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Proceeding Paper

Inoculum Production of *Monascus purpureus* with *Chenopodium quinoa* in Submerged Culture †

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Abstract: *Monascus purpureus* is widely used in different substrates for the production of secondary metabolites of interest and requires an inoculum of easy handling. For this reason, this research provides the information necessary to produce an inoculum of *M. purpureus* for future research. A maximum production of 34.83 mg of glucosamine/g dry weight was obtained under the conditions of NaCl 0.01%, pH 6.0 and agitation of 120 rpm ($p < 0.05$); additionally, in the fungus, an adaptation of the final medium which would give a rapid propagation in solid state fermentation with quinoa, was generated.

Keywords: *Monascus purpureus*; submerged culture; biomass; N-acetyl glucosamine; inoculum

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1. Introduction

Natural dyes reflect freshness and safety in food as well as being indicators of aesthetic and sensory values, unlike synthetic dyes, which have been linked to health problems. For this reason, the popularity of natural dyes is increasing among consumers [1]. One producer of natural pigments that has been used in China for hundreds of years is *Monascus purpureus*, which through solid fermentation is capable of producing food products through fermentation that are used for food coloring, seasoning and preserving. The fungus also produces monacolin K, which regulates the production of cholesterol in humans and animals, as well as other undesirable metabolites, such as citrinin, that at high doses can become toxic [2]. Low NaCl concentration contributes to the biosynthesis of secondary metabolites such as pigments, and inhibits the synthesis of undesirable compounds in fermentation [3].

On the other hand, quinoa has been cultivated since pre-Hispanic times in Peru; it is considered by the Food and Agriculture Organization of the United Nations (FAO) to be a 'food of the future' because of its good adaptability and high protein content [4]. In addition to containing good levels of fatty acids, vitamins, minerals, dietary fibres, bioactive compounds and phenolic compounds, quinoa is a gluten-free pseudocereal, rich in protein for people with celiac sensitivity and disease [5].

Taking into consideration the natural components of *M. purpureus* and the nutritional source of quinoa, a new fermented food that has characteristics of both could be generated; however, this would require a suitable inoculum and rapid propagation. Through

the production of submerged culture, mycelium production time is reduced and allows automation in industrial production. Many species of edible mushrooms produce their "seeds" through submerged culture, such as *Flammulina velutipes*, *Hypsizigus marmoreus*, *Pleurotus eryngii* and *Lentinula edodes* [6]. Therefore, the objective of this research was to produce a mycelial biomass of *M. purpureus* in submerged culture, with quinoa flour as substrate, at varying conditions of pH, agitation and NaCl concentration, so that the best conditions for inoculum preparation could be established for future research.

2. Materials and Methods

2.1. Strain of Interest

M. purpureus 2955 (from the Spanish Type Culture Collection (CECT)) strain was reactivated in potato dextrose broth and incubated for a period of 3 days. Later, the inoculum obtained was seeded in potato dextrose agar (PDA) medium and incubated for 5 days at 30 °C. After the incubation time, cuts were made with a 5 mm diameter punch and seeded in the center of the medium formulated with quinoa, then incubated at 30 °C until the fungus invaded the plate. These plates were refrigerated at 4 °C to obtain the material necessary for the research.

2.2. Evaluation of the Parameters rpm, pH and Sodium Chloride Concentration

In 250-mL flasks, 4 g of quinoa flour was added to 100 mL of distilled water with sodium chloride in different concentrations (0, 0.01, 0.05, 0.1 M), and adjusted to different pH levels (5, 6, 7). Flasks were sterilized, and after they had cooled to room temperature, 0.5 mL of inoculum was added and incubated with agitation of 100, 120, and 140 rpm according to the treatment, at a constant temperature of 30 °C, for 5 days, in darkness. After the time had elapsed, the pellets formed were filtered and dried at 60 °C to a constant weight. The dried samples were ground and stored at 4 °C until the respective analyses of N-acetylglucosamine concentration (mg) and pigments. The flasks were prepared in triplicate.

