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Enhanced production of laccase and pectinase using co-culture of *Trametes hirsuta* and *Phanerochaete* sp. through EVOP-factorial design technique

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Abstract

The present study deals with the coproduction of laccase and pectinase enzymes through solid state fermentation using mixed fungal culture of *Trametes hirsuta* and *Phanerochaete* sp., to minimize the cost and time of the process. Substrates selected for the enzyme production were wheat bran, pulse husk and mustard peel. To get optimum yield of laccase and pectinase in a single fermenter, EVOP factorial design technique with factors like pH, incubation temperature and substrates ratio have been explored. At search level of EVOP, outcomes of the one factor at a time has been considered as incubation temperature 30 °C for 7 days at pH 5 with wheat bran: pulse husk: mustard peel ratio of 2:2:1 (w/w/w) and yield of laccase and pectinase was found as 78.1 U/gds and 105.0 U/gds respectively. In first setup of EVOP factorial both laccase and pectinase activities were not found optimum in same set of experiments; therefore, on the basis of its decision making steps, second set of experiment was performed by taking decisions of first set of EVOP as search level. Optimum yield of laccase and pectinase was achieved about 250 U/gds and 247 U/gds respectively at 34 °C, pH 4.5 with 1.75:1.75:1.5 (w/w/w) wheatbran: pulse husk: mustard peel as substrate, which was 2–3 times higher than the outcomes of one factor at a time method.

Keywords Pectinase · Laccase · Production · Optimization · EVOP · Co-culture

Introduction

Laccases and pectinases, have leading position among the commercially produced industrial enzymes in global market. Laccase (EC:1.10.3.2) belongs to oxidoreductase family, also known as blue copper family of oxidases, which is produced by various microorganisms either extracellularly or intracellularly. Laccase catalyses the oxidation of its substrate molecules, i.e., phenols and amines, by mono-electronic transfer process and converting the substrate into its corresponding free radical moieties (Agrawal et al. 2018). Laccase has extended application in synthetic chemistry, it can be used in various technological processes such as bioremediation, detoxify industrial pollutants, water

detoxification and in nanobiotechnology applications (Couto and Herrera 2006).

Pectinase enzymes (EC no. 4.2.2.10) catalyses the breakdown of α-1,4-glycosidic linkages in pectin by trans-elimination reaction and resultants are unsaturated methyl galactouronates and galactouronates (Rebello et al. 2017). The blend of laccase and pectinase has important applications in wine, beer and fruit juice industries. On using the blend of both, laccase modifies the colour and eliminates the phenolic substances for browning, turbidity development and haze formation, whereas, pectinases facilitates the extraction and filtration process, to intensify the flavour, colour and maximise the juice yield (Chaudhari and Suneetha 2012; Bhat 2000; Jayani et al. 2005). Other industries such as textile, polymer and biofuels etc have also broaden the scope of the blend of these two enzymes such as in textile for bio-bleaching, dyeing, finishing, bio-scouring, wash-off treatment and printing, in biofuels for saccharification of agricultural waste (Pandey et al. 2016; Pusic et al. 2015; Maurya et al. 2015) and in polymer industries for the

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synthesis of biopolymers, generation of monomers and cross-linking of the monomers (Selinheimo et al. 2006; Minussi et al. 2002).

Studies have also been performed which describe the production of the single enzyme or mixture of enzymes using fungal co-cultivations processes, e.g., co-cultivation of *Trichoderma reesei* and *Aspergillus niger* for the production of cellulase enzyme (Maheshwari et al. 1994), Gupte and Madamwar (1997) has reported production of β-glucosidase and cellulase from coculture of *A. fumigatus* and *A. Ellipticus*. Similarly, for the higher yield of lignolytic enzymes, co-cultivation of *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Verma and Madamwar 2002) and *Trametes* and a *Trichoderma strain* (Zhang et al. 2006) were explored. While the production of laccase and pectinase enzymes from the same reactor using co-culture technique, it remained a bottle neck of the process to get common optimal physicochemical conditions for maximum yield of both the enzymes and to achieve it, scientists have approached various statistical and computational methods.

Statistical cum computational methods such as RSM, Factorial design technique, ANN have been used by Negi and Banerjee (2010) for optimization of amylase and protease production, Maurya et al. (2013) for enzymatic saccharification in biofuels. Blend of pectinase and laccase have been produced by Giese et al. (2008) with enzyme activities 46 U/ml of laccase and 32 U/ml of pectinase from the strain *Botryosphaeria rhodina* MAMB-05, however, the yield/activities of these two enzymes were not up to the demand. Therefore, in current study *Trametes hirsuta* and *Phanerochaete* sp. fungal strains have been explored for the production of the blend of laccase and pectinase and optimum physicochemical conditions were achieved using EVOP factorial design technique. The findings of this study would be promising to fulfil the current increase demand of the blend of these enzymes.

