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Optimization of xylanase production by *Mucor indicus*, *Mucor hiemalis*, and *Rhizopus oryzae* through solid state fermentation

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Abstract

Introduction: Xylan is the main hemicellulosic polymer in a number of lignocelluloses which can be hydrolyzed by xylanolytic enzymes. One of the main ways for enzymes production is solid state fermentation (SSF). The ability of three fungal strains (*Mucor indicus*, *Mucor hiemalis*, and *Rhizopus oryzae*) for xylanase production on wheat bran by SSF was investigated.

Materials and methods: The effects of cultivation temperature, medium moisture content, and cultivation time on the enzyme production were investigated. Experiments were designed with an orthogonal central composite design on three variables using response surface methodology (RSM). Analysis of variance was applied and the enzyme production was expressed with a mathematical equation as a function of the three factors. The optimum operating conditions for the enzyme production was obtained.

Results: For xylanase production by *M. indicus*, *M. hiemalis* and *R. oryzae* the optimum temperatures were 40.0, 43.4 and 43.4ºC respectively. These values were 49.8, 54.2 and 71.8% for moisture percent and 51.3, 53.2 and 53.5 h for cultivation time. The highest enzyme activities per g of dry substrate (gds) were 43.1, 43.8 and 25.9 U/gds for *M. indicus*, *M. hiemalis* and *R. oryzae* respectively.

Discussion and conclusion: All the fungi were able to produce xylanase. Maximum xylanase production was predicted by *M. indicus* and *M. hiemalis* at similar optimum conditions, while *R. oryzae* produced relatively lower xylanase activity even at the best condition.

Key words: *Mucor hiemalis*, *Mucor indicus*, Optimization, *Rhizopus oryzae*, Solid state fermentation, Xylanase

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Introduction

Plants cell wall consists of cellulose, hemicelluloses and lignin. Xylan is the main hemicellulosic polymer in a number of lignocellulosic materials e.g., hardwoods, grasses and most of agricultural residues. Xylan is a heteroglycan composed of a linear chain of xylopyranose residues bound by β(1→ 4) linkages, with a variety of substituents linked to the main chain by glycosidic or ester linkages (1). The xylanolytic enzymes (mainly endoxylanase and β- xylosidase) are used for xylan hydrolysis, which split the xylan backbone into xylooligomers and xylose units (2). Xylanase can reduce the pollution of chlorine used for the bleaching in the paper and pulp industry (2). Moreover, the combination of amylase and xylanase improves quality of bread. The mixture of xylanase, cellulase and pectinase can also be used for clarifying must and juices and for liquefying fruits and vegetables (3).

Solid state fermentation (SSF) and submerged fermentation are considered as two common approaches for industrial production of enzymes (4 & 5). In SSF, the free- flowing water does not exist and the growth of the microorganisms (mainly fungi) takes place on the moist solid materials. The solid matrix of the substrate provides the water needed for microbial activities (6- 8). In some occasions, SSF is preferred to submerged fermentation. The capital and operating costs are lower and the required space is less. Furthermore, the equipment and media are simpler and the amount of wastewater produced is less. In solid state fermentations the substrates are solid and insoluble in nature such as grains, wheat bran and vegetables. This process is more suitable for mycelial fungi than yeasts or bacteria. Submerged fermentations involve soluble feed stocks like glucose, molasses, and malt (5, 9- 11). Filamentous fungi can be used for SSF which can grow on complex solid substrates (12). They can produce several extracellular enzymes, amount of which, depends on the fermentation conditions. In order to decrease the cost of enzyme production, it is necessary to optimize the conditions (13).

In this paper, the ability of three fungal strains (*Mucor indicus*, *Mucor hiemalis*, and *Rhizopus oryzae*) for xylanase production on wheat bran by SSF was investigated. These strains have several industrial applications. They are separated from edible sources with high abilities for ethanol production from cellulosic sources. They can convert substrate containing inhibitors. Their biomass has a high nutritional value and contains considerable amounts of chitosan and glucosamine as valuable components (14- 16). In order to design the experiments at different medium temperatures, moisture contents and cultivation times, Response Surface Methodology (RSM) was applied. The production of xylanase by the fungi was described by a quadratic model as a function of three variables. Furthermore, effects of the parameters on xylanase production were studied and their optimum values for the production of the highest amount of xylanase were obtained.

