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Original Article

Cellulase Production Under Solid-State Fermentation by Ethanolic Zygomycetes Fungi: Application of Response Surface Methodology

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ABSTRACT

Background and Objectives: Cellulase is an important enzyme with multiple applications in industries, including food, laundry, pharmaceutical, textile, pulp, paper and biofuel industries. Solid-state fermentation (SSF) is a method for cellulase production, which includes several advantages, compared to submerged fermentation. In this study, cellulase was produced by three filamentous fungi, i.e., *Mucor indicus*, *M. hiemalis* and *Rhizopus oryzae*, through SSF on wheat brans.

Materials and Methods: Effects of cultivation time, temperature, and moisture content of the culture media on cellulase production were investigated using response surface methodology (RSM). Experiments were carried out using an orthogonal central composite design. Based on the analysis of variance, a quadratic model was suggested as a function of the three variables to express cellulase production. The optimum parameters for cellulase production by the fungi were achieved and the highest cellulase activity was reported.

Results: The fungi produced significant amounts of cellulase. Models fitted to the experimental activities of the fungi included high regression coefficients. The optimum media temperature for all fungi was 26.6 ºC. For *M. indicus* and *R. oryzae*, the optimum moisture content and cultivation time of the media were 71.8% and 33.2 h, respectively. These parameters were respectively reported as 38.18% and 66.81 h for *M. hiemalis*. The highest cellulase activities by *R. oryzae*, *M. indicus* and *M. hiemalis* were 281, 163 and 188 U per g of dry wheat bran, respectively. The maximum enzyme production was seen in *R. oryzae*.

Conclusions: In conclusion, these three advantageous fungal strains can successfully be used for cellulase production through SSF with relatively high yields, compared to other fungal strains.

Keywords: Cellulase, *Mucor hiemalis*, *Mucor indicus*, *Rhizopus oryzae*, Solid-state fermentation

Introduction

Cellulose, a linear unbranched homopoly-saccharide, is the world's most abundant organic substance. Cellulose is insoluble in water and is mainly available in plant cell walls (1). This polysaccharide contains glucose monomers and it is industrially important to convert cellulytic materials to glucose efficiently and economically (2). Cellulolytic enzymes are synthesized by microorganisms such as fungi and bacteria. Furthermore, fungi are able to decompose cellulosic substrates (1). Cellulase is a multi-enzyme system, composed of several enzymes acting synergically. Cellulase enzymes contain endo-1,4-β-D-glucanase, β-D-glucosidase and exo-1,4-β-glucanase (3). They are used in wastewater treatments, textiles, detergents, food processes, animal foods, breweries, wines, pulps and paper industries and in the bioconversion of lignocellulosic materials to biofuel ethanol (4). Since markets for these enzymes are increasing, investigations on cellulase production are growing as well (4). Solid-state fermentation (SSF) is an interesting method for the production of enzymes, in which there is no free-flowing water and microorganisms grow on wet solid substrates (5). Water needed for microbial activities exists in solid matrix of the substrate (6–8). The SSF includes

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multiple advantages, compared to submerged fermentation. Examples include lower capital and operating costs, simpler equipment and culture media, smaller required space and lower amount of wastewater output (5, 9). Main drawbacks of SSF include technological challenges with monitoring and control of operational parameters, limiting its large-scale applications. In SSF, substrates are solid and insoluble in nature such as grains, wheat brans and vegetables. In submerged fermentation, substrates are soluble feedstocks such as glucose, molasses and malt (5, 10). Filamentous fungi, compared to other microorganisms which are used in SSF, can grow on complex solid substrates and produce various enzymes (11). Therefore, SSF is more appropriate for mycelial fungi than yeasts or bacteria. *Rhizopus oryzae*, *Mucor indicus* and *M. hiemalis* are three mycelial fungi with unique abilities, making them appropriate for industrial uses. These fungi are promising as newly investigated Zygomycetes fungi for ethanol production from cellulosic sources and can be isolated from edible materials. Moreover, they can grow on substrates with various inhibitors. Their biomass is valuable nutritionally and contains glucosamine and chitosan considerably (12, 13). Several reports have been published on cellulase production by various fungi and effects of several factors on cellulase activity. For example, Shahriarinour and Abdoul Wahab (14) studied effects of dissolved oxygen tension on cellulase production by *Aspergillus terreus*. Javanmard *et al*. (15) investigated cellulase production by *Thermoascus aurantiacus* isolates through submerged fermentation with various carbon and nitrogen sources. Generally, the volume of produced enzyme highly depends on the fermentation conditions; thus, it is necessary to find the optimum fermentation conditions to decrease costs of the enzyme production. In the present study, cellulase production by three fungal species of *R. oryzae*, *M. indicus* and *M. hiemalis* was investigated through SSF on wheat brans. To the best of the authors' knowledge, no studies have been published on cellulase production by these fungal species through SSF. Effects of temperature, cultivation time and moisture content of the media on cellulase production were studied using an experimental design. A mathematical model, using response surface methodology (RSM), was suggested to show the relationship between the enzyme production and the three variables. Furthermore, the interactive effects of the variables on cellulase production were investigated. The optimum values of moisture content, cultivation time and temperature of the media for the highest cellulase production were achieved. Furthermore, the corresponding cellulase activities by the fungi were reported.

