Application of Crude Enzymatic Extract In Mushroom Processing To Recover High Value Products

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Abstract

In the present study, a fungal strain was isolated from mushroom waste dump-site and was described based on the morphological and molecular characteristics. The crude enzymatic extract was prepared by fermenting pineapple peels using the newly isolated fungal strain under solid-state condition. The enzymatic saccharification conditions of mushroom were optimized using the central composite design based on the response surface methodology. The isolate had black colony color, conidial head biseriate and small conidia which are synonymous with *Aspergillus niger*. The phylogenetic analysis using the rDNA ITS sequencing further revealed that the isolate was identical (≥99%) to *A. niger.* The crude extract displayed CMCase, Fpase and xylanase activities of 20.73U/mL, 34.57U/mL and 118.03U/mL respectively. The saccharification using the crude extract at optimal conditions of pH 6.5, temperature 50^{O}C, enzyme loading of 5% (v/v) and time of 12h achieved maximum glucose yield of 1.639 mg mL^{-1} which is 1.1 folds higher than the predicted value. This study demonstrated the potential use of crude enzymatic extract from the newly isolated *A. niger* as a viable and efficient low-cost approach to mushroom processing using enzymes.

Introduction

Edible mushrooms are food items not only rich in nutrients but also other health promoting compounds. Traditionally, mushrooms were eaten for a various reasons including status in the society, nutritional value, taste and aroma and as well as for the treatment of various illnesses (Kotowski 2019). Currently, mushrooms have received widespread application in the production of conventional medicine because of biologically active substances that they possess (Gasecka et al. 2017). These compounds include hemicelluloses, polysaccharides, lipopolysaccharides, peptides, proteins, glycoproteins, nucleosides, triterpenoids, complex starches, lectins, and lipid derivatives (Kalač 2009). In addition, mushrooms have high content of alkaloids, saponins, flavonoids, tannins, sterols, triterpenes, coumarins and cyanogenic glycosides (Lindequist et al. 2005). These compounds have potent immunomodulatory, anticancer, antiproliferative, anti-inflammatory, antiviral, hypotensive and antithrombotic activities (Cheung 2008).

The choice of extraction techniques greatly affects the quality of biologically active compounds from plant or microbial sources including mushrooms, and to that extends, affects their specific applications. The conventional extraction techniques of bioactive compounds have involved the use of highly toxic and inflammable solvents which have adverse effects not only in the environment but also on the quality of the final products (Max et al. 2015). Enzyme-based extraction technique has been recently developed and is considered as a viable alternative to the solvent-based extraction methods (Puri et al. 2012). The increasing popularity of enzymes in industrial processing is due to the fact that it does not cause adverse effect on the environment and the quality of the final product. However, despite many advantages, low product recovery, long extraction period and high cost of commercial enzymes are some of the major bottlenecks associated with enzyme-based processing (Arnau et al. 2019). In addition, it has not been possible to find microbial strain that meets all the production processes.

In an attempt to improve the commercial viability of enzymes in industrial processing, the use of crude enzymatic extracts from the fermentation of low-cost agro-wastes is increasingly being considered (Dale 1999). This is because crude extracts from the fermented agricultural wastes contain enzyme mixtures capable of hydrolyzing lignocellulosic biomass (Gabriela et al. 2015). In fact, in certain instances, crude enzymatic extracts have proved to be more competitive than their commercial counterparts (Griggs et al. 2012). However, the reaction parameters need to be optimized in order to achieve high efficiency of hydrolysis using crude enzyme extracts. The traditional one-variable-at-a-time method has widely been used in many optimization processes; however, it has certain limitations including need for a number of experiments to be performed which is also time consuming (Singh et al. 2011). A response surface methodology is a mathematical and statistical tool which has the capability of modeling reaction parameters to achieve maximum response (Geiger 2014).

In the present study, a fungal strain was isolated from the mushroom waste dump-site and was characterized using cultural, morphological and molecular markers. The crude enzyme extract obtained by the solid state fermentation of pineapple peels was then used to hydrolyze mushroom biomass into glucose. The central composite design based on the response surface methodology was used to obtain optimal reaction condition for maximum glucose release from mushroom saccharification using crude enzyme extract.

Materials And Methods

Sampling

The soil sample was obtained from a mushroom waste dumpsite, near a market place in Nairobi, Kenya. The sterile mushroom-agar media was inoculated with the sampled soil and was incubated at 30^{o}C for 7 days. Several fungal colonies were formed on the medium; however, only a fungal colony with the largest diameter was selected and sub-cultured on fresh Potato Dextrose Agar media. The pure culture of the selected isolate was stored at 20^{o}C for future use.

Strain characterization

Morphological characterization

The fungal isolate was characterized based on the cultural and morphologies features including colony color, appearance, texture, and colony surface (Klich 2002, Samson et al 2007). Microscopic features including conidial shape, vesicle shape, vesicle arrangement, and appearance of the medulla were also examined by a light microscope after staining with lactophenol cotton blue.

