Effect of medium components and time of cultivation on chitin production by *Mucor circinelloides (Mucor javanicus* IFO 4570) - A factorial study

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Summary

Chitin production of mycelia from *Mucor circinelloides* (*Mucor javanicus*) was studied with a two-level factorial design in all combinations of the following factors in the culture medium: time of cultivation, concentration of D-glucose, L-asparagine and thiamine. Chitin was characterised by infrared and nuclear magnetic resonance spectroscopy. The four factors showed statistically significant (95%) positive main effects on chitin production, without interactions between them. The highest chitin yield (23.9%) was obtained with the medium containing 60 g/l of glucose, 3 g/l of asparagine and 0.008 mg/l of thiamine and is comparable to the highest in the literature.

Key words Chitin, Chitosan, Zygomycetes, *Mucor circinelloides*, *Mucor javanicus*

Efecto de los componentes del medio y el tiempo de cultivo sobre la producción de quitina por *Mucor circinelloides (Mucor javanicus* IFO 4570). Un estudio factorial

Resumen

La producción de quitina a partir del micelio de *Mucor circinelloides* (*Mucor javanicus* IFO 4570) fue determinada a través de un estudio factorial de dos niveles, variando el tiempo de cultivo (24 ó 72 h) y las concentraciones de D-glucosa (20 ó 60 g/l), L-asparagina (1 ó 3 g/l) y tiamina (0,002 ó 0,008 mg/l) del medio. La quitina obtenida fue caracterizada a través de espectroscopia de infrarrojos y resonancia magnética nuclear. Los cuatro factores estudiados mostraron efectos estadísticamente significativos (95%) sobre la producción de quitina, sin interacción entre ellos. El rendimiento más alto de quitina (23,9 %) fue obtenido con el medio conteniendo 60 g/l de D-glucosa, 3 g/l de asparagina y 0,008 mg/l de tiamina. La alteración del tiempo de cultivo de 24 a 72 h afectó discretamente los rendimientos. El alto rendimiento de quitina obtenido en este trabajo es equivalente, y en algunos casos superior, a los rendimientos encontrados para otros microorganismos publicados en la literatura.

Palabras clave Quitina, Quitosano, Zygomycetes, *Mucor circinelloides*, *Mucor javanicus*

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Chitin, which is perhaps the second most important polysaccharide after cellulose, is a straight homopolymer composed of ß-1,4-linked N-acetylglucosamine units, with a three-dimensional α-helical configuration stabilised by intramolecular hydrogen bonding [1]. It is an abundant renewable natural resource obtained from insects, algae and marine invertebrates. Almost 10% of the global landings of aquatic species consist of organisms rich in chitinous material (10-55 % on a dry weight basis), including squids, crabs, shrimps, cuttlefish and oysters. Mucoraceous fungi which are known to contain chitin and the deacetylated derivate, chitosan, in cell walls (22 to 44%), have been used for commercial production [2-4]. However, in comparison with marine sources, which yield more than 80,000 metric tons of chitin per year [3,5], chitin production from fungal waste is negligible.

Chitin and its derivatives hold great economic value because of their versatile biological activities and chemical applications. Deacetylated chitin oligomers act as hydrating agents in cosmetic and hair care products [6] and as floculating and clarification agents in the food industry [7]. In agriculture, chitin derivatives have been used as fertilisers and fungicides [6,8]. Recently, some interesting biomedical applications have been proposed. Chitosan has been found to reduce blood cholesterol levels, accelerate wound healing, stimulate the immune system, and also act as an antitumoral and anti-thrombogenic agent [7,9-11].

Conventional preparation of chitin from marine waste material involves demineralisation and deproteination with strong acids or bases [10,12]. However, these chemicals may cause partial deacetylation of chitin and hydrolysis of the polymer, leading to inconsistent physiological properties in the end product [2,10,12,13]. Since the nature of the end product also depends on the processing method, filamentous fungi are an attractive source of chitin for industrial applications because specific products can be manufactured under standardised conditions.

The chitin content of fungal cell walls varies between different species of fungi. The search for alternative sources of chitin has brought to attention the filamentous fungi of the Zygomycetes class, whose cell walls contain native chitin and chitosan, performing protective and supportive functions [14]. It appears that the cell wall's chitin content is generally higher in Zygomycetes, as illustrated by the reported chitin amounts in *Mucor mucedo*, *Rhizomucor miehei*, *Rhizopus oryzae*, *Phycomyces blakesleeanus* [15] and *Cunninghamella elegans* [16].

Several simple models have been used to describe chitin production by microorganisms. Investigations on the influence of growth time on the contents of chitin and chitin derivatives by *Mucor* have been reported. However, these studies usually adopt the univariate approach, studying only one variable at a time, as in the work of Synowieck and Al-Khateeb [17], for example. The alternative multivariate approach, where all factors are considered simultaneously on an equal basis, is becoming increasingly popular for the analysis and optimisation of several experimental systems [18]. Multivariate experimental designs have the considerable advantage of providing information concerning not only the individual effect of each factor but also the possible interactions between all factors, which often prove very significant.