Materials and methods

Microorganisms and fermentation conditions: Three different species of fungi: *M. indicus* (CCUG 22424), *M. hiemalis* (CCUG 16148) and *R. oryzae* (CCUG 28958) were obtained from the Culture Collection, University of Göteborg, Sweden. The strains were maintained on plates containing 40 g l^{-1} glucose, 10 g l^{-1} peptone and 15 g l^{-1} agar and kept at 32ºC for 5 days to grow and then stored at 4ºC until use. Sterile distilled water containing 0.1% (v/v) Tween 80 (Polyoxyethylene Sorbitan Monooleate) was used to separate spores. An amount of 10 g moistened wheat bran was added to different Erlenmeyer flasks and autoclaved at 121ºC for 20 min. After cooling, the flasks were inoculated with the spore suspension and kept at 30ºC for 7 days to grow the spores on the bran surface. Afterwards, 50 ml sterile distilled water containing 0.1% (v/v) Tween 80 and sterile glycerol were added to the flasks to separate the spores from the bran to be kept in micro tubes at -20ºC.

Fermentation was carried out in 250-ml flasks containing 10 g dry wheat bran (with 50% moisture). They were autoclaved, cooled and inoculated with spore suspensions (1000 spores per g dry bran). The medium moisture content as well as cultivation temperature and time were adjusted. The volume of the spore suspension added to the flask was taken into account for determination of the medium moisture content.

Enzyme extraction and assay: Xylanase activity was measured according to the method presented by Konig et al. (17). At the desired fermentation time, the produced xylanase in each flask was extracted by adding 90 ml distilled water and mixing in a rotary shaker (100 rpm) at room temperature for 30 min. Then the samples were filtered and the filtrates were analyzed for xylanase activity. One unit of xylanase activity (*U*) was defined as the amount of the enzyme required to liberate 1 *µmol* of xylose per min under the assay conditions. The yields were expressed as *U* per gram dry substrate (gds).

Experimental design and statistical analysis: In order to study the effect of temperature, medium moisture content and cultivation time on xylanase production and to find the optimum values to get the highest amount of xylanase, Response Surface Methodology (RSM) was used. An orthogonal central composite design with five levels of –1.682, -1, 0, +1, and +1.682 was applied. The ranges of the studied variables are presented in Table 1.

The treatments suggested by SAS (Statistical Analysis System) software are listed in Table 2. All experiments were performed in duplicate and the average values for xylanase activity were reported. A quadratic model expressing xylanase activity as a function of three variables was fitted to the experimental data. PASW Statistics (Version 18.0, SPSS Inc. Chicago, 2009) and Matlab (Version 7.4, The MathWork Inc. Massachusetts, 2007) softwares were used to perform statistical analyses and xylanase production optimization, respectively.

Table 1- The experimental domain (α = 1.682)

Independent variables	Symbol (unit)	Range and level				
		-α	-1	0	+1	+α
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 xylanase production by the fungi: The experiments suggested by the software with the three variables of temperature, medium moisture content and cultivation time were performed and xylanase activity produced by the fungi was measured (Table 2).

A quadratic equation was fitted to the experimental xylanase activity as follows:

Where a_0 is the intercept, a_1, a_2, and a_3 are linear coefficients, a_4, a_7, and a_9 are squared coefficient, and a_5, a_6, and a_8 are interaction coefficients. Analysis of variance (ANOVA) was used to evaluate the significance of the model and the results are presented in Table 3.

The low values of the probability (p-value), which are obtained by the Fisher F-test, as well as high values of R^2 indicated that the model has a high significance and its adequacy is confirmed (18). Generally, $R^2 > 0.75$ shows the aptness of the model. The adequacy of quadratic models for xylanase production was confirmed, since all R^2 values are higher than 0.9 (Table 3). This indicates that the model explained more than 90% of the variability in the response. The coefficients of Equation 1 and their significance were also determined (Table 4). Lower *P values* show that the corresponding coefficients are more significant (19).

(1) $Enzymes\ activity\ (U/gds) = a_0 + a_1T + a_2W + a_3I + a_4T^2 + a_5TW + a_6TI + a_7W^2 + a_8WI + a_9I^2$

Table 2- Experimental design and results for xylanase production (U/gds) by the fungi.

