#### Heliyon 8 (2022) e11312

Contents lists available at ScienceDirect

### Heliyon

journal homepage: www.cell.com/heliyon

**Research article** 

# Strategies for improving hydrolytic efficiency of crude multienzyme extracts in mushroom processing



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#### ARTICLE INFO

Keywords: Bioprocessing Crude enzymes Mushrooms Response surface methodology

#### ABSTRACT

The current study investigated and optimized key process parameters affecting mushroom hydrolysis with crude enzymatic extract. The crude enzyme was prepared by solid-state fermentation of pineapple peels using *Aspergillus niger*. The reaction parameters viz. time, temperature, pH and enzyme concentration were optimized using the central composite design of the response surface methodology. The model predicted glucose yield of 1.49 mg/mL at optimal pH of 6.5, temperature of 50 °C, enzyme loading of 5 % (v/v), and reaction time of 12 h. Mushroom hydrolysis at the same optimal model conditions, increased glucose yield by 10%. More so supplementing SSF media with 0.2% (w/v) Tween-80 and 0.08% (w/v) yeast extract at moisture level of 70–75% significantly (*p* value < 0.05) improved hydrolytic efficiency of the crude enzyme extract by 2.2-fold. This study provides baseline data that will be useful in developing a low-cost enzyme-based process for hydrolyzing mushrooms to recover high-value products.

#### 1. Introduction

Mushrooms are diverse in nature, with over 120,000 species identified and an estimated 3.8 million species still unidentified (Hawksworth and Lücking, 2017; Otieno et al., 2015). Demand for edible mushrooms has recently increased because they are not only nutritious but also have numerous health benefits. Traditionally, eating mushrooms was a sign of social status, however, today, most people eat mushrooms for their health-promoting properties (Buller, 1915; Otieno et al., 2022). Numerous biologically active compounds have been discovered in mushrooms, including bioactive proteins, triterpenes/triterpenoids, and polysaccharides such as glucans, ganoderan etc. (Gasecka et al., 2017; Kalač, 2009; Kotowski, 2019; Lindequist et al., 2005). These compounds have potent anticancer, antiproliferative, anti-inflammatory, antiviral, hypotensive, and antithrombotic properties (Cheung, 2008; Roupas et al., 2012). Consequently, incorporating mushrooms or their extracts into other food products would be a viable strategy to improve their functional properties. It is thus necessary to ensure that the mushroom processing methods are appropriate to maintain the bioactivity of the target extracts.

The extraction techniques used have a significant effect on the functional properties of plant or microbial extracts. Solvent-based extraction techniques remain popular because they are less expensive, take less time, and yield more product. However, their use in many extraction processes is discouraged due to the highly toxic and flammable solvents used, as well as the negative impact on the environment and quality of the final product (Max et al., 2015). Consequently, extraction techniques that are more environmentally friendly and have no negative impact on the quality of the final product were developed (Puri et al., 2012). An enzyme-based technique is one of many environmentally safe techniques that have gained popularity in the natural products industry in recent years, more so because the functional properties of the extracts are unaffected. As a result, enzyme-based techniques are now used to manufacture over 500 high-value industrial products (Johannes and Zhao, 2006; Kumar and Singh, 2013).

Microbial enzymes play a critical role in the development of biochemical processes. Today, enzymes such as proteases, lipases, amylases, oxidases, peroxidases, and cellulases already have applications in detergent industry (Kirk et al., 2002), laccases in textile processing (Araujo et al., 2008; Tzanov et al., 2001), lipases, xylanases and laccases in pulp and paper industry, and cellulases in the production of bioenergy (Rubin, 2008; Wilson, 2009; Zhang, 2011). In addition, phytases, proteases,  $\alpha$ -galactosidases, glucanases, xylanases,  $\alpha$ -amylases, and poly-galacturonases are used in animal feed industry (Pariza and Cook, 2010),

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https://doi.org/10.1016/j.heliyon.2022.e11312