Phylogenetic analysis

The genomic DNA was isolated and amplified using methods discussed in Otieno et al. (2015).

Phylogenetic analysis was performed using MEGA® software version 7. Multiple alignment of sequences and calculations of levels of sequence similarity were carried out by using ClustalW algorithm. A phylogenetic tree was built by calculating distance matrices for Maximum-Likelihood analysis with the Tamura 3-parameter model and bootstrapping analysis with 500 replicates to test the robustness of the internal branches for closely related strains (Kumar et al, 2018).

Physicochemical characterization

The ability of the isolate to grow in media with different pH levels, temperature values and salt (NaCl) concentrations was evaluated. For optimum pH elucidation, media was prepared using buffer solutions of pH ranging from 3 to 13. Temperature regime was evaluated by incubating the isolate at a temperature range of between 15^{o}C and 60^{o}C. Tolerance to [Na] $^{+}$ was assessed by growing the isolate in media with NaCl concentrations ranging from 0-15% w/v.

Solid-state fermentation of pineapple peels

The dry pineapple peels powder (10g) was put into 250mL Erlenmeyer flask and moistened with basal salt solution and then was autoclaved at 121^{o}C for 30 min. The sterile powder was inoculated with 1 mL of spore suspension (~1.0x10^{7} spores/mL) and then was incubated at 30^{o}C. After 7 days, the substrate bed was suspended in 50mM phosphate buffer, pH 6.5 and agitated for 1h at 200rpm in an orbital shaker (Gerhardt, GmbH, Germany). The mixture was centrifuged at 5000rpm for 10 min at 4∘C and the supernatant was stored at 20^{o}C for future use. The total protein content of the supernatant was estimated by the method of Lowry et al. (1951) using BSA as a standard.

Enzyme assay

To determine CMCase activity, 1% (w/v) of CMC solution (prepared using crude enzymatic extract, pH 6.0) was incubated at 50^{o}C for 30 min. Similarly, xylanase activity was determined by incubating 1% (w/v) of xylan solution (prepared using crude enzymatic extract, pH 6.0). Fpase activity was determined by adding small pieces (~50mg) of Whatman filter paper no.1 to crude enzymatic extract. The mixtures were incubated at 50^{o}C for 60min (Miller 1959). Similarly, the xylose content of the Beechwood xylan degradation was determined. The standard graphs were prepared using 0–500 μg of glucose for CMC and filter paper degradation. The standard graph of 0–500 µg xylose was used for xylan degradation. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar equivalent per minute under the specified assay conditions.

Reaction parameters for mushroom saccharification

Initial screening of single parameters on enzymatic saccharification of mushroom (*Pleurotus ostreatus*) was conducted using one variable at a time method (data not shown). Higher yields of glucose were recorded between pH 4.5 and pH 6.5, temperature range of 30–60^{o}C, enzyme loading range of 1–5%, w/v and incubation time of 12h and 60 h. One variable at a time was then optimized by employing response surface methodology based on central composite design to achieve maximum response values.

The range of enzyme loading, reaction pH, incubation time and temperature was determined based on the preliminary experiments shown in Table 1. For each experiment, 1% mushroom powder solution (prepared in phosphate buffer) was mixed with different volumes of crude enzyme extract (1 to 5mL). The pH of the mixtures was varied from pH 4.5 to pH 6.5 and then incubated at different temperatures (30 to 50 ∘C). Aliquots were removed after every 12h and analyzed for changes in glucose concentration using DNS method (Miller 1959).

Experimental design and data analysis

Response surface methodology was employed in determining the optimum reaction parameters for the enzymatic saccharification of mushroom biomass. After determining the initial range of the reaction parameters, a five-level, four-factor CCD with 31 experiments was used in this study (Table 1). The Minitab® software 17 was used to perform experimental design and statistical analysis. The key independent variables optimized were: reaction pH(x_1), incubation time(x_2), reaction temperature (x_3) and enzyme loading(x_4). The design had 16 factorial points, eight axial points, and seven replicates of the center point. The 31 runs were randomized and the response (glucose yield) was recorded in Table 2. The CCD data was analyzed by multiple regression to fit the quadratic polynomial model. The analysis of variance and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined as shown in Table 3. Significant levels were determined at p-values ≤ 0.05. The model was verified and validated by running confirmatory experiment in triplicate using the optimum reaction conditions generated by the RSM. The experimental and predicted values were compared and tested for statistical differences.

Results

Strain characteristics

Morphological characteristics

The culture morphology of the isolate is illustrated in Figure 1.

The fungal isolate exhibited colony color black, reverse colony yellow, colony appearance spread spores, colony texture fluffy, colony surface smooth; conidial head present; vesicle shape ovoid; vesicle arrangement biseriate; the appearance of metula entirely covering medulla; medulla shape oval; exudate absent; decumbent vegetative hyphae thin-walled, hyaline. The culture of the fungal isolate has been deposited at the NRRL Microbial Culture Collection under the voucher number *Aspergillus niger* NRRL 61452.