Materials and Methods

Microorganisms

The experiments were performed using three fungal strains of *R. oryzae* CCUG 28958, *M. indicus* CCUG 22424 and *M. hiemalis* CCUG 16148 provided by the University of Gothenburg, Gothenburg, Sweden. Plates containing 40 g l^{-1} of glucose (Merck, Germany), 10 g l^{-1} of peptone (Merck, Germany) and 15 g l^{-1} of agar (Merck, Germany) were prepared. After inoculation, plates were incubated at 32 $^{\circ}$C for 5 days and then kept at 4 $^{\circ}$C until use. A solution of polyoxyethylene sorbitan monooleate (Tween 80) (Merck, Germany) in distilled water (0.1% v/v) was used to wash the spores. Flasks containing 10 g of moistened wheat brans were autoclaved at 121 ºC for 20 min and inoculated with the spore suspensions after cooling down. These were incubated at 30 ºC for 7 days to provide optimum conditions for the growth of spores on the surface of wheat brans. To separate spores from the substrate, a solution of Tween 80 in distilled water (50 ml, 0.1% v/v) was added to the flasks. Sterile glycerol was added to the flasks and the contents were stored at -20 ºC.

Fermentation conditions

To prepare culture media for the fungi, dry brans (10 g with 50% moisture) were added to a 250-ml flask. After autoclaving and cooling, the media was inoculated with spore suspensions (1000 spores per g of dry brans). Culture temperature and time were set up as well as moisture content of the media using designed experiment approaches. To calculate moisture content of the media, volumes of the spore suspensions added to the flask were considered.

Enzyme extraction and assay

To extract the enzyme, 90 ml of distilled water (D.W.) were added to each flask. Flasks were shaken agitatedly at 100 rpm for 30 min at room temperature. After filtration, enzyme activity of the filtrates was calculated. The cellulase activity was calculated according to a method by Konig *et al*. (16) using 1% carboxymethyl cellulose solution (CMC, sodium salt, medium viscosity) (Sigma, USA) in 0.1 M of acetate

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buffer (Merck, Germany). The quantity of cellulase needed to release 1 µmol of glucose per min was considered as one unit of cellulase activity (U). The enzyme activity was present as U per gram of dry substrate (gds).

Experimental design and interactive effects of the operating parameters

To study the interactive effects of temperature (*T*), time (*I*) and moisture content (*W*) on cellulase production by the three fungal strains and to develop a statistical model, RSM was used. The experiments were suggested based on an orthogonal central composite design with five levels coded as -1.682, -1, 0, +1 and +1.682. Studied temperature, moisture content and time are listed in Table 1. The experiments (containing nine replicates at the center point) suggested by SAS software were performed in duplicate and the average values were reported. A quadratic model based on the following equation was fitted to the cellulase production (1):

$$Cellulase\ activity\left(\frac{U}{gds}\right) = a_0 + a_1T + a_2W + a_3I + a_4T^2 + a_5TW + a_6TI + a_7W^2 + a_8WI + a_9I^2$$

Where a_0 was the intercept; a_1, a_2 and a_3 were linear coefficients; a_4, a_7 and a_9 were squared coefficient and a_5, a_6 and a_8 were interaction coefficients. All statistical calculations were carried out using SPSS software (PASW Statistics 18).