NO.	Uncoded level			*M. indicus*	*M. hiemalis*	*R. oryzae*
	T	W	I			
1	30.00	45.00	40.00	30.00 ± 0.64	11.06 ± 0.06	6.00 ± 0.50
2	30.00	45.00	60.00	27.00 ± 1.00	17.32 ± 0.05	7.50 ± 0.50
3	30.00	65.00	40.00	13.00 ± 0.25	7.08 ± 0.09	17.00 ± 0.40
4	30.00	65.00	60.00	7.50 ± 0.10	17.36 ± 0.20	22.50 ± 0.90
5	40.00	45.00	40.00	37.00 ± 0.80	35.66 ± 0.61	12.00 ± 0.00
6	40.00	45.00	60.00	39.50 ± 0.50	31.33 ± 0.51	12.50 ± 0.70
7	40.00	65.00	40.00	30.50 ± 0.20	22.61 ± 0.42	21.50 ± 0.31
8	40.00	65.00	60.00	28.50 ± 0.15	36.27 ± 1.00	23.00 ± 0.28
9	26.59	55.00	50.00	18.50 ± 0.50	9.40 ± 0.08	12.00 ± 0.00
10	43.41	55.00	50.00	42.50 ± 0.50	42.43 ± 0.10	19.00 ± 0.08
11	35.00	38.18	50.00	37.00 ± 0.14	27.19 ± 0.00	8.00 ± 0.10
12	35.00	71.82	50.00	20.00 ± 0.00	24.23 ± 0.21	23.00 ± 0.00
13	35.00	55.00	33.18	31.50 ± 0.50	20.25 ± 0.70	14.00 ± 0.00
14	35.00	55.00	66.82	29.00 ± 0.00	32.41 ± 0.01	15.00 ± 0.08
15	35.00	55.00	50.00	38.00	29.06	14.00
16	35.00	55.00	50.00	39.00	31.54	14.00
17	35.00	55.00	50.00	40.00	32.52	18.00
18	35.00	55.00	50.00	32.00	32.65	14.00
19	35.00	55.00	50.00	41.00	34.90	18.00
20	35.00	55.00	50.00	41.00	34.24	14.00
21	35.00	55.00	50.00	38.00	35.73	17.00
22	35.00	55.00	50.00	39.00	37.68	17.00
23	35.00	55.00	50.00	33.00	30.75	15.00

Table 3- Regression analysis (ANOVA) for xylanase production by the fungi.

Fungus	SS_M	SS_R	DF_M	DF_R	MS_M	MS_R	*F value*	*P value*	R^2
M. indicus	1835.9	117.3	9	13	203.9	9.0	22.6	0.000	0.940
M. hiemalis	1948.5	117.7	9	13	216.5	9.0	23.9	0.000	0.943
R. oryzae	449.5	34.9	9	13	49.9	2.7	18.6	0.000	0.928

SS: Sum of Square; DF: Degree of Freedom; MS: Mean Square. Subscripts: M: Model; R:Residual

Table 4- Regression coefficients of equation 1 for xylanase production by the fungi

Fungus	*M. indicus*		*M. hiemalis*		*R. oryzae*	
Term	Coefficient	*P value*	Coefficient	*P value*	Coefficient	*P value*
a_0	-220.8	0.015	-356.18	0.001	-77.25	0.095
a_1	6.545	0.028	12.463	0.000	1.956	0.198
a_2	2.422	0.072	2.521	0.063	0.773	0.273
a_3	2.851	0.035	2.562	0.055	0.561	0.412
a_4	-0.126	0.001	-0.130	0.001	-0.001	0.933
a_5	0.048	0.043	-0.010	0.632	-0.015	0.218
a_6	0.022	0.309	-0.018	0.412	-0.013	0.301
a_7	-0.039	0.000	-0.033	0.001	0.000	0.933
a_8	-0.009	0.425	0.028	0.023	0.006	0.301
a_9	-0.032	0.001	-0.031	0.001	-0.004	0.362

For xylanase production by *M. indicus*, the second order term of temperature, moisture content and time were highly significant ($p<<0.05$). The linear term of moisture content, the interaction term of moisture content and time and the interaction term of temperature and time were insignificant and the remaining terms were equally significant.

Xylanase production by *M. hiemalis* was strongly dependent on the linear term of temperature and the second order term of temperature, moisture content and time. Furthermore, the interaction effect of moisture content and time was significant and the effects of the remaining terms were insignificant.

For *R. oryzae*, all the terms involved in Equation 1 were insignificant.