Recently, in a preliminary study, the influence of carbon and nitrogen sources on chitin production by *C. elegans* was evaluated with a factorial design analysis. It was found that chitin production was affected mostly by L-asparagine, followed by D-glucose and thiamine. No interaction effects between these three factors were observed. A fourth factor, time of cultivation, displayed only a minor effect [16].

Here we report on a similar study based on *Mucor javanicus* (Zygomycetes). The nature and amount of carbon and nitrogen sources, represented by the concentrations of of D-glucose, L-asparagine and thiamine, plus time of cultivation, were chosen as the basis of a two-level full factorial design centered on the medium proposed by Hesseltine and Anderson [19] for Mucorales cultivation.

Materials and methods

Materials. The standard chitin was obtained from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade.

Microorganisms. Strains of *M. javanicus* (IFO 4570) were obtained from the live collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Japan. The organism was maintained on potato dextrose agar (Difco).The spores were harvested from cultures grown for 5-7 days at 28 °C, in Petri dishes containing Difco's potato dextrose agar. A stock suspension was prepared and adjusted to 1.0 x 10^7 spores/ml, using an improved Neubauer haemacytometer chamber for counting. The solution was then stored at 4 °C.

Growth conditions. The cultures were grown in synthetic media with compositions defined by variations around the medium proposed by Hesseltine and Anderson [19], which contains 40 g/l glucose, 2 g/l asparagine, 0.005 mg/l thiamine, 0.50 g/l potassium phosphate, and 0.25 g/l magnesium sulphate. This medium was set as the central point of the experimental design, and the concentrations of D-glucose, L-asparagine, thiamine and the time of cultivation were varied symmetrically around this point according to the 2^4 factorial design given in Table 1. An estimate of pure experimental error was calculated from eight replicate runs, at synthetic media corresponding to a half-fraction of the complete factorial. The recorded responses of interest were biomass yields and percent chitin yields.

Mycelia were produced in 250 ml Erlenmeyer flasks containing 100 ml of the desired medium and autoclaved for 20 min at 121 °C. The flasks were inoculated with one millilitre spore suspension (1.0 x 10^7 spores/ml) at 25 °C on a reciprocal orbital shaker (Taitec, Br–300L model) at 120 strokes/min. After the desired growth time, the mycelia of *M. javanicus* were harvested by filtration through a silkscreen nylon filter, and washed with distilled water until a clear filtrate was obtained. The mycelia were then lyophilized and the biomass determined.

Isolation of chitin. Chitin extraction was carried out by an alkali-acid treatment, using the procedure described by Sangar and Dugan [20] and modified by Campos-Takaki [21]. One gramme of lyophilised mycelia was first deproteinized by treatment with 5 ml of 2M NaOH for 15 h at room temperature. The alkali-insoluble fraction was separated by centrifugation (2000 x *g*, 10 min) and the residue washed three times with distilled water, after which it was treated with 5 ml of 0.5M H_2SO_4 (16 h, 50 °C) and centrifuged (2000 x *g*, 10 min). The new residue was washed three more times with distilled water. The sample was treated with 5 ml of 2M NaOH for 30 min at room temperature, filtered, washed three times with distilled water and centrifuged (2000 x *g*, 10 min). The residue, crude chitin, was finally dialysed for 72 h against water, yielding the pure fragments.

Table 1. Design matrix for the factorial experiments used to study the influence of four factors on chitin production by *Mucor javanicus*. The first 16 runs correspond to all possible combinations of the two levels of the three concentration factors, plus the growth time. The seventeenth is the central point, with experimental conditions set at the average values of the two extreme levels [19].

Run	Factor levels			
	G[a]	A[b]	T[c]	t[d]
1	–1	–1	–1	–1
2[e]	+1	–1	–1	–1
3[e]	–1	+1	–1	–1
4	+1	+1	–1	–1
5[e]	–1	–1	+1	–1
6	+1	–1	+1	–1
7	–1	+1	+1	–1
8[e]	+1	+1	+1	–1
9[e]	–1	–1	–1	+1
10	+1	–1	–1	+1
11	–1	+1	–1	+1
12[e]	+1	+1	–1	+1
13	–1	–1	+1	+1
14[e]	+1	–1	+1	+1
15[e]	–1	+1	+1	+1
16	+1	+1	+1	+1
17[f]	0	0	0	0

[a] Concentration of D-glucose: 20 g/l at level –1; 40 g/l at level 0; 60 g/l at level +1
[b] Concentration of L-asparagine: 1 g/l at level –1; 2 g/l at level 0; 3 g/l at level +1
[c] Concentration of thiamine: 0.02 mg/l at level –1; 0.05 mg/l at level 0; 0.08 mg/l at level +1
[d] Growth time: 24 h at level –1; 48 h at level 0; 72 h at level +1
[e] Replicated runs
[f] Medium of Hesseltine and Anderson [19]

Analytical procedures. Chitin samples were characterised by infrared and ^{13}C-nuclear magnetic resonance (^{13}C–NMR) spectral analysis.