Received 23 May 2022; Received in revised form 24 June 2022; Accepted 26 October 2022



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and lipases in fruit juice processing, baking, and vegetable fermentations (Mendez and Salas, 2001; Okanishi et al., 1996). More applications of enzymes are in dairy industry, whereas proteases are used in cheese production (Bodie et al., 1994; Pariza and Johnson, 2001), pharmaceutical industry where enzymes are used to prepare chiral medicines and esterases, lipases, proteases, and KREDs (ketoreductases) used in the synthesis of chiral alcohols, carboxylic acids, amines, and epoxides (Kirk et al., 2002; Kirchner et al., 1985; Zheng and Xu, 2011). However, there are currently very few studies in which crude enzymes have been used in food processing. Mahamud and Gomes (2012) used crude enzymes in the saccharification of sugarcane bagasse to produce low-cost bioethanol, and Kumar and Sharma (2012) clarified fruit juice with crude enzyme extract. In fact crude enzyme extracts outperformed commercial enzymes in the latter study, and the combination of crude and commercial enzymes produced even better results.

Despite the many benefits of enzyme-based extraction techniques, bottlenecks such as low product recovery, a long extraction period, and the high cost of commercial enzymes, limit the use of enzymes at industrial scale (Arnau et al., 2020). As a result, crude enzyme extract from low-cost agro-wastes is gradually gaining traction in many bioprocesses as alternative to the high cost of commercial enzymes (Dale, 1999). In addition, crude enzyme extracts contain enzyme sets capable of hydrolyzing any lignocellulosic biomass (Gabriela et al., 2015). In fact, crude enzyme extracts have been shown to be more competitive than commercial enzymes in the hydrolysis of lignocellulosic biomass in some cases (Kumar and Sharma, 2012). However, the most likely limitations of using crude enzyme extract in mushroom processing would be the inhibitory effect of mushroom phytochemicals on enzyme activities, resulting in low catalytic efficiency, decreased product titer, and a lengthy extraction period. The objective of the current study was to investigate and optimize key reaction parameters that affect mushroom hydrolysis with crude enzyme extract.

#### 2. Materials and methods

#### 2.1. Fruit wastes and microbial strains used in this study

Fruit processing wastes i.e. mango peels, pineapple peels, passion fruit peels, and orange peels were obtained from the local fruit vendor in Nairobi, Kenya. The pure culture of *Aspergillus niger* NRRL\_61452 was obtained from the microbial culture collection, Department of Biochemistry, University of Nairobi, Nairobi, Kenya. The fungal culture was sub cultured on fresh PDA for 5 days at 30 °C. The fruiting body of fresh *Pleurotus ostreatus* mushroom was obtained from the Food Research Division, Kenya Industrial Research and Development Institute, Nairobi, Kenya.

#### 2.2. Preparation of crude enzyme extracts

The ability of A. niger to produce mushroom hydrolyzing enzymes under solid-state fermentation of fruit waste materials (mango peels, pineapple peels, passion fruit peels, and orange peels) was evaluated. These wastes were chosen because they are abundant and readily available. Fresh peels were washed with clean water and dried to constant weight. Dried peels were then milled into fine powder (mesh size  $\sim$ 32) using laboratory blender (Sumeet Inc. Mumbai, India). Ten grams of each powdered substrate were placed in separate 250 mL Erlenmeyer flasks and moistened with a basal salt solution containing g/L: 1 % (w/v)KH<sub>2</sub>PO<sub>4</sub>, 1 % (w/v) MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.005 % (w/v) CaCl<sub>2</sub>.2H<sub>2</sub>O, 4 % (w/v) NaCl, 1 % (w/v)  $Na_2CO_3$ , and trace elements: 0.005 FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.0016 MnSO<sub>4</sub>.H<sub>2</sub>O, 0.0014 ZnSO<sub>4</sub>.7H<sub>2</sub>O, 0.002 CoCl<sub>2</sub>.6H<sub>2</sub>O using method described by Kumar et al. (2016). The substrates were then autoclaved for 30 min at 121  $^\circ\text{C}$  before being inoculated with 1 mL of spores (1.0  $\times$  $10^7$  spores/mL) from a three-day-old fungal culture. The inoculated substrates were then incubated for 7 days at 30 °C. Phosphate buffer (pH 6.8) was added to the substrate beds in a 1:10 (w/v) ratio and agitated for

1 h at 200 rpm. The fermented mashes were passed through a muslin cloth and then centrifuged for 10 min at 5000 rpm. Clear supernatants were used as crude enzyme extract.

