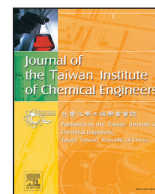




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Optimization and up scaling of ionic liquid tolerant and thermo-alkali stable laccase from a marine *Staphylococcus arlettae* S1-20 using tea waste

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ABSTRACT

Laccase are versatile oxidoreductase enzyme having a capability of degrading variety of phenolic and non phenolic compounds. Therefore, in this work cost effective process is developed for the higher production of extracellular, thermo-alkali stable laccase (S1-20LAC) from *Staphylococcus arlettae* S1-20 using tea waste. To enhance the enzyme yield, multiple sequential statistical tools *i.e.* RSM and ANN linked GA are employed which led to 16 fold enhancement in enzyme yield. Further, a pilot scale bioprocess is developed using optimum conditions shown by GA *i.e.*, 2.50% tea waste, 4.95 mM NaCl, 37 °C Temperature and 5.65 mM L-DOPA which led to 72 fold (879 nkatml⁻¹) improve in the enzyme yield. The optimum temperature and pH for S1-20LAC is 85 °C and 9.0. S1-20 retained significant amount of activity even in the presence of 20% (v/v) Ionic liquids (ILs). The detailed kinetic and thermodynamic studies revealed that the overall quality of laccase is spontaneous process at high temperature. The outcome of this study shows that high production and remarkable properties of S1-20LAC offers a significant opportunity for the degradation and synthesis of wide range of compounds, which makes it an attractive green biocatalyst for the future aspects.

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1. Introduction

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) are multi-copper-oxidoreductase enzymes that catalyze the oxidation of wide range of phenolic substrates by a radically catalyzed reaction mechanism accompanying reduction of oxygen to water. Catalytic action in degradation processes by laccase make them suitable candidates in enormous field such as food, textile, pulp and paper industry, nanobiotechnology, soil bioremediation, synthetic chemistry, cosmetics, etc [1,2]. Laccases also have ability to catalyze polymers and hence referred to as green biocatalysts. These enzymes are mainly produced by fungi, but fungal laccases generally fails to work in extreme environmental conditions. Till date, only few bacterial laccases have been characterized, which can produce some extracellular laccase. Hence there is an utmost requirement for the production of extracellular laccase [2,3].

Production of this enzyme, in a cost effective manner is a prerequisite, for their use in the industrial processes. Hence agricultural waste is one of the best alternative substrates which not only solve the problem of disposal of agricultural waste, as well as it

also help in providing essential nutrients for the production of enzyme in high titre [1,3].

Optimization and physic-chemical factors are some of the best method used in increasing the yield of these enzymes. Statistical experimental designs have been used as optimization strategy and they are better recognized by the traditional one variable at a time method [2]. Response surface methodology (RSM) is an effective statistical design, for optimization of various factors, in order to predict the best result with the less number of experiments. It is also suitable for studying the main and interactive effects of various factors on growth or metabolite formation during fermentation. Sometimes application of RSM for all modelling and optimization studies is difficult, hence for that possible alternatives are, Artificial neural network (ANN) modelling and Genetic algorithm (GA) optimization [4,5]. They can mimic different features of biological information process for data modelling and can prove to be useful in media optimization. GAs are optimization algorithms, which are unconventional and help in the direct search for elucidating the problem by imitating part of the process of natural evolution. GA performs direct searches through a given set of alternatives, with the aim of finding the best alternative, with respect to the given criteria of goodness of fit. It is expressed in terms of objective function (also referred to fitness function). The use of advanced ANNs and GAs is well established and used frequently

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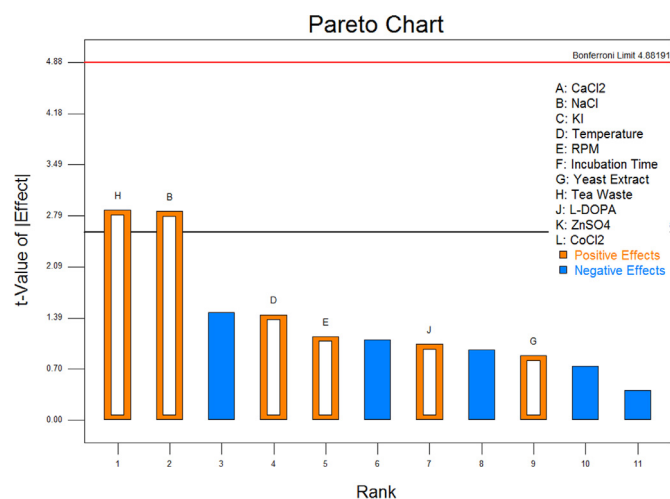


Fig. 1. Pareto chart showing parameters used in the Plackett-Burman design.

in environmental biotechnology and biochemical engineering for modelling and optimization [5–8].

