites elegans* KSG32: Tween 20 concentration has found to be less influence on laccase production

The influence of two individual factors at different levels of interaction is depicted by severity index (SI). The predicted interactions between each factors and SI were given in Table 3. The higher SI (90.16 %) was represented by the interaction between beef extract and least influencing factor tween 20. While low SI (1.99 %) was for the interaction between sugarcane bagasse length and maltose concentration.

Table 3: Predicted interactions and severity index for different fermentation factors

Sl. No.	Factors	Columns	SI (%)	Reserved column	Levels
1	Beef extract x Tween 20	5 x 6	90.16	3	[1, 1]
2	Tween 20 x Pyrogallol	6 x 7	77.92	1	[1, 1]
3	Beef extract x Pyrogallol	5 x 7	76.05	2	[1, 1]
4	Maltose x Beef extract	4 x 5	70.5	1	[2, 2]
5	Maltose x Tween 20	4 x 6	68.27	2	[2, 2]
6	Maltose x Pyrogallol	4 x 7	66.71	3	[2, 2]
7	Sugarcane bagasse x pH	2 x 3	52.63	1	[1, 1]
8	Incubation period x pH	1 x 3	47.36	2	[1, 1]
9	Incubation period x Sugarcane bagasse	1 x 2	44.13	3	[1, 1]
10	pH x Pyrogallol	3 x 7	33.28	4	[1, 2]
11	Sugarcane bagasse x Tween 20	2 x 6	31.72	4	[1, 2]
12	Incubation period x Beef extract	1 x 5	29.49	4	[1, 2]
13	Sugarcane bagasse x Beef extract	2 x 5	23.94	7	[1, 1]
14	pH x Maltose	3 x 4	22.54	7	[1, 2]
15	Incubation period x Tween 20	1 x 6	22.07	7	[1, 1]
16	pH x Tween 20	3 x 6	9.83	5	[1, 1]
17	Sugarcane bagasse x Pyrogallol	2 x 7	7.72	5	[1, 1]
18	Incubation period x Maltose	1 x 4	6.44	5	[1, 2]
19	pH x Beef extract	3 x 5	2.83	6	[1, 1]
20	Incubation period x Pyrogallol	1 x 7	2.05	6	[1, 1]
21	Sugarcane bagasse x Maltose	2 x 4	1.99	6	[1, 2]

The variations contributed by each fermentation factors were analyzed by analysis of variance (ANOVA) (Table 4). The results showed that incubation period has significant effect (35.979%) on laccase production followed by sugarcane bagasse size (29.137%) and pH (25.288%). All other factors contribute 9.414% to laccase production. Since, tween 20 concentration had less effect on production, it was selected for pooling. This helped to avoid saturation of the system. All the factors and interactions in the designed system were statistically significant at 95% confidence limit.

Table 4: Analysis of variance (ANOVA)

Sl. No.	Factors	DOF	Sum of squares	Variance	F-Ratio	Pure Sum	Percentage
1	Incubation period (days)	1	51.189	51.189	1408.371	51.153	35.979
2	Sugarcane bagasse size (m)	1	41.461	41.461	1140.717	41.424	29.137
3	pH	1	35.989	35.989	990.179	35.953	25.288
4	Maltose concentration (%)	1	8.956	8.956	246.418	8.92	6.274
5	Beef extract concentration (%)	1	0.428	0.428	11.8	0.392	0.276
6	Tween 20 concentration (%)	Pooled					
7	Pyrogallol concentration (mM)	1	4.109	4.109	113.054	4.072	2.864
	Other Error	1	0.036	0.036			0.182
	Total	142.171					100%

D Optimum level determination and validation

Based on Taguchi DOE design the optimum conditions for maximum laccase production were highlighted in Table 5. The expected laccase production at optimum conditions was 1519.848 IU/mL. The variation reduction plot (Fig. 2) graphically represents the performance distribution of current and improved laccase production. The upper and lower control limits (UCL and LCL) were 439.705 IU/mL and 1679.062 IU/mL respectively. From the graph the improved condition causes percentage elimination of non-necessary media components such as tween 20.