### 2.3. Selecting enzyme extract with highest mushroom hydrolyzing capability

Fresh mushroom was dried to a constant weight and then milled into fine powder. The mushroom powder (200 mg) was placed in four 250 mL Erlenmeyer flasks and then autoclaved at 121 °C for 30 min. The crude enzyme extracts were added to the mushroom powder in a 1:10 (w/v) ratio and then incubated at 50 °C for 1h at 100 rpm (Geiger, 2014). The resulting hydrolysates were centrifuged for 10 min at 5000 rpm and the levels of glucose in supernatants were determined by dinitrosalicylic method (Miller, 1959). The efficiency of the extract to hydrolyze mushroom was determined by calculating the enzyme hydrolysis efficiency using the following formula:

Hydrolytic efficiency (%) = 
$$\frac{\text{Reducing sugars}, \frac{\text{mg}}{\text{mL}} \times 0.9 \times 100}{\text{Initial substrate}, \text{mg/ mL}}$$
 (1)

The factor 0.90 was used to convert polysaccharide to monosaccharide to account for water uptake during hydrolysis.

#### 2.4. Mushroom hydrolysis using crude enzyme extract

The crude enzyme extract obtained by solid-state fermentation of pineapple peels using *A. niger* exhibited the highest efficiency in mushroom hydrolysis and was chosen for further experiments. Mushroom hydrolysis was performed under optimal reaction conditions (i.e., which included enzyme loading, medium pH, temperature, and incubation time) as predicted by statistical design of experiments (Table 2). The resulting hydrolysates were centrifuged for 10 min at 5000 rpm and the levels of glucose in the supernatants were determined by dinitrosalicylic method (Miller, 1959).

#### 2.5. Experimental design

Screening of single parameters was conducted using one variable at a time method (data not shown). The selected reaction parameters were optimized based on the central composite design (CCD) of the response surface methodology (RSM). Glucose yield was used as a response variable. The range and the levels of the experimental variables in coded and uncoded form are given in Table 1.

The test factors were coded according to the following equation:

$$X_i = \frac{X_i - X_0}{\Delta x_i} \tag{2}$$

where  $x_i$  is the coded value of the independent variables,  $X_0$  is the value of the independent variable on the center point, and  $\Delta X_i$  is the step change.

A five-level, four-factor CCD was used to fit a second-order response model. Each variable and their interactions are described by applying the following quadratic equation:

 Table 1. Coded and actual levels of the independent variables for the design of experiment.

Independent variables	Range and level					
	$-\alpha$	-1	0	1	$+\alpha$	
рН (Х1)	4.5	5.0	5.5	6.0	6.5	
Time (X <sub>2</sub> , h)	12	24	36	48	60	
Temperature (X <sub>3</sub> , °C)	30	35	40	45	50	
Enzyme loading (X <sub>4</sub> , %v/v)	1	2	3	4	5	

Table 2. Central composite design and experimental responses of dependent variable (glucose).