Lack of available scientific literature on statistical optimization and modelling studies, using *Staphylococcus arlettae* S1-20, for extracellular, thermo-alkalizable laccase (S1-20LAC) production, gave the opportunity to do the proposed work. Here significantly affect variables are optimized through hybrid statistical optimization tools (RSM, ANN, GA) for enzyme production, and thereafter enzyme is characterized for application point of view.

2. Material and methods

2.1. Bacteria cultivation and enzyme production

Staphylococcus arlettae strain S1-20 was isolated earlier in our laboratory (Genbank accession no. KU321288.1) and cultivated on Zobell Marine Agar media (Hi-media company, India). For enzyme production, overnight grown culture (16 h ; Cells $7.89 \times 10^7\text{ cfu ml}^{-1}$) was used as an inoculum for 100 ml of Zobell Broth and incubated at 37°C for 48 h. The supernatant was used as a crude enzyme source.

2.2. Role of agricultural residues on laccase production

Various agricultural substrates (apple peel, banana peel, orange peel, tea waste, potato peel, corn husk, sugar cane, lemon peel, wheat bran, saw dust, sea weed) were screened in the concentration of 1% in M162 medium [2] containing 100 μM copper and laccase production was evaluated after 24 and 48 h respectively.

2.3. Optimization of S1-20LAC production using central composite design (CCD)

Plackett-Burman Design (PBD) was used for shortlisting important variables such as (CaCl_2 , NaCl, KI, temperature, rotation speed, incubation time, yeast extract, tea waste, L-DOPA, ZnSO_4 , CoCl_2) (Fig. 1). Further, Central composite design (CCD) was used using selected variable i.e. [A] tea waste (0.5–2.5%), [B] NaCl (1–5 mM), [C] temperature (26.50–40.50 $^\circ\text{C}$) and [D] L-DOPA (2–10 mM) at five levels to investigate the effect of each factor and interaction amongst them for laccase production (Table S1). The behavior of the organization was demonstrated with the help of following quadratic equation.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where Y represents feedback (laccase nkat ml^{-1}), β_0 is the interception coefficient, β_i coefficient of the linear effect, β_{ii} the coefficient of quadratic effect and β_{ij} the coefficient of interaction effect. The importance of each coefficient was decided by student-t-test and p values. The variability in the dependent variable was demonstrated by R^2 .

A 2^3 -factorial design, with eight axial points ($\alpha = 2$) and six replications at the centre points ($n_0 = 6$) leading to a sum of 30 experiments in which enzyme yield was used as a response. Design expert version 8.0 (Stat-ease corporation, USA) was used for multiple regression analysis and to generate the diagram of data.

2.4. Optimization of S1-20LAC production by artificial neural network (ANN) linked genetic algorithm (GA)

Artificial Neural Network (ANN) technique was performed by a sequence of steps these are compilation of data, to generate the network, configuration of network, training the network, network validation and explore that network. The artificial neural network mechanisms have three layers with inputs, outputs and neurons to generate the best fit model. The input data and output data were explored from the RSM design matrix. Every input has three variables and three replications. The various parameters obtained from ANN were used in genetic algorithm (GA) to predict the optimum reaction variables.

The GA optimization begins with initialization of the population of solutions P(t). The population size was 16 (4 x no. of variables) and the initial population type chosen was double. In the present optimization for the process of the selection among the available methods, Rank method opted. The scattered option was used as crossover operator and other constraints used for reproduction and mutations are 0.8 crossover rate and constraint dependent mutations function. Other parameters were approximated as follows, migration direction -Intermediate, migration fraction- 0.2, migration interval-20, distance measure function - distance crowding, Pareto front population fraction-0.35. For stopping criteria following values were taken i.e. Maximum number of iterations = $100 \times$ No. of variables i.e 400. Time limit = ∞ , fitness limit = ∞ , stall generations = 100 and function tolerance was 10^{-4} [4,5].

2.5. Scale up of laccase production

A 100 L fermenter containing 50 L of production medium was utilized for large-scale production of laccase. The fermenter was maintained at 37°C and was aerated by forced air at 0.2 $\text{v}^{-1}\text{v}^{-1}\text{min}$. The contents were stirred by a marine impeller at 150 rpm. Bioreactor and production medium were sterilized in standard conditions. The 2000 ml of culture medium based on condition optimized (2.50% tea waste, 4.95 mM NaCl, 37°C temperature, 5.65 mM L-DOPA) by ANN linked GA was inoculated with grown culture (Cells $7.89 \times 10^7\text{ cfu ml}^{-1}$; 16 h). Samples were taken at regular intervals and analyzed for activity, growth and pH.

2.6. Activity assay

The enzymatic activity was determined according to the method described by Chauhan and Jha [1] using di-methoxy phenol as a substrate. Protein concentration was estimated by Bradford protein estimation kit (Sigma-Aldrich, USA).

2.7. Effect of temperature on S1-20LAC

The temperature versus activity profile of laccase was studied at different temperature ranging from 60 to 100°C . The thermostability profile was studied by incubating the enzyme upto 3 h in the

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