Materials and methods

Screening of pectinase and laccase producing strains

Samples were collected aseptically from the soil containing Pine decompost of Uttarakhand region and microbes capable of producing laccase and pectinase enzymes extracellularly were screened. Serial dilution was performed. After serial dilution, pour plate technique was used to screen out the microbes. For laccase producing strains selective media having composition (in g/l): Peptone (3.0), Glucose (10.0), KH_2PO_4 (0.6), $ZnSO_4$ (0.001), K_2HPO_4 (0.4), $FeSO_4$ (0.0005), $MnSO_4$ (0.05), $MgSO_4$ (0.5), Agar (20), pH-6, supplemented with 0.02% guaiacol was used

and for pectinase producing microbes media containing (g/l) KCl (0.5), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.01), $NaNO_3$ (2.0), KH_2PO_4 (4.5), Peptone (0.5), Beef extract (0.3), Agar (15), Pectin (4.0) was used. Laccase and pectinase producing microorganisms were confirmed by performing their respective quantitative assays. The microbial isolates found to be more efficient for the desired enzymes were characterized by 18S r-RNA sequencing using forward and reverse primers ITS1 and ITS4, respectively, from Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India.

SSF for enzymes production

For the production of Laccase and pectinase enzymes through solid state fermentation (SSF), co-culture of *Trametes hirsuta* and *Phanerochaete* sp. strains were inoculated on substrate containing different ratio of wheat bran: pulse husk and mustard peel (WB:PH:MP). Substrate was taken into a 250 ml flasks and the minimal salt media (modified Chapex-Dox) having composition (in g/l): KCl (0.5), $MgSO_4·7H_2O$ (0.5), $FeSO_4·7H_2O$ (0.01), $NaNO_3$ (2.0), KH_2PO_4 (4.5) was added to moistened the substrate (1:2 w/v ratio) and autoclaved. The inoculation was done with the spore suspension containing 1.8×10^8 spores/ml of the fungal culture. Spore count was measured using haemocytometer. After inoculation the flasks were kept for 7 days at 28 ± 2 °C under static condition. The flask containing substrate without inoculation was used as control.

Enzyme extraction

After one week of incubation, enzyme extraction was done by adding 50 ml of acetate buffer (pH 4.5, 50 mM) into the fermented flask and the material inside the flask were kept for soaking on orbital shaker at 30 ± 2 °C for 2 h at 100 rpm. The broth was filtered and solid material was separated using muslin cloth. After filtration, the filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C to remove remaining solid residues and get a clear supernatant (Negi and Banerjee 2009). Supernatant obtained after centrifugation was used as crude enzyme. All the tests were performed in duplicate and the given data are the mean values of the results obtained.

Enzyme assay

Pectinase assay

Pectinase activity was determined using the reaction between thiobarbituric acid and end product of pectin degradation.

Supernatant was added to a mixture of thiobarbituric acid, HCl and dist. water, heated in boiling water for 30 min. and absorbance was measured at 550 nm (Pitt 1988). One unit of activity is the amount of enzyme required to change in the absorbance of 0.01 under the assay conditions.

Laccase assay

Laccase activity was determined by observing the change in absorbance at 420 nm, change related to the rate of oxidation of 1 mM 2,2-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in acetate buffer (50 mM, pH 4.5). Assay was carried out at 25 ± 1 °C with 50 μl of diluted liquid media containing culture (Elisashvili et al. 2008). One unit of enzyme activity is the amount of enzyme required to transform 1 μmol of substrate per minute per ml in to product.

Optimization of the physico-chemical parameters by one factor at a time method

Different physico-chemical parameters for, e.g., substrate ratio (WB:PH:MP), size of the inoculum, pH, temperature, and inoculum age for the coproduction of the enzymes were studied. Ratio of the substrates (WB:PH:MP) were varied while keeping the final weight of the total substrate constant, i.e., 5 gm (3:1:1, 2:2:1, 1:3:1, 1:2:2, 1:1:3, 1.4:1.4:1.4, 5:0:0, 0:5:0, 0:0:5) w/w, effect of pH and temperature was studied in the range between 3 and 9 and 25–50 °C, respectively. Inoculum size was varied from 1 to 3 ml and incubation time was studied between 5 and 10 days. Optimization was done using 'one factor at a time method' in which only one factor at a time was changed while keeping other factors constant for seeking the optimum level (Negi and Banerjee 2009).