Phylogenetic relationships

The isolate had an ITS rDNA sequence length of 572 base pair. The BLAST search against the NCBI database revealed >99% maximum homology of the fungal isolate with the GenBank *Aspergillus niger*. The matrix for this analysis had 562 characters, 560 characters were conserved and only 2 were variable

while none was phylogenetically informative. The phylogenetic relationship of this isolate with the GenBank homologs is shown in Figure 2. The sequence was deposited in GenBank under the accession number: MW237706. The isolated fungal strain was designated as *A.niger* KWM.

Bootstrap support of branches indicated on the node was obtained using 500 replicates. Only statistically significant bootstrap values (≧50%) are indicated. Branch lengths are indicated as 0.01 substitutions per positions according to the scale bar underneath the tree. Number in parentheses denotes GenBank accession numbers.

Physico-chemical characteristics

The isolate exhibited good growth at temperature range of 25-30^{o}C with optimal growth recorded at 30^{o}C; however, extreme temperatures of ≦ 20^{o}C or ≧40^{o}C highly restricted growth. Growth was recorded in media with pH range of 5 and 11 with the optimal growth being exhibited at pH 6 to 8; the growth was greatly limited at extreme pH≤4 or ≧ 12. Similarly, good growth rate was exhibited at 0-10% (w/v) NaCl concentration; best growth was recorded at 3-5 % (w/v) NaCl concentration. The growth was slow between 6-10 % (w/v) salt concentrations for the first 5 days, but then highly improved after 7 days of growth. However, growth was extremely restricted beyond10% (w/v) NaCl concentration.

Enzyme activities

The crude extract was obtained from solid-state fermentation of pineapple peels by the fungal isolate. The enzyme activities of 20.73U/mL, 54.57U/mL and 118.03U/mL for CMCase, Fpase and xylanase respectively were displayed in the crude enzymatic preparations.

Statistical analysis and model fitting

In the present study, a total of 31 runs were employed for optimizing the four independent variables. The CCD and their coded, experimental and predicted values are shown in Table 2. The results showed that the glucose yield ranged from 0.00 to 1.046 mg mL^{-1}. The experimental maximum glucose yield (1.046 mg mL^{-1}) was found in conditions of pH 5.5, temperature 40^{o}C, time 36h and enzyme loading 5%. The model predicted maximum glucose yield of 0.934mg/mL in conditions of pH 6.0, temperature 45^{o}C, enzyme loading 4% (v/v) and time 48h. The results were fitted with a second-order polynomial model described in equation 1:

$$Y_{(\text{Glucose yield})} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 \quad (1)$$

Where Y is the measured response; β_0 is the intercept term; β_1, β2, β_3, and β_4 are linear coefficients; β_{11}, β_{22}, β_{33}, and β_{44} are quadratic coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are interaction coefficients; and x_1, x_2, x_3, and x_4 represent the independent variables: temperature, pH, time and enzyme loading respectively.

Table 2 Matrix of CCD for evaluation of effect of independent variables on the glucose yield

The model expressed by equation (2) represents glucose yield (Y) as a function of pH (X_1), time (X_2), temperature (X_3), and enzyme loading (X_4).

$$Y_{(Glucose\ yield)} = 0.367714 + 0.044625x_1 + 0.062208x_2 + 0.117292x_3 + 0.147958x_4 - 0.041085x_1^2 - 0.019710x_2^2 + 0.022290x_3^2 + 0.081415x_4^2 - .028438x_1x_2 + 0.025438x_1x_3 + 0.012062x_1x_4 - 0.005563x_2x_3 + 0.052563x_2x_4 - 0.094938x_3x_4 \quad (2)$$

The statistical significance of the regression model was evaluated by the *P*-value and F-test, and the ANOVA for the response surface quadratic model (Table 3). The coefficient of determination was (R^2) was 0.87. The linear and quadratic terms in second order polynomial model (Eq. 2) were highly significant ($p<0.01$). The *t* and *p*-values for linear, quadratic and combined effects of the variables are given in Table 4. The time, temperature, pH and enzyme loading were linear variables which had significant ($p<0.05$) positive effects on mushroom hydrolysis. With regard to the quadratic terms, the medium pH and reaction time had negative effect on the glucose levels obtained. The temperature and enzyme loading positively affected mushroom hydrolysis. The effect of enzyme loading on glucose yield was significant ($p < 0.05$).

Table 3 Analysis of variance of second order polynomial model for optimization of enzymatic saccharification of mushroom.