Runs	$X_1$	$X_2$	X <sub>3</sub>	$X_4$	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

where Y is the measured response;  $\beta_0$  is the intercept term;  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ , and  $\beta_4$  are linear coefficients;  $\beta_{11},$   $\beta_{22},$   $\beta_{33},$  and  $\beta_{44}$  are quadratic coefficients;  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{14}$ ,  $\beta_{23}$ ,  $\beta_{24}$ , and  $\beta_{34}$  are interaction coefficients; and  $x_1$ ,  $x_2$ ,  $x_3$ , and x4 represent the independent variables: temperature, pH, time and enzyme loading respectively. The term  $\varepsilon$  represents experimental error. The significance of the model was evaluated by determining the coefficient R<sup>2</sup>. The model parameters were optimized using CCD model optimizer of RSM. All independent variables were kept within a certain range, and the response was maximized based on the process requirements. Optimum values were established using the desirability function method. Experiments were performed to validate the optimum response using the maximum conditions predicted by the model. Threedimensional surface plots were drawn to visualize effect of the independent variables on the response, and quadratic polynomial equation was proposed to describe the mathematical relationship between the variables and the response.

#### 2.6. Feedstock preparation for solid state fermentation

The effect of substrate moisture levels (55-90 %, w/v) was evaluated on hydrolytic efficiency of crude enzyme extract on mushroom hydrolysis. Similarly, the effect of surfactants viz. Tween-80, Tween-60, Tween-40, Tween-20, SDS and Triton-x-100 and nitrogen sources: beef extract, malt extract, peptone, yeast extract, soybean, and tryptone were also evaluated. Finally, the combined effect of moisture level, surfactant, and nitrogen source was determined.

#### 2.7. Kinetic analysis of mushroom hydrolysis

The kinetics of crude extract produced by solid-state fermentation of pineapple peels was investigated. The reaction velocity was measured at various substrate concentrations to determine the  $K_m$  and  $V_{max}$  values. The kinetic parameters of enzyme-catalyzed reactions were obtained from the Lineweaver-Burk plot derived from the Michaelis-Menten equation:

$$V = V_{max}[S]/(K_m + [S])$$
 (4)

where S is mass concentration of the substrate (g/L), V is starting rate (g/ (L.min)),  $V_{max}$  is the maximum reaction rate (g/(L.min)), and Km is the Michaelisian constant (g/L). The relation between the reaction velocity and the substrate concentration was determined with non-regression analysis.

#### 2.8. Statistical analysis

Experiments and analyses were carried out in triplicate. Data were subjected to one-way analysis of variance (ANOVA). Means were compared by Tukey's test at confidence interval of 95 % (p < 0.05) using SPSS v.16 software.

#### 3. Results and discussion

## 3.1. Suitable fruit waste for producing enzymes capable of hydrolyzing mushrooms

The suitability of various fruit peels as feedstock for the production of mushroom hydrolyzing enzymes by A. niger under solid-state fermentation (SSF) was investigated. The efficiency of crude enzyme extracts in bioconversion of mushroom substrates was determined using Eq. (1). The study noted that crude enzyme extract obtained from SSF of pineapple peels with A. niger had the highest mushroom bioconversion rate of 28.4%, closely followed by extracts from the fermentation of orange peels at 25.9%. Meanwhile, fermentation of mango peels and passion fruit peels with A. niger under solid-state conditions produced crude enzyme extracts with the lowest hydrolytic efficiencies of 18.6 and 20.3 %, respectively. The hydrolytic efficiency of crude enzyme extracts obtained from fermentation of fruit wastes viz. pineapple peels, orange peels, passion fruit peels, and mango peels differed significantly at p value < 0.05. Although we were unable to establish how fruit wastes affected the hydrolytic efficiencies of crude enzyme extract (because it was beyond the scope of this study), we were certain that the chemical diversity of the

**Table 3.** ANOVA for the regression model and the estimated regression coefficient with corresponding p-values representing glucose yield.

Source	DF	Seq SS	Adj. MS	F-value	<i>p</i> -value
Regression	14	1.50048	0.10718	7.39	0.00
Linear	4	0.99625	0.24906	17.17	0.00
Square	4	0.28970	0.07243	4.99	0.01
Interaction	6	0.21453	0.03576	2.46	0.07
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			
Adjusted R <sup>2</sup>		0.827			

Values in bold are statistically significant. DF-degree of freedom; Seq SS-sequential sum of squares.

fruit wastes played a role. As a result, we recommend therefore that proper fruit waste selection for solid state fermentation be followed in order to obtain enzyme consortia suitable for specific applications. Our findings are also consistent with those of Elisashvili et al. (2008), who noted changes in the production and activities of extracellular enzymes including cellulase, xylanase, laccase, and manganese peroxidase by white-rot fungi during solid state fermentation of different fruit wastes.