Table 1. The experimental ranges of the variables

Variable (symbol/unit)	Range and level				
	-α (-1.682)	-1	0	+1	+α (+1.682)
Temperature (T/ºC)	26.59	30	35	40	43.4
Moisture content (W/W) (W/%)	38.18	45	55	65	71.81
Time (I/h)	33.18	40	50	60	66.81

Results

Empirical models for cellulase production

The experimental design and cellulase activities are listed in Table 2. The experimental cellulase activities were fitted to a quadratic model. The values calculated by this model and the relative deviations were shown in Table 2. Cellulase activities vary in various conditions. Therefore, it is necessary to find the most appropriate values for the cultivation parameters to achieve the highest enzyme production. Analysis of variance (ANOVA) was used to show the significance of the model. When the probability (*P* value) calculated by the Fisher F-test is low, the regression model includes high significance and the quadratic model adequacy is confirmed (17). Furthermore, R^2 values greater than 0.75 indicate that the model is appropriate. As shown in Table 3, low *P* values and high R^2 values obtained for cellulase production by the fungi confirmed the adequacy of the quadratic models. Coefficients of equation 1 were calculated for cellulase enzymes produced by the fungi using RSM. The significance of all coefficients was calculated using student's t-test (Table 4). Lower *P* values and higher *t*-values of coefficients indicated further significance (18). For the cellulase production by *M .indicus*, R^2 value (Table 3) showed a good consistency between the experimental data and predicted values. This indicated that 96.4% of the sample variations were explained by the independent variables of cultivation time, moisture content and temperature. As shown in Table 4, *P* values of the coefficients showed that the cellulase activity of *M. indicus* was strongly dependent on temperature and incubation time. Furthermore, the interaction effect of time and moisture content was not significant. Coefficients of the model showed that cellulase production decreased with increased temperature and culture time. However, moisture content of the media demonstrated a quadratic effect on cellulase production.

For cellulase production by *M. hiemalis*, the R^2 value (0.973) showed a good agreement between the calculated and experimental activities. According to Table 4, the cellulase activity of *M. hiemalis* was strongly dependent on moisture content and incubation time of the media as the *P* values of the model showed. Furthermore, the linear effect of temperature on cellulase production was not significant. Coefficients of the model showed that cellulase production increased with decreased moisture content and increased cultivation time. However, the quadratic effect of temperature and the interaction term of temperature and moisture content or temperature and cultivation time affected cellulase production. Fitting the model presented for cellulase production by *R. oryzae* resulted in a low *P* value and a high R^2 value (0.99). This means that the quadratic model included a satisfactory adjustment for explaining experimental data. Relatively, the model explained nearly 99% of the variability in response. For cellulase production by *R. oryzae*, the linear coefficient of time and the second order term of temperature, time and moisture content and the interaction term of moisture content and temperature were highly significant (P<0.001). The interaction term of moisture content and time was insignificant and the remaining terms were equally significant. For

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high significant terms, small changes in their values result in considerable changes in the enzyme activity (19). Effects of the variables on cellulase production can be understood from magnitude and sign of the corresponding coefficients.

Table 2. Experimental design and results (Exp.), calculated values (Cal.) and relative deviation (RD) for the cellulase production (U/gds) by the fungi