Analysis of response surface plots

Three-dimensional contour plots were used to visualize the interaction effect of the dependent and independent variables on the response while keeping the other two factors constant (Fig. 2a-f). Figure 2a depicts increase in glucose yield (0 -1.50mg/mL) with increasing enzyme loading (1-5.0% v/v) and temperature (30-50^oC) while keeping time and pH constant at 36h and 5.5 respectively. Figure 2b shows effect of enzyme loading and incubation time on the glucose yield at a constant temperature of 40^oC and pH 5.5. Under these conditions, the glucose range of 1.00 to1.25mg/mL was reported with increase in incubation time and enzyme loading. Figure 2c shows interaction effect of the temperature and incubation time at constant pH and enzyme loading of 5.5 and 3% (v/v) respectively. The glucose yield increased with increase in reaction time from 12 to 60h and temperature from 30-50^oC. Figure 2d describes the interaction between pH and enzyme loading at a constant reaction time (36h) and temperature (40^oC). Increase in enzyme loading and pH resulted in increase in glucose yield. Figure 2e shows interaction effect of temperature and pH on the glucose yield. An increase in glucose yield was recorded by the increase in pH and temperature. However, further increase in pH and temperature did not cause any change in glucose yield. Figure 2f depicts interaction effect of incubation time and pH at constant temperature (40^oC) and enzyme loading (3%, v/v). Glucose yield increased with increase in incubation time and pH.

The validity of the model equation for predicting the optimum response value was evaluated under the following optimal model conditions: pH 6.5, temperature 50 ∘C, time 12h and enzyme loading 5% (v/v). This set of optimum conditions was determined by a response optimizer (Figure 3) and was used to validate the experimental and predicted yields of the responses using the model equation. A mean value of 1.639 ±0.04 mg mL^{-1} (n = 3) was obtained from the experiment which is 1.1 folds higher than the predicted value.

Discussion

The identity of the fungal isolate was determined based on the cultural and morphological characteristics (Figure 1). The black colony color, conidial head biseriate and small conidia 2.7-3.5µm exhibited by this isolate are typical of *Aspergillus niger* as reported previously by Klich (2002) and Samson et al. (2007). The phylogenetic analysis using rDNA ITS sequences and the BLAST search against the GenBank reference strains revealed that the isolate was a close relative of *Aspergillus* species with the closest sister being the *Aspergillus niger* (MT620753). Li et al. (2021) had reported the applications of *A. niger* (MT620753) in rice wine saccharification. However, despite being 99% identical to the GenBank *Aspergillus niger* strains, our isolate separated distinctively from her closely related GenBank strains (Figure 2) suggesting that the isolate could probably have underwent unique evolutionary events in the recent past.

The physicochemical characteristics of the isolate were evaluated on media with different pH values, NaCl concentrations and temperature levels. The fungal isolate showed good growth in pH range of 4-12, temperature 25-30^{o}C and 0-12% (w/v) NaCl concentrations. The wide ambient pH and temperatures exhibited by the isolate indicated that it was a strong haloalkalitolerant strain of *A. niger*, it could as well be that the strain was under transition in alkaline soil since it was also able to grow well in neutral media. The alkaliphiles have been reported to have the ability to survive under salt concentrations ≥5% w/v and high pH ≥9 (Grum-Grzhimaylo et al. 2016).

Pineapple peels are produced in high quantities across many fruit processing factories worldwide and their disposal still remain a great challenge. Their utilization as raw materials for the production of value added products such as enzymes may be a welcome move by the bioprocessing companies. In the present study, the fermentation of pineapple peels proved to be a viable cost-effective strategy of producing enzymes with widespread industrial applications. Crude enzymatic extract displayed strong cellulolytic and xylanolytic activities of 20.73U/mL, 34.57U/mL and 118.03U/mL for CMCase, Fpase and xylanase respectively. The cellulolytic and xylanolytic activity of crude extract from this isolate was higher than most filamentous fungi reported in the literature (Jampala et al. 2017). Similarly, xylanase and CMCase activities of 91.9 U/mL and 5.61 U/mL respectively were reported in fermentation broth of barley straw. In this study, the pineapple peels substrates must have influenced the high enzyme activities displayed in the fermentation broth. Elisashvili et al (2008) had previously reported that the growth substrate have great influence on the production enzyme production and activities.

The response surface methodology was used to develop the experimental design for evaluating the optimum conditions and the interaction effects of the process parameters on the response. The probability (*p*) F<0.05, the *F*-value = 7.39 and a low *p*-value (p=0.000) suggested that the model terms were significant. The coefficient of determination (R^2 =0.87) indicated that the experimental and predicted values were in good agreement, and that the model can well be used to predict process performance and optimization. The lack-of-fit (F-value of 1.1) for regression of Eq. 2 was not significant (*p*-value=0.475). Non-significant lack-of-fit is a good proof that the model equation is adequate to predict the response under any combination of values of the variables. Non-interactive effect of the variables ($p > 0.05$) on mushroom hydrolysis (Table 3) implies that these variables had additive effects on mushroom hydrolysis. Similar results had been reported by de Almeida et al. (2016) where non-interactive effect of reaction variables resulted in additive effects on the enzymatic saccharification of pineapple peels.