#### 3.2. Model parameters for mushroom hydrolysis

Optimal reaction parameters for mushroom hydrolysis with crude enzyme extract was established using central composite design of the response surface methodology. Eq. (2) was used to calculate the coded values of the independent variables that were used in developing the central composite design (CCD). Table 2 illustrates the experimental and predicted values of the response variable (glucose). Thirty-one experimental runs were conducted, with seven replications at the central points, eight at the axial points, and sixteen at the factorial points.

The statistical analysis of the experimental data permitted the validation of an empirical model for glucose released as a function of reaction temperature, pH, time and, enzyme loading. The CCD results were expressed as the second-order polynomial equation (Eq. (4)) by applying a quadratic regression analysis of the experimental data

 $\begin{aligned} & \text{Glucose yield} = 0.367714 + 0.044625x_1 + 0.062208x_2 + 0.117292x_3 + 0.147958x_4 - 0.041085x_1^2 - 0.019710x_2^2 + 0.022290x_3^2 + 0.081415x_4^2 \\ & - 0.028438x_1x_2 + 0.025438x_1x_3 + 0.012062x_1x_4 - 0.005563x_2x_3 + 0.052563x_2x_4 - 0.094938x_3x_4 \end{aligned} \tag{5}$ 



Figure 1. Response surface plots for mushroom hydrolysis as a function of (a) temperature and enzyme loading; (b) time and enzyme loading; (c) time and temperature; (d) pH and enzyme loading; (e) pH and temperature; (f) pH and time.



Figure 2. Optimization plot for mushroom hydrolysis with crude enzyme extract.

The model expressed by Eq. (3) represents glucose yield as a function of pH ( $X_1$ ), time ( $X_2$ ), temperature ( $X_3$ ), and enzyme loading ( $X_4$ ). The *p*-values for linear, quadratic and interaction effects of the variables are given (Table 3).

The model was validated by analysis of variance (ANOVA) with F test (calculated value 2.92 higher than the tabled one), making the model valid at 95 % confidence level. Meanwhile, 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 equation as not significant (p-value = 0.475). The model Eq. (3) had non-significant lack-of-fit which is a good proof that it is adequate to predict the response under any combination of values of the variables. The linear and quadratic terms in second order polynomial model were significant at p < 0.05.

All linear variables had positive coefficients, indicating positive influence on activities of enzyme extract and mushroom hydrolysis. Except for temperature, the effects of all other linear variables were significant, indicating that they should be taken into account when designing a process for mushroom hydrolysis using crude extracts from *A. niger*. Regarding the quadratic terms, both time and enzyme loading positively influenced mushroom hydrolysis, with enzyme loading having the most significant (p < 0.05) effect. Similarly, time and enzyme loading had a positive effect on mushroom hydrolysis.

Figure 1 depicts three-dimensional response surface plots used to visualize the interaction effect of the dependent and independent variables on the response while keeping the other two factors constant. Figure 1a depicts glucose yield as a function of enzyme loading (1.5–5.5 % w, v) and reaction temperature. Glucose yield increases with increasing temperature from 30 °C to 50 °C, with optimum yield recorded at 50 °C above which glucose yield decreases. A similar study by Daniel and Danson (2013) linked a decrease in product yield to a decrease in enzyme activities caused by thermal inactivation of enzymes. Meanwhile increasing enzyme load from 1.5 to 5.5 increases glucose yield; however, enzyme loading above the optimum level has no effect on glucose yield, most probably because the available glycosidic bonds had been depleted. Previous research by Akhter et al. (2011) and Sandri and Silveira (2018) found that high substrate loading reduces the amount of available enzymes and free water in the reaction system, resulting in lower enzyme activity. On the other hand, Sattler et al. (1989) reported a similar effect of enzyme loading and temperature, in which increasing enzyme loading and temperature resulted in an increase in glucose from bioconversion of cellulose from poplar wood. In addition, Kaur et al. (1998), Pan et al. (2005) and Manonmani and Sreekantiah (1987) discovered that increasing the temperature and enzyme loading to optimum levels favored the enzymatic hydrolysis of cellulose from softwood. sugarcane bagasse, and rice straw. Figure 1b visualizes the potential effect of incubation time and enzyme loading on mushroom hydrolysis.