2.3. Quantification of N-Acetylglucosamine

A dry sample (0.1 g) was taken and submitted to acid hydrolysis (H_2SO_4 72%) for 4 h, then diluted to 3% and kept at 100 °C for 2 h. It was cooled down and neutralized with NaOH 10 N, then centrifuged. A volume of 0.5 mL of the supernatant was taken, and 0.25 mL of solution A (4% (*v/v*) acetylacetone in 1.25 N sodium carbonate) was added and then left at 90 °C for 1 h. Subsequently, it was cooled, and 0.8 mL of 96% ethanol was taken and added to 0.25 mL of Ehrlich's reagent. The amount of N-acetylglucosamine was estimated from the calibration curve of the N-acetylglucosamine standard.

2.4. Statistical Analysis

The results for N-Acetylglucosamine (mg/g) were analysed by ANOVA using the software Statgraphics 19 (Statpoint Technologies Inc., Warrenton, VA, USA). The means were analysed using Tukey's test, considering a significance level of $p < 0.05$ throughout the study.

3. Results and Discussion

Table 1 presents the results from the analysis of variance (ANOVA) and Tukey's test, showing significant differences in mean effects and interactions.

All F-ratios are based on the residual mean square error. Since seven *p*-values are less than 0.05, these factors have a statistically significant effect on N-acetylglucosamine (N-AcG) at the 95.0% confidence level.

Table 1. Analysis of variance for N-AcG (mg/g)—type III sums of squares.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
main effects					
A:rpm	100.82	2	50.41	855.15	0.0000
B:pH	5.11	2	2.56	43.42	0.0000
C:NaCl (M)	164.28	3	54.76	928.97	0.0000
interactions					
AB	19.52	4	4.88	82.82	0.0000
AC	1.12	6	0.186	3.17	0.0081
BC	7.45	6	1.240	21.05	0.0000
ABC	12.03	12	1.003	17.02	0.0000
residual	4.24	72	0.059		
total (corrected)	314.60	107			

In Table 2, two homogeneous groups are identified by columns of Xs. Within each column, the levels containing Xs form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Tukey's honestly significant difference (HSD) procedure. With this method, there is a 5.0% risk of calling one or more pairs significantly different when their actual difference equals 0. There are no significant differences between the rpm of 120 and 140; therefore, the use of 120 is considered to be favorable for energy saving and avoiding fungus stress. Furthermore, there is no significant difference between pH 5 and 6 (Table 3); therefore, pH 6 was chosen, as it is the closest value to the initial pH (~5.86) of the formulated culture medium, which would imply less time and cost for future scale-up.

Table 2. Multiple range tests for N-AcG (mg/g) by agitation rpm.

RPM	Count	LS Mean	LS Sigma	Homogeneous Groups
100	36	29.9755	0.0404651	X
140	36	31.964	0.0404651	X
120	36	32.081	0.0404651	X

Method: 95.0 percent Tukey HSD. X for homogeneous group.

Table 3. Multiple range tests for N-AcG (mg/g) by pH.

pH	Count	LS Mean	LS Sigma	Homogeneous Groups
7	36	31.0329	0.0404651	X
5	36	31.4769	0.0404651	X
6	36	31.5107	0.0404651	X

Method: 95.0 percent Tukey HSD. X for homogeneous group.

In the interaction of pH and NaCl, it can be seen that the highest production of N-AcG was at 120 rpm at pH 6, while the lowest production occurred at all pH levels (5, 6, 7) at 100 rpm (Figure 1A). This is due to the fact that at a lower agitation speed (100 rpm), the hyphae do not receive good oxygenation; therefore, the production of N-AcG is lower, while at a high agitation speed (140 rpm), instability in pellet formation is generated. In the interaction of NaCl and rpm, a decrease in the production of N-AcG can be seen from a concentration of NaCl higher than 0.5 M (Figure 1B). Similarly, in the interaction of NaCl and pH, a concentration greater than 0.05 M decreases the production of N-AcG at all pH levels (Figure 1C). This is because a concentration higher than 0.05 M is detrimental to the fungus, causing a salt stress that alters its metabolism, thereby decreasing the production of N-AcG. On the other hand, at a concentration lower than or equal to 0.01 M, the fungus can produce a greater amount of N-AcG (Table 4), being favorable at pH 6 and an rpm of 120.