The effect of independent variables and their interaction on mushroom saccharification were visualized using three dimensional response surface plots (Figure 3). The interaction effect of temperature, incubation time, pH and enzyme loading had influence on glucose yield. In a similar study, Sattler et al. (1989) reported 31% increase in glucose yield from bioconversion of cellulose from pretreated poplar wood with increase in the enzyme loading and temperature. Pan et al. (2005), Manonmani and Sreekantiah (1987) and Kaur et al. (1998) also noted that the increase in temperature and enzyme loading up to the optimum levels favored the enzymatic hydrolysis of cellulose from softwood, sugarcane bagasse and rice straw respectively. However, the temperature and enzyme load required to achieve a complete conversion of cellulose into glucose vary with raw material used. Increasing enzyme loading beyond optimum level resulted in minimal or no increase in glucose yield because all the glycosidic bonds available for hydrolysis may have been exhausted. Saini et al. (2013) reported that the hydrolysis of the enzyme-susceptible cellulose linkages occur simultaneously when enzyme is absorbed into the suspended substrate particles. Increasing temperature beyond 50^{o}C resulted in a decrease in glucose yield. This is in agreement with the study by Daniel and Danson (2013) that reported enzyme inactivation at elevated temperature due to thermal inactivation. Variation in pH of a reaction mixture beyond optimal pH value negatively affected mushroom hydrolysis and glucose yield. Reactions at pH and temperatures beyond optimum levels of pH 5.5 and temperatures of 50^{o}C respectively affect enzyme activity and conformation (Althuri and Banerjee 2016). This corresponded well with the present study where temperature of 50^{o}C and pH 6.5 displayed maximum glucose yield.

A response optimizer (Figure 4) was used to create optimum conditions of temperature 50^{o}C, incubation time of 12h, pH 6.5 and enzyme loading of 5% (v/v) with predicted yield of 1.494 mg mL^{-1}. A validation experiment under the optimal model conditions produced 1.639 ±0.04 mg mL^{-1} of glucose after saccharification, which is 1.1 folds higher than the predicted value; suggesting that the predicted and experimental response values are in good agreement. This further validates the RSM model, showing that the model was adequate for the optimization of enzymatic saccharification of mushroom experiments. The reported glucose yield (1.639 ±0.04 mg mL^{-1}) in the present study is above glucose yields obtained from *Pleurotus* species previously reported (Sławińska et al. 2020, Zhou et al. 2912). This could have

resulted from the efficient hydrolysis of mushroom biomass and the presence of adequate balance between different enzymes in the crude extract. Van Dyk and Pletschke (2012) noted that biomass degradation is a function of a balanced enzyme proportions that act in synergy to breakdown the complex structure of the lignocellulose. The accessory enzymes that are important in mushroom cell-wall degradation include glucanases, chitinases and proteases (Harman et al. 2004, Kubicek et al. 2014). These enzymes are also important during mushroom pathogenesis by the pathogenic fungi such as *Trichoderma harziunum* (Wang et al. 2016). The extract contains antifungal properties that may be employed in the management of many fungal related complications.

Enzymes have been used in mushroom processing to recover high value products. Banjongsinsiri et al. (2016) used commercial bromelain and papain enzymes to enhance recovery of protein from mushroom biomass. Similarly, Poojary et al (2017) digested mushroom biomass with commercial enzymes to recover amino acids responsible for umami taste. However, for a long time, the cost of commercial enzymes has remained a major bottleneck in industrial bioprocesses. Increasing research into the applications of the crude enzyme extracts in the bioconversion processes is being driven majorly by the need to make enzyme-based processing more competitive. Mahamud and Gomes (2012) applied crude enzymes in saccharification of sugarcane bagasse for the production of bioethanol production; the crude enzyme extract of *Trichoderma* sp. displayed CMCase, Fpase and xylanase activities of 0.977, 0.110 and 9.280 U/ml which had overall degree of saccharification 45.71%. Similarly, Kumar and Sharma (2012) used crude enzyme extract in juice clarification. In the same study, the crude enzymatic extract was more competitive compared to commercial enzymes and the combination of crude and commercial enzymes produced even better results. However, despite potential of crude enzymes as a viable approach to lowering the cost of bioprocessing, limited or probably no study is available on its application in mushroom processing. This study therefore provides baseline information necessary for the application of crude enzymatic extracts in mushroom processing.

Declarations

Acknowledgement

Not applicable

Authors Contribution

ODO and MFJ conceived and planned the project. ODO, MFJ, OG and MJ designed the project. ODO implemented the study. MFJ, OG and MJ guided implementation of this study. All authors read, edited and approved the final manuscript.