No.	Uncoded level			*Mucor indicus*			*Mucor hiemalis*			*Rhizopus oryzae*		
	T	W	I	Exp.	Cal.	RD	Exp.	Cal.	RD	Exp.	Cal.	RD
1	30.00	45.00	40.00	44.50	46.34	3.97	26.98	23.92	-12.80	55.50	51.83	-7.08
2	30.00	45.00	60.00	16.00	11.32	-41.30	93.49	93.32	-0.18	36.50	33.79	-8.01
3	30.00	65.00	40.00	86.00	80.81	-6.42	18.78	18.64	-0.74	152.00	147.36	-3.15
4	30.00	65.00	60.00	34.00	35.80	5.02	76.47	72.14	-6.00	143.00	141.07	-1.37
5	40.00	45.00	40.00	17.50	12.82	-36.52	23.05	22.91	-0.61	33.00	28.84	-14.44
6	40.00	45.00	60.00	18.00	20.30	11.35	18.81	14.48	-29.92	29.00	27.55	-5.27
7	40.00	65.00	40.00	13.50	15.29	11.71	39.16	34.86	-12.34	37.00	33.61	-10.08
8	40.00	65.00	60.00	17.50	12.78	-36.97	11.93	10.52	-13.39	46.50	44.07	-5.50
9	26.59	55.00	50.00	51.00	53.31	4.34	59.47	61.89	3.91	109.00	113.76	4.19
10	43.41	55.00	50.00	4.00	5.77	30.62	5.32	9.22	42.30	9.00	12.86	29.99
11	35.00	38.18	50.00	15.00	16.71	10.23	37.01	39.43	6.15	12.00	16.19	25.89
12	35.00	71.82	50.00	37.00	39.37	6.02	27.77	31.67	12.31	106.00	110.43	4.01
13	35.00	55.00	33.18	42.00	44.32	5.24	14.25	16.64	14.36	56.00	62.49	10.39
14	35.00	55.00	66.82	11.00	12.76	13.79	50.61	54.54	7.21	54.00	56.12	3.78
15	35.00	55.00	50.00	21.00	16.70	-25.75	20.13	22.59	10.90	32.00	36.50	12.34
16	35.00	55.00	50.00	21.00	16.70	-25.75	28.52	22.59	-26.24	30.00	36.50	17.82
17	35.00	55.00	50.00	11.00	16.70	34.13	18.53	22.59	17.98	37.00	36.50	-1.36
18	35.00	55.00	50.00	17.00	16.70	-1.79	23.43	22.59	-3.71	38.00	36.50	-4.10
19	35.00	55.00	50.00	21.00	16.70	-25.75	24.84	22.59	-9.95	40.00	36.50	-9.58
20	35.00	55.00	50.00	18.00	16.70	-7.78	28.45	22.59	-25.93	37.00	36.50	-1.36
21	35.00	55.00	50.00	14.00	16.70	16.17	21.62	22.59	4.30	42.00	36.50	-15.06
22	35.00	55.00	50.00	12.00	16.70	28.15	17.27	22.59	23.56	39.00	36.50	-6.84
23	35.00	55.00	50.00	16.00	16.70	4.19	21.62	22.59	4.30	35.00	36.50	4.12

$$\text{Relative deviation (\%)} = 100 * \frac{\text{calculated result} - \text{experimental result}}{\text{calculated result}}$$

Table 3. Regression analysis (ANOVA) for the cellulase production

Fungus	Model SS	Residual SS	Model DF	Residual DF	Model MS	Residual MS	F-value	*P* value	R^2
M. indicus	6865.2	253.2	9	13	762.8	19.5	39.2	0.000	0.964
M. hiemalis	9447.9	258.6	9	13	1049.8	19.9	52.8	0.000	0.973
R. oryzae	31217.7	320.2	9	13	3468.6	24.6	140.8	0.000	0.990

SS, Sum of Square; DF, Degree of Freedom; MS, Mean Square

Table 4. Calculated coefficients (coef.) of the equation 1 and the relative t and *P* values

Term	*Mucor indicus*			*Mucor hiemalis*			*Rhizopus oryzae*		
	Coef.	t-value	*P* value	Coef.	t-value	*P* value	Coef.	t-value	*P* value
a_0	568.95	4.93	0.000	-58.06	-0.49	0.627	408.32	3.14	0.008
a_1	-17.36	-4.47	0.001	-1.24	-0.31	0.757	-11.77	-2.69	0.018
a_2	3.11	1.71	0.110	-6.30	-3.43	0.004	6.79	3.32	0.005
a_3	-11.19	-6.28	0.000	12.34	6.85	0.000	-12.80	-6.39	0.000
a_4	0.18	4.10	0.001	0.18	4.09	0.001	0.38	7.61	0.000
a_5	-0.16	-5.12	0.000	0.09	2.73	0.017	-0.45	-12.92	0.000
a_6	0.21	6.81	0.000	-0.39	-12.30	0.000	0.08	2.38	0.033
a_7	0.04	3.62	0.003	0.05	4.09	0.001	0.10	7.61	0.000
a_8	-0.03	-1.60	0.133	-0.04	-2.52	0.026	0.03	1.67	0.118
a_9	0.04	3.78	0.002	0.05	4.10	0.001	0.08	6.47	0.000

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Effects of operating parameters on enzyme production

Effects of cultivation time, moisture content and temperature of the media on cellulase production were studied. In Figures 1–6, the value of one variable was chosen equal to its central point and the simultaneous effect of the other two parameters on cellulase production was plotted.