When a term of Equation 1 has a high significance, it would be a limiting factor. This means that a small change in its value would considerably change the enzyme production (20). The magnitude and the sign of coefficients indicated the effects of the parameters on xylanase production.

Effect of temperature: Temperature is reported to be the most important factor influencing the SSF (21). The effect of temperature on xylanase production by three fungi was investigated. For this purpose, the moisture content was chosen as 55% (the central point of the studied domain) and the variation of xylanase activity with time for different values of temperatures were studied.

Fig. 1 (a, b, and c) shows the xylanase activity as a function of time at five coded levels of temperatures for *M. indicus*, *M. hiemalis* and *R. oryzae*, respectively.

According to Fig. 1, at all cultivation times, xylanase production increased with increasing the temperature for the three fungi. For *M. hiemalis*, the result for the temperature of 26.6ºC was not plotted, since the corresponding xylanase activities showed negative values. The optimum temperature for the highest xylanase production was about 40ºC.

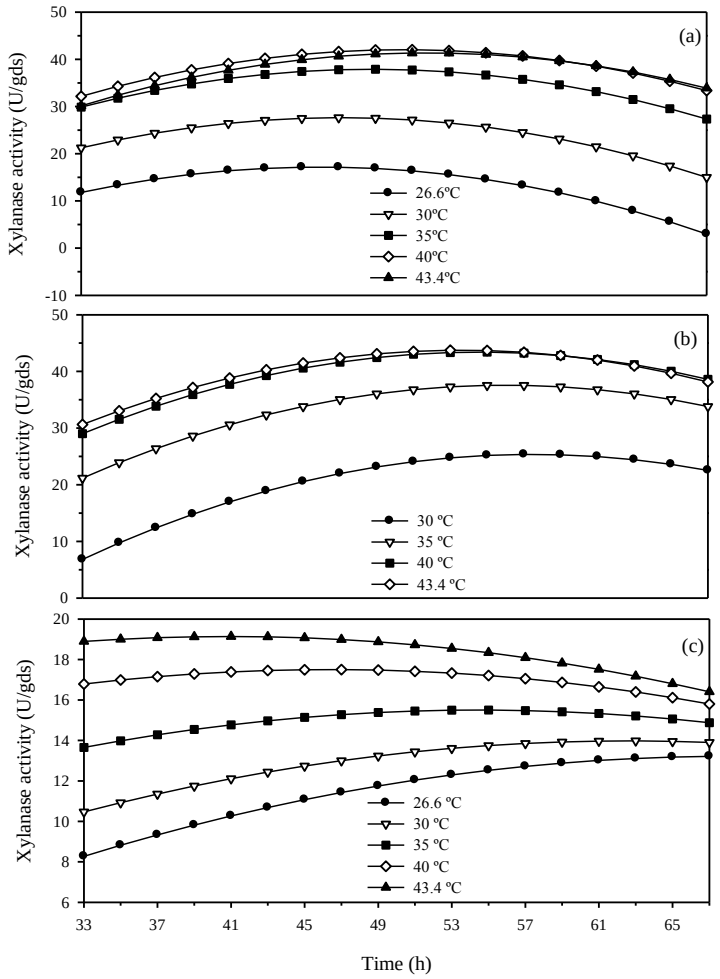

Fig. 1- The effect of temperature and time on xylanase production by (a) *M. indicus,* (b) *M. hiemalis,* and (c) *R. oryzae* at w=55%

Effect of medium moisture content: For xylanase production, medium moisture content has been reported to be an important factor in SSF (10). Low and high medium moisture contents influence the growth of the fungus, enzyme biosynthesis (22 & 23) and the physical properties of the solid substrate (24). Therefore, it is necessary to find the optimum moisture content, which is dependent on the type of the substrate end product, and nutrient requirements of the fungus (25). In this paper, the effect of moisture content on xylanase production was studied in the range of 38 to 72%. At the fixed temperature of 35ºC (the central point of the studied temperature range), xylanase production by *M. indicus*, *M. hiemalis* and *R. oryzae* was studied as a function of cultivation time and medium moisture content and the results were shown in Fig. 2.