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Tables

Table 1

Independent variables	Symbol	Coded levels				
		-2	-1	0	1	2
pH	X_1	4.5	5.0	5.5	6.0	6.5
Time (h)	X_2	12	24	36	48	60
Temperature (^oC)	X_3	30	35	40	45	50
Enzyme loading (%v/v)	X_4	1	2	3	4	5

Table 2 Matrix of CCD for evaluation of effect of independent variables on the glucose yield

Std Order	X_1	X_2	X_3	X_4	Glucose yield(mg/mL)	
					Experimental	Predicted
1	5.0(-1)	24(-1)	35(-1)	2(-1)	0.203	0.190
2	6.0(1)	24(-1)	35(-1)	2(-1)	0.154	0.261
3	5.0(-1)	48(1)	35(-1)	2(-1)	0.233	0.277
4	6.0(1)	48(1)	35(-1)	2(-1)	0.202	0.234
5	5.0(-1)	24(-1)	45(1)	2(-1)	0.023	0.195
6	6.0(1)	24(-1)	45(1)	2(-1)	0.421	0.367
7	5.0(-1)	48(1)	45(1)	2(-1)	0.321	0.260
8	6.0(1)	48(1)	45(1)	2(-1)	0.345	0.319
9	5.0(-1)	24(-1)	35(-1)	4(1)	0.011	0.166
10	6.0(1)	24(-1)	35(-1)	4(1)	0.249	0.286
11	5.0(-1)	48(1)	35(-1)	4(1)	0.435	0.464
12	6.0(1)	48(1)	35(-1)	4(1)	0.512	0.470
13	5.0(-1)	24(-1)	35(-1)	4(1)	0.608	0.551
14	6.0(1)	24(-1)	45(1)	4(1)	0.687	0.772
15	5.0(-1)	48(1)	45(1)	4(1)	0.804	0.826
16	6.0(1)	48(1)	45(1)	4(1)	0.945	0.934
17	6.5(-2)	36(0)	45(1)	3(0)	0.207	0.114
18	7.0(2)	36(0)	40(0)	3(0)	0.304	0.292
19	5.5(0)	12(-2)	40(0)	3(0)	0.328	0.164
20	5.5(0)	60(2)	40(0)	3(0)	0.354	0.413
21	5.5(0)	36(0)	30(-2)	3(0)	0.344	0.222
22	5.5(0)	36(0)	50(2)	3(0)	0.674	0.691
23	5.5(0)	36(0)	40(0)	1(-2)	0.445	0.397
24	5.5(0)	36(0)	40(0)	5(2)	1.046	0.368
25	5.5(0)	36(0)	40(0)	3(0)	0.433	0.368
26	5.5(0)	36(0)	40(0)	3(0)	0.365	0.368
27	5.5(0)	36(0)	40(0)	3(0)	0.387	0.368

28	5.5(0)	36(0)	40(0)	3(0)	0.398	0.368
29	5.5(0)	36(0)	40(0)	3(0)	0.125	0.368
30	5.5(0)	36(0)	40(0)	3(0)	0.366	0.368
31	5.5(0)	36(0)	40(0)	3(0)	0.500	0.368

Table 3 Analysis of variance of second order polynomial model for optimization of enzymatic saccharification of mushroom.

Source	DF	Seq SS	Adj. MS	F-value	*p*-value
Regression	14	1.50048	0.10718	7.39	0.000
Linear	4	0.99625	0.24906	17.17	0.000
Square	4	0.28970	0.07243	4.99	0.008
Interaction	6	0.21453	0.03576	2.46	0.070
Residual Error	16	0.23210	0.01451		
Lack-of-Fit	10	0.15013	0.01501	1.10	0.475
Pure Error	6	0.08197	0.01366		
Total	30	1.73258			

DF-degree of freedom; Seq SS-sequential sum of squares; Adj. MS-adjusted mean squares;

Table 4

Terms	Coef	SE Coef	t-value	p-value
β_0	0.367714	0.04552	8.078	0.000
β_1	0.044625	0.02458	1.815	0.088
β_2	0.0662208	0.02458	2.530	0.022
β_3	0.117292	0.02458	4.771	0.000
β_4	0.147958	0.02458	6.018	0.000
β_{11}	-0.041085	0.02252	-0.824	0.087
β_{22}	-0.019710	0.02252	-0.875	0.394
β_{33}	0.022290	0.02252	0.990	0.337
β_{44}	0.081415	0.02252	3.615	0.002
β_{12}	-0.028438	0.03011	-0.944	0.359
β_{13}	0.025438	0.03011	0.845	0.411
β_{14}	0.012062	0.03011	0.401	0.694
β_{23}	-0.005563	0.03011	-0.185	0.856
β_{24}	0.052563	0.03011	1.746	0.100
β_{34}	0.094938	0.03011	3.153	0.006