Optimization of variables by EVOP-factorial design technique

Evolutionary optimization factorial design (EVOP) is a method which depends upon two rules. (1) It looks for optimum conditions first for each and every parameter. (2) Depending upon optimum levels of different parameters, it set up new trials with in the upper and lower limit of the optimum conditions. If we want to determine the integrated effect of various parameters including the minor one also then we have to follow statistical approach. In case of three variable systems, the effects can be calculated using the calculation worksheet given in Table 1. Based on the size of the effects, changes in main effect value, and error limit values calculations, decision making process can be done so as to find the optimum conditions (Negi and Banerjee 2006). The experimental set up for three variables system contains, one upper (+ 1) limit and one lower (− 1) limit from its corresponding optimum conditions(0) in ten separate experiments The effects were calculated and compared with the error limit and change in mean effect values to get the final conclusion of the optimization process. As per the decision making step, if the calculated values for effects are smaller than the error limits and change in mean effect value is negative and large then only the optimum conditions for the process will be achieved.

Decision making process

Decision making in EVOP-factorial design technique is a crucial step. During this process error limits and change in mean effect values are taken into consideration and compared with the magnitude of the effects. This step further provides us the direction to change the parameters so as to get improved response. If the calculated value of effects is found to be higher than the error limit value then a slight change in the parameters can achieve optimum level. In this study the parameters such as temperature, substrate ratio and pH were taken into account for optimization using Evolutionary operation factorial design (EVOP) technique.

Table 1 Calculation table for three variables

Exp. parameter	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Substrate (WB + PH + MP)	0	_	_	+	+	0	+	_	+	_
Temperature	0	_	+	_	+	0	+	_	_	+
pH	0	_	+	+	_	0	+	+	_	_
Response	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
	Block1					Block2				

Total no. of experiments performed were ten in 2 cycles. (0) is the optimum search level, (+) is the upper limit and (−) represent the lower limit

WB wheat bran, *PH* pulse husk, *MP* Mustard peel
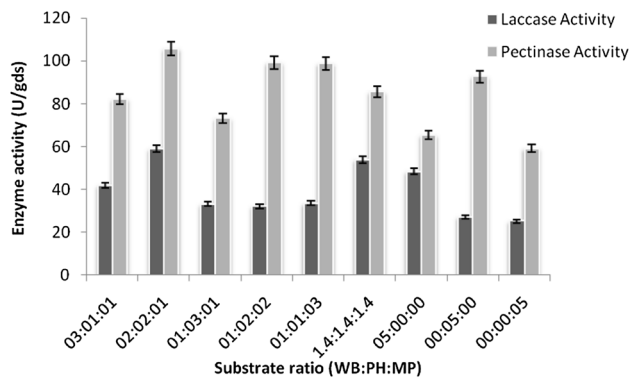
Fig. 1 Optimization of substrates (Wheat bran: Pulse husk: Mustard peel) ratio by one factor at a time method
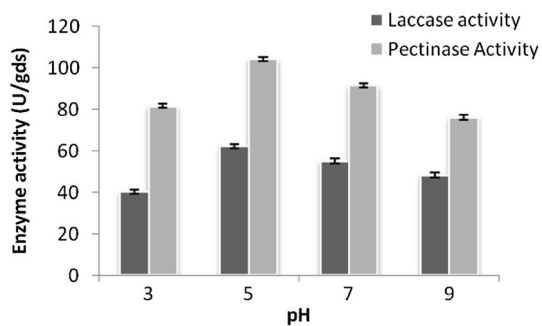