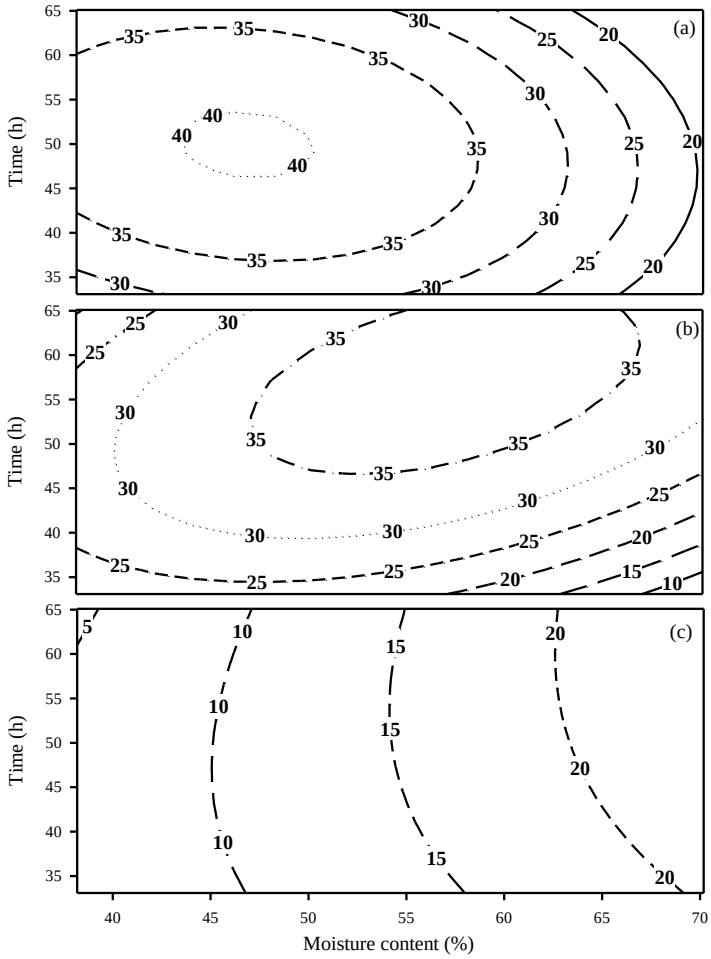

Fig. 2- The effect of medium moisture content and time on xylanase production by (a) *M. indicus*, (b) *M. hiemalis*, and (c) *R. oryzae* at T=35ºC

For all times, with increasing moisture content, xylanase production by *M. indicus* and *M. hiemalis* increased to a maximum value and then decreased. According to Fig. 2c, xylanase production by *R. oryzae* increased with increasing the moisture content for all values of cultivation time.

Effect of cultivation time: According to Fig. 1A, xylanase production by *M. indicus* increased with increasing cultivation time to a maximum value and then decreased. The maximum xylanase activity was observed at the intermediate stages of cultivation time. Xylanase production by *M. hiemalis* and *R. oryzae* showed a parabolic behavior with a maximum point of time. By increasing the medium moisture content, the descending branch of the parabola, gradually disappeared and ascending behavior with time was observed for xylanase production (Fig. 1B- C).

Optimum conditions for xylanase production: According to Table 2, xylanase production by the fungi varies considerably with cultivation time, temperature and moisture content. Thus, it is necessary to find the conditions at which the highest enzymes activity could be obtained.

Table 5- Optimum conditions and enzyme activity for production of xylanase by the fungi.

Parameter's values	*M. indicus*	*M. hiemalis*	*R. oryzae*
T (ºC)	40.0	43.4	43.4
W (%)	49.8	54.2	71.8
I (h)	51.3	53.2	53.5
Activity (U/gds)	43.1	43.8	25.9

The optimum conditions for xylanase production by the fungal strains are presented in Table 5. Accordingly, xylanase production by *M. indicus* and *M. hiemalis* at the optimum conditions was the highest (43 U/gds), while *R. oryzae* showed the lowest xylanase activity (26 U/gds). The optimum temperature and cultivation time for the fungi were in the range of 40-43.4ºC and 51.3- 53.5 h, respectively. The optimized medium moisture content for both *Mucors* was in the range of 49.8-54.2%, while it was at its highest value (71.8%) for *R. oryzae*.

Discussion and conclusion

Since the fungal growth was affected by temperature, the enzyme production is dependent on temperature (21, 26 & 27). The physiological changes due to high temperatures in enzyme production are not well known. However, it is reported that high temperatures may limit the synthesis of essential proteins for fungal growth and other physiological processes (26). The optimum temperature for the highest xylanase production was about 40ºC. Similar trend and value (the optimum temperature of 40ºC) were observed for xylanase production by *Aspergillus niger* via SSF (27).