Figure 3. Effect of moisture content in SSF on hydrolytic efficiency of crude extracts from *Aspergillus niger*. SSF conditions: Incubation time-7 days, temperature-30 °C, initial moisture level-65-70.0% (control), and initial pH-6.5.0; error bars show standard deviation.



Figure 4. Effect of surfactants in SSF on hydrolytic efficiency of crude extracts from *Aspergillus niger*. SSF conditions: incubation time-7 days, temperature-30 °C, initial moisture level-65-70.0% (control), and initial pH-6.5.0; error bars show standard deviation.

The model predicts that extending mushroom hydrolysis beyond the optimal period results in lower glucose levels. This prediction is consistent with the findings of Saini et al. (2019), who noted that when enzyme is absorbed into suspended substrate particles for an extended period of time, the hydrolysis of enzyme-susceptible cellulose linkages occurs simultaneously. Figure 1c illustrates effect of time and temperature on mushroom hydrolysis with crude enzyme extract. The glucose yield increased as the temperature and time were increased. Beyond optimal time, there is no increase in yield, and yield is reduced above optimal temperature. Figure 1d depicts glucose yield as a function of pH and enzyme loading. The glucose yield increases with pH up to an optimum, after which the yield decreases. A similar effect of pH on enzyme activities had been reported by Yang et al. (2011) who noted that enzymes function best at optimal pH, which when exceeded has a negative effect on enzyme conformation and activity. Griggs et al. (2012) also reported that too much product can inhibit enzyme activity and disrupt enzyme reactions. Figure 1f illustrates glucose yield in response to medium pH and reaction time. The glucose yield increases with increasing pH from 4.8 to optimum pH 6.5, after which it decreases, confirming previous research that enzymes function best at optimal pH, above which enzyme conformation and activity are compromised (Yang et al., 2011). In the meantime, increasing the incubation time beyond the optimal time of 12 h does not affect glucose yield; this confirms the findings of Saini et al.

Table 4. Effect of nitrogen sources on hydrolytic efficiency of crude extracts.

Nitrogen	Degree of hydro	Degree of hydrolysis (%)					
	0.04	0.08	0.12	0.16			
Beef	$28.4\pm0.1^{\rm b}$	$35.4\pm0.2^{\rm c}$	$32.5\pm0.2^{ab}$	$\textbf{27.2}\pm0.9^{b}$			
Malt	$28.6 \pm \mathbf{0.3^{b}}$	$31.8\pm0.5^{c}$	$26.8\pm0.4^{b}$	$21.6\pm0.3^a$			
Peptone	$26.2\pm0.8^a$	$30.5\pm0.6^{b}$	$32.2 \pm 0.2^{b}$	$28.3\pm0.1^{a}$			
Yeast	$\textbf{37.7}\pm0.4^{a}$	$44.2\pm0.7^{c}$	$39.7\pm0.1^{ab}$	$35.4\pm0.5^a$			
Soybean	$27.9\pm0.4^{a}$	$29.4\pm0.4^a$	$28.8\pm0.7^{a}$	$26.7\pm0.8^a$			
Tryptone	$23.5\pm0.7^a$	$27.7 \pm 0.5^{b}$	$\textbf{26.4} \pm \textbf{0.3}^{b}$	$25.9\pm0.7^{ab}$			
Control	$23.2\pm0.6^{\rm a}$						

SSF conditions: Incubation time-7 days, temperature-30  $^\circ\text{C}$  , initial moisture level-65-70%, and pH 6.5  $\pm$  SD.