Infrared spectroscopy - Two milligrammes samples of fungal chitin and standard chitin (Sigma Chemical Co., USA) which had been dried overnight at 60 °C under reduced pressure were thoroughly blended with 100 mg of KBr, to produce 0.5 mm thick disks. The disks were dried for 24 h at 110 °C under reduced pressure. Infrared spectra were recorded with a Bruker 66 FT-infrared spectrometer (DQF - Federal Pernambuco University, Recife, Brazil) using a 100 mg KBr disk for reference. The intensity of maxima of the infrared absorption bands were determined by the baseline method.

^{13}C-NMR spectroscopy - Chitin samples were dissolved in D_2O in 5 mm tubes at pD 4 (70 mg/ml). Deuterium resonance was used as a field-frequency lock and chemical shifts were referenced to internal sodium 3-(trimethylsilyl) propionate-d_4. ^{13}C-NMR spectra were recorded with a 75.4 MHz Varian-Unity Plus 300 Spectrometer (DQF - Federal Pernambuco University, Recife, Brazil), using 64K data points and a spectral width of 16.000 Hz. Relative resonance intensities were determined by the instrument's software.

Results

Table 2 shows the biomass and chitin yields obtained in the experiments. Runs 1-8 correspond to a cultivation time of 24 h. Runs 9-16 are for 72 h of cultivation, and run 17 corresponds to the medium of Hesseltine and Anderson [19].

The analysis of a two-level factorial design begins with the calculation of the main and interaction effects of all factors [18,22]. All these effects are calculated as differences between two averages, each one containing half of the experimental responses. With four factors, each average contains eight responses. The four main effects are simply the difference between the average response at the higher level of the factor in question and the corresponding average at the lower level. To obtain the main effects, one thus applies to the responses of Table 2 the signs of the corresponding columns in Table 1, performs the sum, and divides the result by 8. For example, the main effect of factor A (L-asparagine) on chitin yield is given by

$$A = [-(7.0) - (8.6) + (18.0) + (17.5) - (11.0) - (12.3) + (19.5) + (21.3) - (8.5) - (10.2) + (18.5) + (19.8) - (12.6) - (16.1) + (21.6) + (23.9)\ ] / 8 \cong 9.23$$

Where the average values of the eight duplicate responses have been substituted. The interaction effects are linear combinations of the form

$$E = \frac{1}{8}\sum_i^n a_i y_i \qquad (1)$$

where y_i is the average response in run i and the coefficient a_i is set equal to plus or minus one, depending on the sign of the product of the columns of the factors involved. For example, to calculate the four-factor interaction GATt, the sign of run no.7 is given by (G)(A)(T)(t) = (–1)(+1)(+1)(–1) = +1. The values of all main and interaction effects are presented in Table 3.

Since each effect is given by a linear combination of independent observations, the variances at each experimental setting can be combined into a single value representing the variance of an effect:

$$\hat{V}(effect) = \sum_i a_i^2 s_i^2 \qquad (2)$$

where $a_i = \pm 1/8$ is the coefficient of the ith response on the right-hand side of Equation 1 and s_i^2 is an estimate of the variance of that response, obtained in this case from the duplicate runs given in Table 2. The square root of $\hat{V}(effect)$ is the standard error of an effect [18,22].

Substituting into Equation 2 the variances calculated from the duplicate runs in Table 2, a standard error of 0.56% is obtained for the value of an effect. According to a t-test based on this error, only the four main effects are statistically significant at the 95% confidence level. Their values are shown in boldface in Table 3.

Table 2. Biomass and chitin yields from *Mucor javanicus* mycelia. Experimental conditions as defined in Table 1.

Run	Biomass (g/l)	Chitin Yield (%)
1	0.98	7.00
2[a]	1.76 1.60	9.1 8.0
3[a]	2.25 1.95	17.1 15.2
4	3.61	17.5
5[a]	1.38 1.22	11.8 10.2
6	2.32	12.3
7	3.75	19.5
8[a]	6.29 6.75	20.5 22.1
9[a]	1.36 1.84	7.7 9.3
10	2.80	10.2
11	3.80	18.5
12[a]	6.37 5.95	21.0 18.5
13	1.91	12.6
14[a]	3.28 3.72	15.0 17.2
15[a]	5.00 5.42	20.9 22.3
16	8.71	23.9
17[a, b]	2.45 3.03	17.1 19.3

[a] Duplicate runs
[b] Control medium [19]

Table 3. Main and interaction effects calculated from the chitin yield values given in Table 2. Units are percent chitin yield relative to total biomass. Statistically significant effects are shown in boldface. Notation as in Table 1.