For xylanase production, different values have been reported as the optimum moisture content. For instance, the optimum initial moisture content in production of xylanase by *A. niger* was 43%, while this amount was 83% using *Paecilomyces thermophila* (28 & 29). Generally, moisture content is an important factor affecting enzymes production. Low moisture contents reduce mass transfer and solubility of nutrients and increase water tension, which decreases metabolic and enzymatic activity. In contrast, high moisture contents decrease oxygen transfer and porosity of the medium. It also changes the structure of substrate particles and clumps the medium affecting aeration and fungal growth (20, 30- 32). There are several reports on the favorable effect of high moisture contents on enzyme production. It may be resulted from the fact that at high moisture contents, the fungal growth is faster and the enzyme production initiates earlier (31 & 33).

According to the results, cultivation time affects xylanase production by the fungi. Short cultivation times provide conditions for the economical enzymes production. Generally, xylanase production increased with increasing time up to a certain level and then decreased. Similar trend was observed in the production of xylanase by *A niger* (27). This reduction may be due to the rapid degradation of xylanase by non-specific protease enzyme secreted by the microorganism (27). Prolonged agitation and extraction of denaturing agents from the wheat barn may lose the enzyme activity (34). Accordingly, stopping the fermentation at a desirable time is of a

considerable importance. Existence of some byproducts may inhibit the fungal growth and affect the enzyme formation (35).

Maximum xylanase production was predicted by *M. indicus* (43.1 U/gds) and *M. hiemalis* (43.8 U/gds) at similar optimum conditions, while *R. oryzae* produced relatively lower xylanase activity (25.9 U/gds) even at the best conditions.

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بهینه‌سازی تولید زایلاناز توسط *موکور ایندیکوس*، *موکور هیمالیس* و *رایزوپوس ارایزه* با استفاده از تخمیر حالت جامد

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چکیده

مقدمه: زایلان یکی از مهم‌ترین پلیمرهای همی‌سلولزی موجود در برخی از مواد لیگنوسلولزی است که با کمک آنزیم‌های زایلانولیتیک هیدرولیز می‌شود. یکی از مهم‌ترین روش‌ها برای تولید آنزیم‌ها تخمیر حالت جامد است. توانایی سه گونه قارچی *موکور ایندیکوس*، *موکور هیمالیس*، و *رایزوپوس ارایزه* برای تولید زایلاناز بر روی سبوس گندم با کمک تخمیر حالت جامد بررسی شد.

مواد و روش‌ها: اثر دمای محیط کشت، میزان رطوبت آن و زمان کشت بر تولید آنزیم بررسی شد. آزمایش‌ها با استفاده از طراحی ترکیب مرکزی ارتوگونال بر روی سه متغیر با استفاده از روش سطح پاسخ طراحی شد. تحلیل واریانس به کار گرفته شد و تولید آنزیم با استفاده از یک معادله ریاضی به عنوان تابعی از سه متغیر بیان شد. شرایط بهینه عملیاتی برای تولید آنزیم به دست آورده شد.

نتایج: برای تولید زایلاناز با *م. ایندیکوس*، *م. هیمالیس*، و *ر. اورایزه*، مقدار بهینه دما به ترتیب ۴۰، ۴۳/۴ و ۴۳/۴ درجه سانتی‌گراد به دست آمد. بهینه رطوبت به ترتیب ۴۹/۸، ۵۴/۲ و ۷۱/۸ درصد و مقدار بهینه زمان کشت ۵۱/۳، ۵۳/۲ و ۵۳/۵ ساعت بود. بالاترین فعالیت آنزیمی توسط سه گونه قارچی یاد شده به ترتیب ۴۳/۱، ۴۳/۸ و ۲۵/۹ واحد به ازای هر گرم سوبسترا به دست آمد.

بحث و نتیجه‌گیری: همه گونه‌های قارچی قادر به تولید زایلاناز بودند. بیش‌ترین میزان تولیدی زایلاناز توسط *م. ایندیکوس* و *م. هیمالیس* در شرایط بهینه مشابهی انجام شد، در حالی که *ر. اورایزه* حتی در بهترین شرایط میزان کمابیش کمتری زایلاناز تولید کرد.

واژه‌های کلیدی: بهینه‌سازی، تخمیر حالت جامد، *رایزوپوس ارایزه*، زایلاناز، *موکور ایندیکوس*، *موکور هیمالیس*

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