Means in the same row followed by the same letters are not significantly different at  $p \leq 0.05. \label{eq:product}$ 

(2019), who noted that enzymes are absorbed into suspended substrate particles for a period of time until the optimum time is reached, after which no further hydrolysis occurs because all enzyme-susceptible cellulose linkages have been depleted.

### 3.3. Optimum parameters for mushroom hydrolysis with crude enzyme extract

The CCD model optimizer of RSM predicted the optimum glucose yields based on the regression model Eq. (4). Figure 2 depicts the maximum response (glucose) predicted by the CCD model. When the CCD model optimizer was set to 1.0, the model predicted a glucose yield of 1.49 mg/mL at optimal temperature of 50 °C, pH of 6.5, enzyme loading of 5% (v/v), and a time of 12 h. Experiments were conducted to validate the maximum glucose yield predicted by the model. The experimental glucose yield of 1.64 mg/mL obtained after process optimization did not differ significantly (p value  $\leq 0.05$ ) from the predicted value, indicating that the predicted and experimental response values agree well. This further validates our RSM model, demonstrating that it is adequate for optimizing any experiment involving mushroom hydrolysis using crude enzyme extract under similar conditions. The glucose yield in this study is higher than 0.5-1.2 mg/mL reported by Sławińska et al. (2021) and Zhou et al. (2012). The improved glucose yield in this study could therefore be attributed to adequate balance of accessory enzymes involved in the degradation of mushroom cell-wall polysaccharides in the crude extract. The findings of the present study are thus consistent with previous studies that found effective enzymatic degradation of lignocellulosic biomass to be a function of balanced enzyme proportions working in synergy to break down the complex structure of lignocellulose (Harman et al., 2004; Kubicek et al., 2014; Van Dyk and Pletschke, 2012).

#### 3.4. Suitable conditions for solid-state fermentation

The preparation of substrate for solid state fermentation has an impact on enzyme production and activity. Figure 3 depicts the effect of substrate moisture content on the hydrolytic efficiency of crude extracts. A substrate moisture range of 70–75 % (w/v) was identified as ideal for producing crude enzyme extract with 32 % efficiency in mushroom hydrolysis. In a similar study a substrate moisture content of 65 % was found to be optimal for maximum biomass and enzyme production by



Figure 5. Reaction kinetics of mushroom with crude enzyme extract.

A. niger under solid-state conditions (Patil and Dayanand, 2006). Low moisture levels, on the other hand, were found to reduce substrate swelling, nutrient diffusion, and solid substance solubility. In fact, it has been demonstrated that low moisture content reduces nutrient supply, resulting in decreased growth and enzyme production (Prior et al., 1992). On a similar study, moisture content above 80% reduced microbial growth and enzyme activities. This is because excessive moisture content has been shown to decrease substrate porosity, decreased heat and mass transfer through the culture, and decreased air exchange, all of which result in decreased growth and product formation (Kumar et al., 2011).

Figure 4 illustrates the effect of surfactants on the hydrolytic efficiency of crude enzyme extracts. Surfactants in bioreaction are important because they may decrease, increase, or change the yield of reaction rates (Samiey et al., 2014). In the current study, concentrations of all the surfactants except SDS (0.3%, w/v) had a positive effect on reaction rate of mushroom hydrolysis with crude enzyme extract. The crude enzyme extract with the highest (41.2%) efficiency in mushroom hydrolysis was obtained by supplementing the SSF media with 0.2% (w/v) Tween-80, which was higher than the 23.8 % hydrolytic efficiency reported in the control experiment.