Coef-coefficient; SE Coef-standard error coefficient

Figures

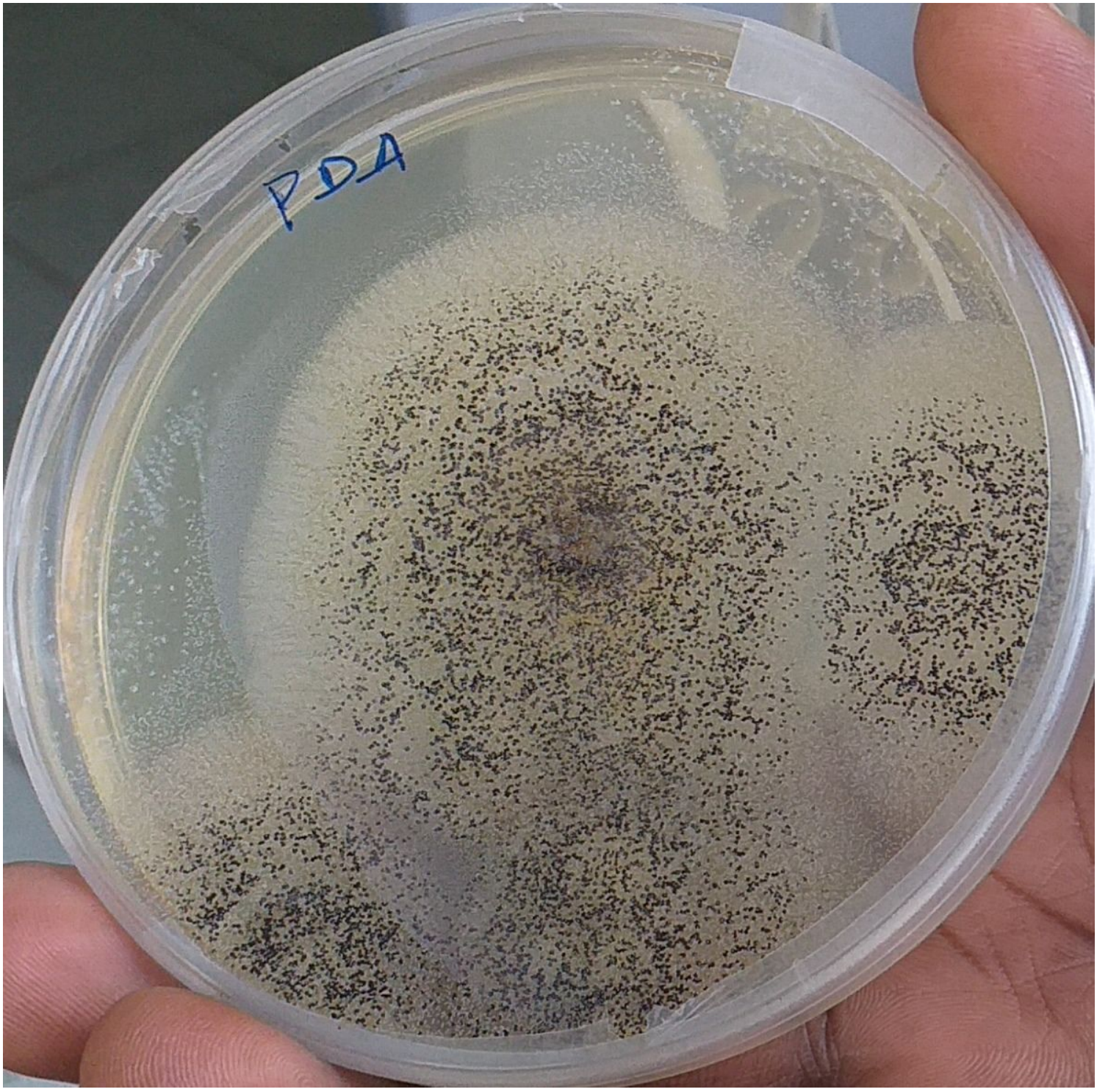

Figure 1

Cultural and morphological characteristics of the fungal isolate on PDA media

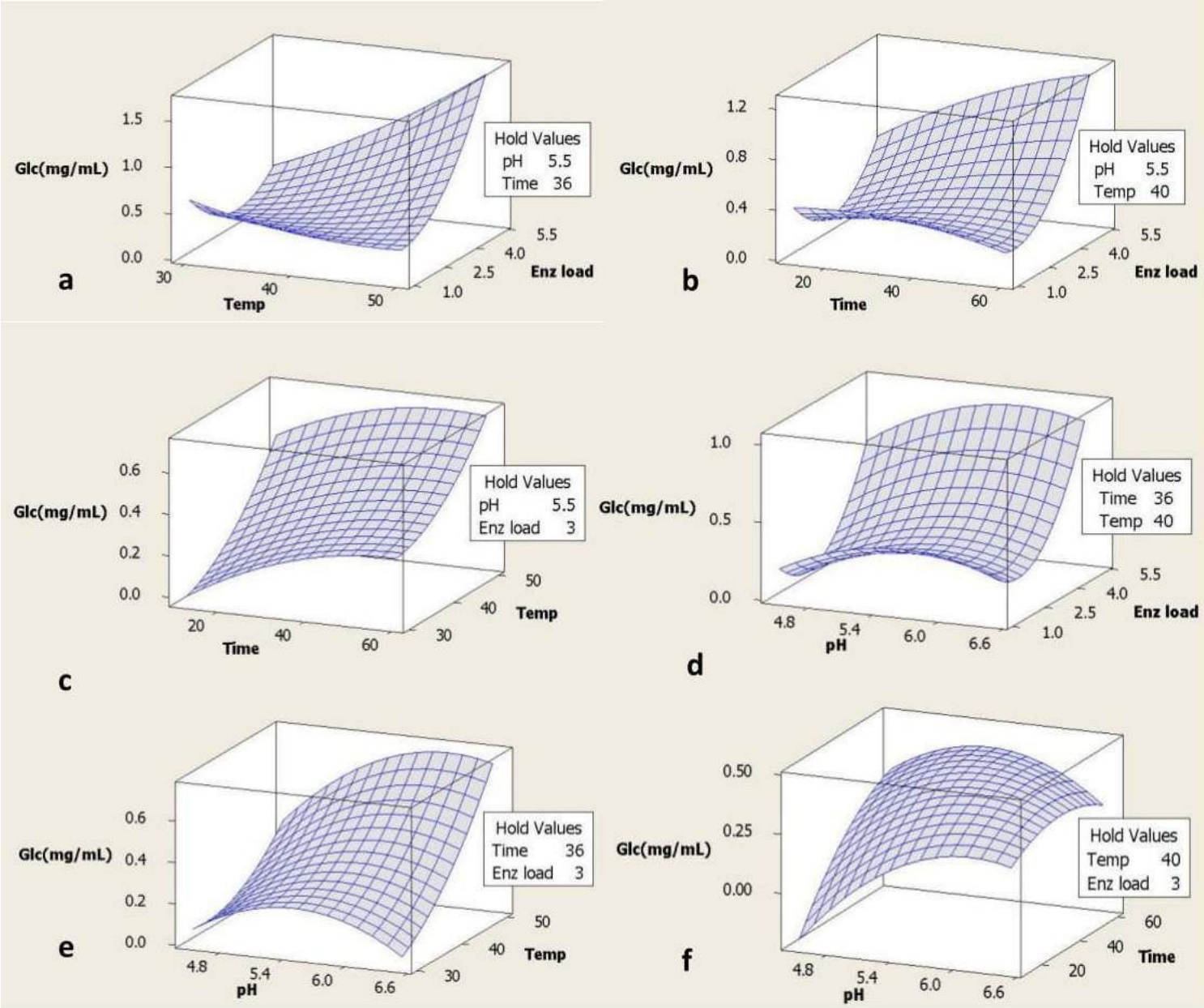

Figure 2

Effect of reaction variables on glucose yield during mushroom saccharification using crude enzymatic extract.