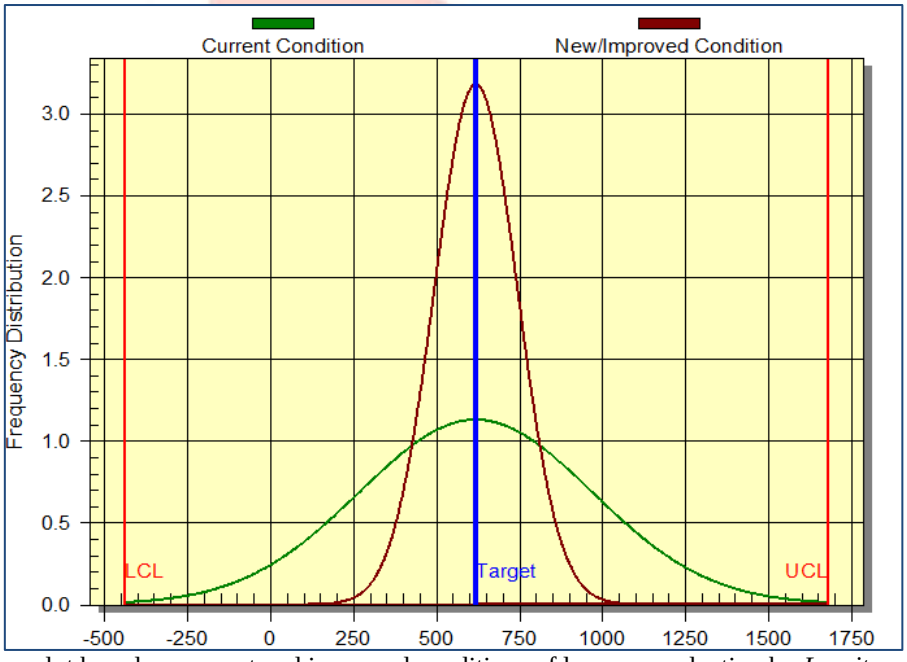

Fig. 2: Variation reduction plot based on current and improved conditions of laccase production by *Lenzites elegans* KSG32

For validation of the results SSF was conducted with optimized factors. The resulted production of 1603±12.6 IU/mL was comparable to the predicted value. After Taguchi DOE experiments 39.74% increase in laccase activity than OFAT method was observed. Laccase production by various basidiomycetes under SSF was noted in Table 6. The relatively high laccase production by *Lenzites elegans* KSG32 offers the potential use in industrial applications like dye decolourization (Lakshmi et al., 2017)

Table 5: Predicted optimum culture condition and their contributions on the selected levels

Sl. No.	Factors	Level description	Level	Contribution
1	Incubation period (days)	3	1	2.529
2	Sugarcane bagasse size (m)	0.02	1	2.276
3	pH	3	1	2.12
4	Maltose concentration (%)	2.5	2	1.058
5	Beef extract concentration (%)	0.1	1	0.231
6	Pyrogallol concentration (mM)	0.25	1	0.716
	Total contributions from all factors			8.929
	Current Grand average of performance			54.706
	Expected result at optimum conditions			63.636

Table 6: Laccase production comparison of *Lenzites elegans*KSG32 with other white rot fungi under SSF

Sl. No.	Organism	Substrate	Laccase activity (IU/mL)	References
1	*Trametes pubescens* CBS 696.94	Banana skin	1.57	[16]
2	*Ganoderma lucidum* IBL-06	Rice straw	338	[17]
3	*Marasmius* sp.	Rice straw	1116.11	[18]
4	*Ganoderma lucidum* GD 88	Pineapple leaf	472.3	[19]
5	*Pleurotus ostreatus* IBL-04	Wheat straw	517	[20]
6	*Phanerocrete chrysosporium* MTCC 787	Wheat straw	10.5	[21]
7	*Coriolus versicolor*	Rice bran	980	[22]
8	*Pleurotus ostreatus*	Pineapple leaf	1632.63	[23]
9	*Marasmiellus palmivorus* LA1	Pineapple leaf	667.4	[24]
10	*Lenzites elegans* KSG32	Sugarcane bagasse	1603±12.6	Present study

II. CONCLUSION

Laccase production by *Lenzites elegans* KSG32 was enhanced by optimizing the SSF factors by Taguchi orthogonal array experimental design methodology. Both cultural and nutritional factors were optimized by Taguchi method taking into consideration of the interactions between factors. The predicted optimum conditions were validated by performing fermentation experiments. After statistical optimization, the laccase production was increased upto 39.74 % than classical optimization. The study concludes that the experimental design by Taguchi method enhanced the production of laccase which provides insight into the industrial scale up.

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