Fig. 2 Optimization of pH of media by one factor at a time method

Results and discussion

Screening of pectinase and laccase producing strains

Seven fungal isolates were screened for the laccase and pectinase enzymes. Pectinase producing fungus (F6) was isolated which shows cottony growth and had white colour spores and on molecular characterization has shown 96% sequence similarity with *Phanerochaete* sp. on NCBI data base analysis. The isolated strains for laccase production shows negligible laccase activity in comparison with the commercial strain *Trametes hirsuta* (TH). Therefore, for coproduction of pectinases and laccases, mixed culture of *Phanerochaete* sp. and *Trametes hirsuta* (TH) strain was used as inocculum. There are very few studies available which show coproduction of laccase and pectinase enzymes, Giese et al. (2008) reports coproduction of these enzymes using orange bagasse under solid state fermentation from *Botryospheria rhodina* MAMB-05. But the yield of Laccase and Pectinase was found lower (46 U/ml and 32 U/ml respectively) than the reported in present work.
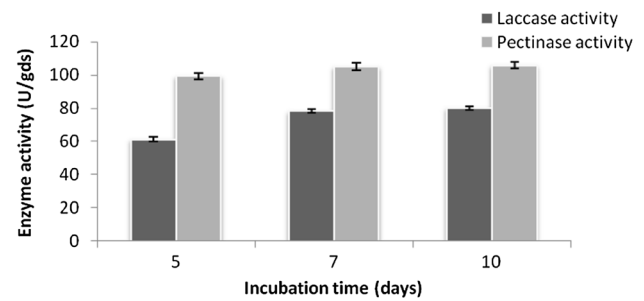
Fig. 3 Optimization of incubation time period by one factor at a time method

Table 2 Experimental setup of optimization (EVOP1)

Parameters	(+)	(0)	(−)
Substrate	3:1:1	2:2:1	1:3:1
pH	6	5	4
Temperature	33	30	27

'0' optimum search level, '+' upper limit, '−' lower limit

In this study, we are using the combination of the two fungal strains *T. hirsuta* and *Phanerochaete* sp. which has not been explored yet for the coproduction of these enzymes.

Optimization of the physico-chemical parameters by one factor at a time approach

Different physico-chemical parameters such as substrate ratio, inoculum age, inoculum size, pH and temperature for coproduction of the enzymes were studied and optimized first by "one factor at a time" approach (Figs. 1, 2, 3). Maximum laccase and pectinase activity were found to be 78.25 U/gds and 105.31 U/gds, respectively with wheat bran: pulse husk: mustard peel (WB:PH:MP) in 2:2:1 ratio as substrate at pH 5.0 temperature 30 °C and incubation time of 7 days. Similar optimum conditions were reported for the laccase produced by *T. hirsuta* and *T. versicolor* cultured individually on the substrates grape seed and barley bran under solid state fermentation (Couto et al. 2006) and for pectinase enzyme from the source *Penicillium viridicatum* RFC3 on agro-industrial wastes and mixed culture of *Aspergillus* species on pine apple residues (Silva et al. 2002; Singh and Mandal 2006).

EVOP Factorial design technique to achieve optimum conditions

To optimize three variable systems EVOP technique needs lesser number of experimental setups, which make

Table 3 Experimental design table (EVOP1)

Exp. parameter	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Substrate (WB + PH + MP)	2:2:1	1:3:1	1:3:1	3:1:1	3:1:1	2:2:1	3:1:1	1:3:1	3:1:1	1:3:1
Temperature	30	27	33	27	33	30	33	27	27	33
pH	5	4	6	6	4	5	6	6	4	4
Response	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10

Table 4 Effects and limits for experimental (EVOP1)

Effect of variables		Laccase activity (U/gds)	Pectinase activity (U/gds)
E1 = effect of (S-TP)		− 1082	− 20.35
E2 = effect of (S-TP)		1342	15.255
E3 = effect of (S-TP)		− 1106	− 41.175
E4 = effect of (S-TP)		− 311.2	10.1712
E5 = effect of (S-TP)		1462	33.05
E6 = effect of (S-TP)		1084	58.48
E7 = effect of (S-TP)		1062	33.05
E8 = effect of (S-TP)		230.4	− 13.234
Effect of S	Effect of substrate ratios (S)	190	6.36
Effect of T	Effect of temperature (T)	1213	36.86
Effect of P	Effect of pH (P)	− 22	37.112
Effect of ST		1084	− 4.06
Effect of SP		− 129	21.612
Effect of TP		1272	26.7
Changes in mean effect		− 40.4	− 1.53
Standard Deviation		405.39	22.887
Error Limits			
For Averages		± 573.39	± 32.362
For Effects		± 406.60	± 22.95
For Change in mean		± 361.12	± 20.388

Table represents the calculated value of effects for different variables

S substrate ratio, P pH, T Temperature in first round of EVOP method

it a cost effective and less time taking process (Negi and Banerjee 2006; Kumar et al. 2011; Kumari and Negi 2014; Pandey et al. 2016). It is a real time experiment based optimization process in which we perform different combinations of experiments and then calculate the combinatorial effect of different parameters on yield. The chances of error are less since each combination were performed manually and provides interfactorial impact on the yield of the product. The aim of this study was to increase the production of laccase and pectinase enzyme concomitantly which should be cost effective and having high yield in a single step using EVOP-factorial design. The parameters such as substrate ratio, pH and temperature shows significant effect on enzyme activities in "one