Effects of temperature: In SSF, temperature is reported as the most important variable influencing the process (20); therefore, effects of temperature on cellulase production by the three fungi were studied. Time distributions of cellulase activity for various temperatures at moisture content of 55% are shown in Figures 1–3 for *M. indicus*, *M. hiemalis* and *R. oryzae*, respectively. At short culture times, lower temperatures favored cellulase production by *M. indicus* while at long incubation times, high temperatures were preferred (Figure 1). For a culture time of 50 h (the central point of the studied region), a temperature of 26.6 ºC was the optimum temperature for cellulase production. This was controversial for *M. hiemalis* (Figure 2). For a culture time of 50 h, lower temperatures were more efficient. For *R. oryzae*, cellulase production increased with decreased temperature for all culture times.

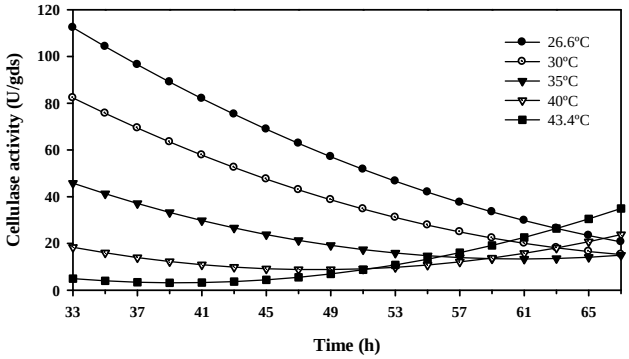

Figure 1. Cellulase production by *Mucor indicus* as a function of time and temperature at 55% moisture content

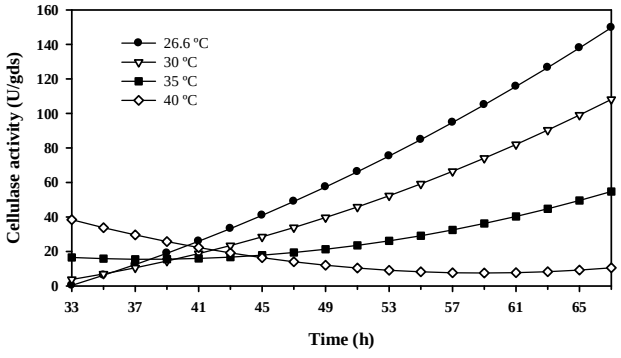

Figure 2. Cellulase production by *Mucor hiemalis* as a function of time and temperature at 55% moisture content

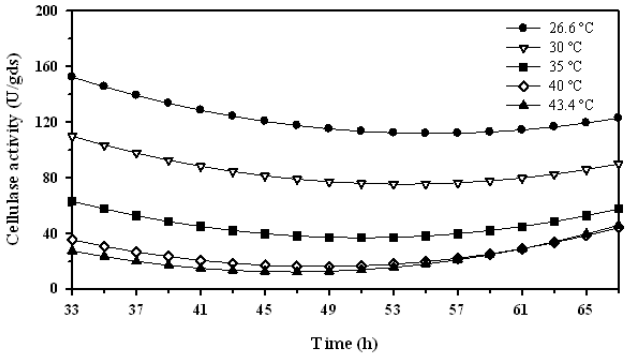

Figure 3. Cellulase production by *Rhizopus oryzae* as a function of time and temperature at 55% moisture content

Effects of moisture content of the media: Moisture content of the media is an important factor for the enzyme production using SSF (10) since it influences the growth of microorganism and product biosynthesis (21). Low and high moisture contents of the substrate affect the fungal growth, resulting in low cellulase production. Moisture may affect the physical properties of the solid substrate (22). At higher moisture contents, growth of the microorganisms is faster and subsequently enzyme production initiates earlier (23). The optimum moisture content is dependent on the requirements of microorganism and type of substrates and end products (24). In this study, five moisture contents, ranging from 38–72%, were developed. Simultaneous effects of cultivation time and moisture content of the media on cellulase production at 35 ºC for *M. indicus*, *M. hiemalis*, and *R. oryzae* are shown in Figures 4–6. Cellulase production by *M. indicus* decreased to a minimum value and subsequently increased with increased moisture content. The highest cellulase production was seen at the highest value of moisture content (Figure 4). Cellulase production by *M. hiemalis* decreased to the lowest value and subsequently increased with increased moisture content of the media. For culture times shorter than 42 h, the maximum production was achieved at the highest moisture level. For higher culture times, the maximum production was achieved at the lowest moisture level (Figure 5). Cellulase production by *R. oryzae* increased with increased moisture content (Figure 6).