Table 4 shows effect of different nitrogen sources and their concentrations on hydrolytic efficiency of crude enzyme extracts. This follows a study by Sumantha et al. (2006) who reported that variations in carbon source, nitrogen source, or their ratios had a significant impact on enzyme production and activity. Similarly, our study found that adding yeast extract at a concentration of 0.08 % (w/v) to the fermentation media resulted in enzyme extract with the highest (44.2%) efficiency in mushroom hydrolysis. Gautam et al. (2018) made a similar observation, noting changes in microbial growth, enzyme production, and activities in response to nitrogen sources and concentrations. Meanwhile, the control extract had the lowest (23.2%) efficiency in mushroom hydrolysis, implying that selecting the right nitrogen source is critical if improved enzyme activities are to be achieved for mushroom hydrolysis.

Further experiments were performed to evaluate the effect of different combinations of surfactants and nitrogen sources at various moisture levels on the hydrolytic efficiency of crude enzyme extracts. The SSF conditions of 30 °C temperatures, initial moisture level of 70–75 %, and yeast extract (0.08 % w/v) and 7 days incubation period resulted in a 2.2-fold mushroom conversion rate. This was the highest mushroom bioconversion rate compared to other reaction combinations evaluated in this study suggesting strong synergy between surfactants and nitrogen at ideal moisture levels in order to produce enzymes with improved hydrolytic effect on mushroom. The experimental results on the substrate formulation for SSF revealed that the appropriate combination of surfactants and nitrogen source at ideal moisture levels is critical in

preparing crude enzyme extracts with improved activities suited for mushroom hydrolysis.

#### 3.5. Kinetics of mushroom hydrolysis

Figure 5 depicts the kinetic parameters (K<sub>m</sub> and V<sub>max</sub> values) measured using the Lineweaver-Burk plot derived from Michaelis-Menten equation (Eq. (5)). Our data obtained a straight line from a plot of the reciprocal of substrate decomposition rate (1/V) versus the reciprocal of substrate concentration 1/[S] that agreed well with the Michaelis-Menten model. According to the Michaelis-Menten model, a plot of the reciprocal of substrate decomposition rate versus the reciprocal of substrate concentration should produce a straight line with an intercept 1/V<sub>max</sub> and slope K<sub>m</sub>/V<sub>max</sub> (Roskoski, 2015). The line equation, y = 0.0145x + 0.0046 of the Lineweaver-Burk plot, and the coefficient of determination (R<sup>2</sup> = 0.998) revealed a positive relationship between substrate concentration and enzyme activity, implying a direct relationship between enzyme activities and substrate concentration. On-regression analysis revealed that K<sub>m</sub> and V<sub>max</sub> were 3.6 mg/mL and 192.3 U/g, respectively.

In conclusion, this study presents crude enzyme extract as a viable alternative to expensive commercial enzymes that may be used in competitive processing of mushroom at an industrial scale. As a result, it suggests cost-effective strategies for improving the activities of crude enzyme extract for hydrolyzing mushroom and, by extension, other lignocellulosic biomass. When hydrolyzing mushrooms with crude enzyme extracts, one of the strategies for reducing extraction time and increasing product yield has been presented: optimize reaction conditions. This is in addition to using the appropriate fruit waste as fermentation feedstock to produce enzymes with enhanced efficiency in mushroom hydrolysis. The study also noted that when preparing fruit waste substrates for SSF with *A. niger*, the appropriate combination of surfactants and nitrogen source at ideal moisture levels is critical in producing enzyme extracts with improved activities for mushroom hydrolysis.

#### Declarations

#### Author contribution statement

Ojwang D. Otieno: Conceived and designed experiment; Performed the experiment; Analysed and interpreted the data; Wrote the paper.

Mulaa F. Jakim; Obiero George; Midiwo Jacob: Conceived and designed experiment; Contributed reagents, materials, analysis tools or data; Wrote the paper.

#### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### Data availability statement

Data included in article/supplementary material/referenced in article.

#### Declaration of interest's statement

The authors declare no conflict of interest.

#### Additional information

No additional information is available for this paper.

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