Table 5 Experimental setup for optimization using new range (EVOP2)

Parameters	(+)	0	(−)
Substrate	2.25:2.25:0.5	2:2:1	1.75:1.75:1.5
pH	6.5	5.5	4.5
Temperature	34	32	30

Experimental set up for second round of EVOP optimization with new range

at a time method" therefore these variables were further taken into consideration for further optimization using EVOP (Table 2). In this study the substrate ratio (S),

Table 6 Experimental design table (EVOP2)

Exp. parameter	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Substrate (WB+PH+MP)	2:2:1	1.75:1.75:1.5	1.75:1.75:1.5	2.25:2.25:0.5	2.25:2.25:0.5	2:2:1	2.25:2.25:0.5	1.75:1.75:1.5	2.25:2.25:0.5	1.75:1.75:1.5
Temperature	32	30	34	30	34	32	34	30	30	34
pH	5.5	4.5	6.5	6.5	4.5	5.5	6.5	6.5	4.5	4.5
Response	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10

Temperature (T) and pH (P) variables were considered (Table 3). In the first cycle of EVOP after performing the experiments on the basis of decision making process it was found that most of the effects are positive and larger than the error limits and change in mean effect is smaller (Table 4), which shows that to achieve optimum condition either we have to increase the range of variables or new variables and new EVOP should be performed. Hence a new EVOP was performed using a new range (Table 5) of the variables and set up new experimental design shown in Table 6. In the second round of EVOP experiments, all the calculated values for the effects of different variables had smaller magnitude than the error limit and the change in mean effect value were negative and higher than the effects (Table 7). Based on these observations and decision making procedure, it was concluded that the optimum conditions for the coproduction of laccase and pectinase enzymes in single bioreactor were found as: pH 4.5, incubation temperature 34 °C and with substrate containing 1.75:1.75:1.5 (w/w/w) wheat bran: Pulse husk: Mustard Peel ratio. With these optimum conditions, laccase and pectinase activities were enhanced up to 250 U/gds and 247 U/gds, respectively; whereas it was found 78.102 U/gds and 105.013 U/gds at 30 °C for 7 days with wheatbran:Pulsehusk:MustardPeel ratio 2:2:1 (w/w/w) by conventional method. On achieving two–threefold increased enzyme activities, it can be concluded that the Evolutionary operation factorial (EVOP) technique is useful tool to achieve the goal with very less time and efforts by taking in to consideration, the inter factorial impact on output. As the method involves the production of two different enzymes at the same time and in the single reactor system, therefore, it will reduce the cost and time duration of the production at industrial scale.

Conclusion

Evolutionary operation optimization technique (EVOP) was found promising to achieve same range of physico-chemical conditions for optimum yield of both the enzymes. It also provides interfactorial impact of different parameters on yield. Coproduction of more than one enzyme concept would get more preference in industry where more than one enzymes are used simultaneously, for e.g., in wine industries and polymer industries. The results based on present study would be helpful in improving the cost effectiveness and minimize the time of the laccase and pectinase production in a single step for various industrial applications.

Table 7 Effects and limits for experimental (EVOP-2)

Effect of variables		Laccase activity (U/gds)	Pectinase activity (U/gds)
E1 = effect of (S-TP)		− 40	97.12
E2 = effect of (S-TP)		− 168	36.99
E3 = effect of (S-TP)		− 51	159.55
E4 = effect of (S-TP)		− 168.4	− 222.912
E5 = effect of (S-TP)		193.5	315.635
E6 = effect of (S-TP)		26.5	− 54.335
E7 = effect of (S-TP)		− 76.5	− 38.195
E8 = effect of (S-TP)		− 31	− 167.874
Effect of S	Effect of substrate ratios (S)	76.75	206.375
Effect of T	Effect of temperature (T)	− 70.75	8.674
Effect of P	Effect of pH (P)	− 12.75	98.8725
Effect of ST		63.75	60.6775
Effect of SP		97	45.6625
Effect of TP		116.75	109.257
Changes in mean effect		168.5	295.391
Standard deviation		73.35	51.3325
Error Limits			
For averages		± 103.74	± 72.586
For effects		± 173.66	± 251.536
For change in mean		± 65.341	± 45.729

Table represents the calculated value of effects for different variables

S substrate ratio, P pH, T Temperature in second round of EVOP method

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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