Effects of culture time: Cellulase production by *M. indicus* decreased with increased culture time to a minimum value and subsequently increased. The maximum cellulase production was achieved at the initial stages of culture time (Figure 4). Cellulase production by *M. hiemalis* increased at low moisture contents with increased culture time. Cellulase production by *M. hiemalis* decreased at high moisture contents to a minimum value and subsequently increased. Generally, the highest cellulase production

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was achieved at the longest culture time (Figure 5). Increased culture time decreased cellulase production by *R. oryzae* to a minimum value and subsequently increased it (Figure 6). The highest cellulase activity was achieved at the lowest culture time.

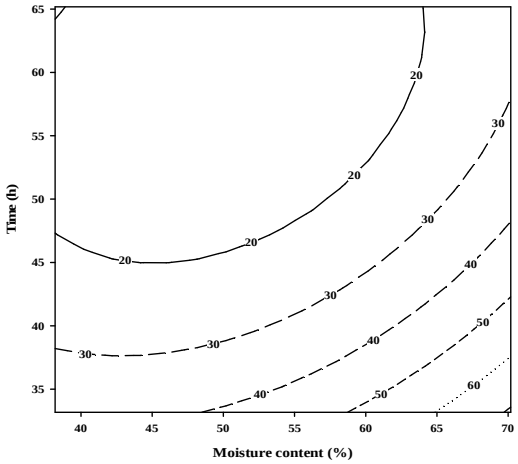

Figure 4. Cellulase production by *Mucor indicus* as a function of time and moisture content of the media at 35 ºC

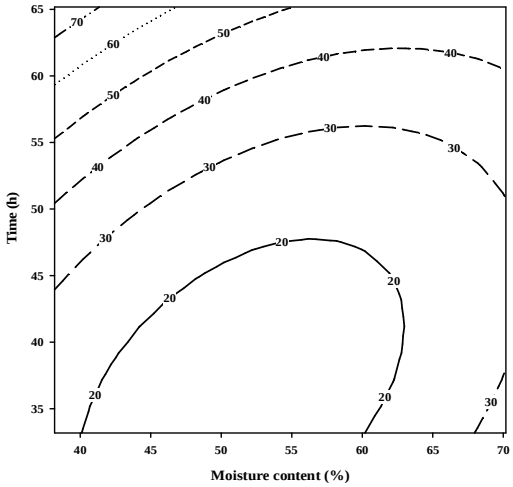

Figure 5. Cellulase production by *Mucor hiemalis* as a function of time and moisture content of the media at 35 ºC

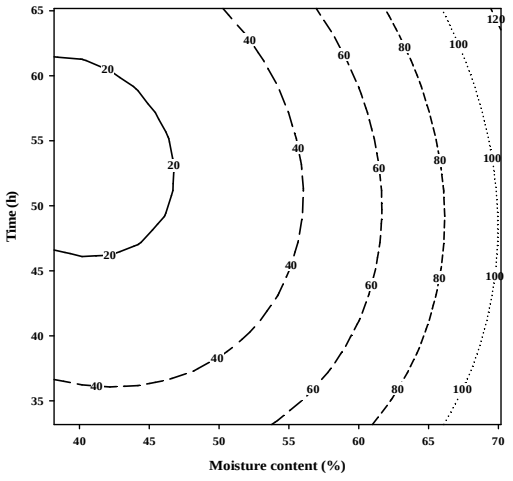

Figure 6. Cellulase production by *Rhizopus oryzae* as a function of time and moisture content at 35 ºC

The optimum conditions for cellulase production

The minimum and maximum experimental cellulase productions by *M. indicus* included 4.0 and 86.0 U/gds, respectively (Table 2). The corresponding values for *M. hiemalis* and *R. oryzae* included 5.3 and 93.5 U/gds and 9.0 and 152.0 U/gds, respectively. Results indicated that the enzyme production was considerably affected by the operational conditions. Furthermore, it was important to use the optimum conditions to achieve the highest cellulase production by the fungi. Table 5 shows the optimum variables for cellulase production by the fungi. The maximum cellulase production (281 U/gds) by *R. oryzae* was achieved at optimum conditions. The highest cellulase activity in *M. hiemalis* was higher than that in *M. indicus* ((188 instead of 163 U/gds). The optimum temperature for the three fungi was 26.6 ºC. The highest moisture content and the lowest culture time positively affected cellulase production by *M. indicus* and *R. oryzae.* However, the lowest moisture content and the highest culture time affected *M. hiemalis*. Jatinder *et al*. (25) optimized cellulases production by *Scytalidium thermophilum* using RSM and respectively reported 62.5 and 151 U/g for endoglucanase and β-glucosidase under optimum conditions.