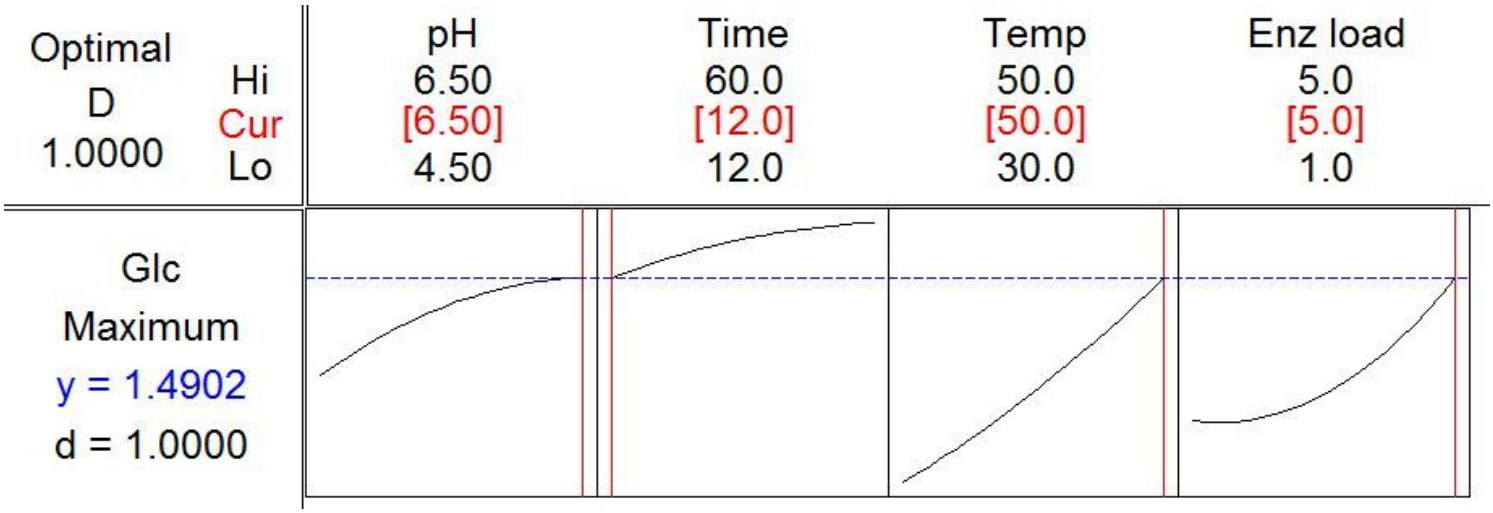

Figure 3

Optimization plot for enzymatic saccharification of mushroom

Image not available with this version

Figure 4

A response optimizer (Figure 4) was used to create optimum conditions of temperature 50oC, incubation time of 12h, pH 6.5 and enzyme loading of 5% (v/v) with predicted yield of 1.494 mg mL−1