Factor	Chitin yield (%)
Overall average	15.91
Main effects	
G	3.12
A	10.27
T	5.29
t	3.50
Two-factor interactions	
GA	1.10
GT	1.65
Gt	1.62
AT	0.85
At	1.37
Tt	1.57
Three-factor interactions	
GAT	–0.01
Gat	–0.24
GTt	0.33
Att	–0.27
Four-factor interaction	
GATt	-0.42

Discussion

Since there are no significant interactions, the main effects can be interpreted separately, one at a time. They are all positive, indicating that chitin yield increases when any of the four factors is changed from the lower to the higher level. The most significant effect is due to L-asparagine (+10.27%), after which come those of thiamine (+5.29%), and of growth time and D-glucose (3.50 and 3.12, respectively). The highest yield (23.9%), is obtained with run 16, when, not unexpectedly, all four factors are at their higher levels. However, this value is quite close to the average yield of run no. 8 (21.3%). Since the two runs differ only in their cultivation times, and that of run no. 8 is only 24 h (as compared with 72 h for run 16), on practical terms run 8 should undoubtedly be preferred.

Results from traditional chitin sources like mollusk and crustacean shells have been widely reported [23,24]. However, recent advances in fermentation technology suggest that large-scale culturing of an organism containing chitin and deacetylated chitin might be an attractive route for producing this polymer. Several investigations have indeed shown that some strains of *Mucor* [25,26], *Aspergillus* [27], *Absidia* [28] and *Cunninghamella* [16] contain significant quantities of chitin and/or chitosan.

Screenings of a number of chitin-producing fungi showed that the mycelia of *Mucor* have been frequently reported to contain significant amounts of chitin and derivates as cell-wall components [2,17,29]. Studies on the use of chitin in wound healing also demonstrated relatively high yields of chitin plus chitosan (about 52%) from *M. mucedo* cell walls, at different cultivation conditions [15]. Among the *Mucor* chitin-producing species producers, *Mucor rouxii* has been the most extensively researched. Synowiecki and Al-Khateeb [17] found that mycelia of *M. rouxii* from two days old cultures might be used as a source of chitin, yielding 8.9% on a dry mycelia weight basis. Knorr [30] obtained a similar chitin content of 9% dry cell wall, while Bartinick-Garcia and Nickerson [25] suggested that 8.5% of chitin can be extracted from *M. rouxii* yeasts.

As reported here, *M. javanicus* yields up to 23.9% chitin, on a dry mycelia weight basis, a result superior to most *Mucor* yields reported in the literature. It should be noted, however, that still better results (28,8%) have been obtained in our own laboratory in a study using *C. elegans*, also based on a factorial design [16]. Reports that this organism can be readily cultivated on simple nutrients have led us to study the production and isolation of microbial chitin as an alternative to the shellfish-derivated product.

The presence of chitin in fungal materials from *M. javanicus* was supported by the characteristic amide absorbance at 1655 cm^{-1} and 1560 cm^{-1}, the amide I and II bands in the FT-infrared spectra, respectively, which are also seen with standard chitin. Infrared spectra of the chitin samples were similar to those reported in the literature [2,15,16,31].

On the other hand, the most significant parts of these spectra are those showing the amide bonds at approximately 1655, 1560 and 1313 cm^{-1}, which could be assigned to the C=O stretching, the N-H deformation in the CONH plane and the CN bond stretching plus CH_2 wagging. This is in agreement with the report by Shigemasa et al. [31] that the chitin structure contains two types of amide group and both form C=O---H-N intermolecular bonds, but one is also an acceptor for the CH_2OH group.

The presence of chitin in *M. javanicus* mycelia was further confirmed by a 75.4 MHz peak in the ^{13}C-NMR spectrum, which is seen also with standard chitin (Sigma). From a comparison with the work of Varum et al. [32,33], the resonance regions were identified as C-1 (95.8-97.7 ppm), C-2 (56.9-59.7 ppm), C-3 (72.3-74.2 ppm), C-4 (78.6-78.8 ppm), C-5 (75.1-76.7 ppm), C-6 (63.1-63.5 ppm), acetyl (24.6-25.0 ppm) and carbonyl (177.2-177.4 ppm) from N-acetylglucosamine units. The presence of these well-resolved peaks is taken as a confirmation of the occurrence of chitin chains.

The present work indicates that *M. javanicus* mycelia are a promising source of chitin. The yields reported here could also be suitable for optimization of the isolation parameters of fungal chitin as an alternative to the shellfish-derived product.

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