Table 5. Optimum conditions and activities for the production of cellulase

Parameter	*Mucor indicus*	*Mucor hiemalis*	*Rhizopus oryzae*
T (ºC)	26.6	26.6	26.6
W (%)	71.81	38.18	71.8
I (h)	33.18	66.81	33.18
Activity (U/gds)	163.59	188.77	281.55

Discussion

The temperature affects the cellulase production by influencing the microbial growth. In enzyme production, high temperatures result in physiological changes which are not well known. However, a limited number of proteins necessary for the growth and other physiological processes of microorganisms are synthesized at high temperatures (26). Studies have reported that low temperatures favor cellulase production by various fungi. For example, the optimum temperature recorded for cellulase production by *A. niger* was 30 °C (27). In SSF of two *Trichoderma reesei* mutants, temperatures of 25–30ºC were reported as the optimum values for cellulase production (28). Trinh *et al*. (29) reported that the maximum carboxymethyl cellulase production by *Peniophora* sp. was achieved at 28 ºC.

Several investigations have reported that high moisture contents increase enzymes production. In a study by Kalogeris *et al*. (9) on wheat straws using *T. aurantiacus*, high moisture levels favored cellulase production. The highest endoglucanase yield was achieved at the highest moisture level (80%). Similar

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results were reported for other fungal cellulases (27, 30). The low moisture content of 40% has been reported as the optimum moisture content for cellulase production by *T. viride* using SSF. Low moisture contents are known to decrease metabolic and enzymatic activities. This is possibly linked to low swelling, high water tension and reduced solubility of nutrients from the solid substrate at low moisture contents (23, 31). In contrast, high moisture contents decrease porosity, alter substrate particle structure and lower oxygen transfer (31). Furthermore, high quantities of water in media result in the media clump which does not suit aeration and hyphae growth (27). Therefore, finding the best moisture content of the media is necessary to achieve the highest enzyme production.

As results show, enzyme production varied with incubation time in the three fungi. Short incubation times promise low-cost production of cellulase. In the current study, lower culture times favored cellulase production by *M. indicus* and *R. oryzae*. Lower activities at longer times might be seen due to catabolite repression by glucose released from wheat bran hydrolysis; similar to what seen for cellulase production by two mixed strains of *A. niger* using SSF. The optimum culture time was recorded 30–35 h (27). At longer times (after the maximum production of cellulase), production of other byproducts may inhibit the growth of fungi, which consequently affects enzyme formation (32). Results are different from the results from study on *M. hiemalis*, in which the maximum cellulase activity was achieved during the fungus autolysis at the longest time. These results are similar to results by Mekala *et al*. (33), who reported the ideal incubation time of 66 h for cellulase production by *T. reesei*.

In summary, SSF is a promising method for the production of enzymes. Although technological challenges exist with monitoring and control of the operational parameters in this method, SSF includes multiple advantages over the submerged fermentation. In the present study, three strains of filamentous zygomycetes fungi of *M. indicus*, *M. hiemalis* and *R. oryzae*, which can grow on complex solid substrates in comparison with other microorganisms, were used for the production of cellulase as an extracellular enzyme. These strains include unique abilities making them appropriate for the industrial use. Normally, SSF was used on wheat brans as an insoluble substrate due to the advantages for mycelial fungi than yeasts and bacteria. Results have shown that cellulase can be produced considerably by *M. indicus*, *M. hiemalis* and *R. oryzae* using SSF on wheat brans. The highest cellulase production by the three fungal strains was achieved at 26.6 ºC. The optimum moisture content was reported at 71.8% for *M. indicus* and *R. oryzae* and 38.2% for *M. hiemalis*. The optimum culture time was achieved at 33.2 h for *M. indicus* and *R. oryzae* and 66.8 h for *M. hiemalis*. In general, *R. oryzae* included the highest (281 U/gds) and *M. hiemalis* (188 U/gds) and *M. indicus* (163 U/gds) included the lower cellulase activities. Furthermore, cellulase production by these fungi were higher than that by fungi such as *T. reesei* (1.31 U/gds) (8) and **A. nige**r (24.0 U/gds) (34).

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