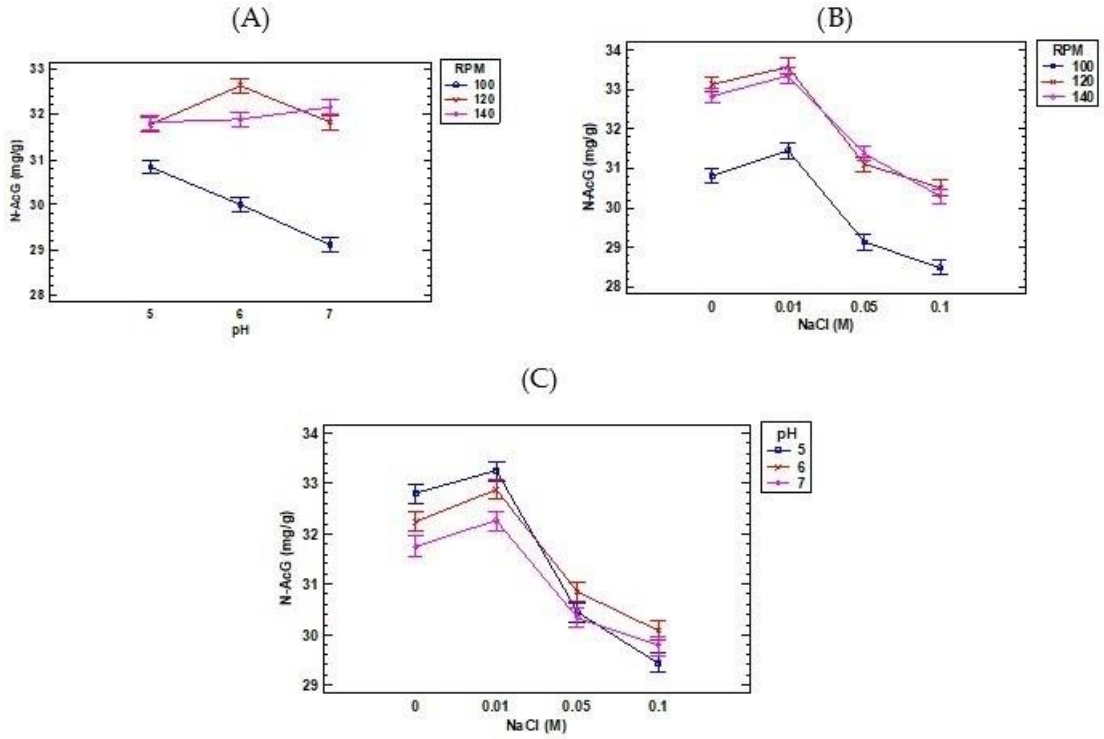

Figure 1. Interactions and 95.0 percent Tukey HSD intervals. The figure shows the interactions of pH and rpm (**A**), NaCl and rpm (**B**), and NaCl and pH (**C**) in relation to N-AcG production in mg/g dry matter.

Table 4. Multiple range tests for N-AcG (mg/g) by NaCl (M).

NaCl (M)	Count	LS Mean	LS Sigma	Homogeneous Groups
0.10	27	29.7666	0.046725	X
0.05	27	30.5383	0.046725	X
0.0	27	32.2596	0.046725	X
0.01	27	32.7961	0.046725	X

Method: 95.0 percent Tukey HSD. X for homogeneous group.

4. Conclusions

In this study, an inoculum was produced in submerged culture with quinoa flour with a maximum production of 34.83 mg glucosamine/g dry weight under the conditions of 0.01% NaCl, pH 6 and 120 rpm agitation, with a greater stability in the formation of pellets in a short period of time, giving the possibility of scaling up in future research.

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