

PRODUCTION OF FUNGAL CHITOSAN IN LIQUID CULTIVATION USING APPLE POMACE AS SUBSTRATE

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ABSTRACT

In this work, we propose the reuse of apple pomace as a substrate for fungal chitosan production by liquid cultivation of *Gongronella butleri* CCT4274. Different concentrations of reducing sugars and sodium nitrate were added to the aqueous extract of apple pomace and the best result was obtained with 40 g/L of reducing sugars and 2.5 g/L of sodium nitrate. The results indicate the possibility of producing 1.19 g/L of chitosan per liter of culture medium after 72.5 hours of cultivation, representing around 21% of the biomass content.

Key words: apple pomace, fungal chitosan, *Gongronella butleri*, liquid cultivation

INTRODUCTION

Chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine units, derived from the deacetylation of chitin in the presence of hot alkali (14). This polymer can be used as a suitable functional material, because it possesses desirable properties such as: biocompatibility, biodegradability, non-toxicity and adsorption of fats. Also, it can be used as a flocculating and chelating agent, as a permeability control agent, as a support to immobilize enzymes and as an encapsulating agent, among other applications found in different areas (6).

Commercially available chitosan is obtained from the deacetylation of shrimp and crab shell chitin with the use of strong alkali (14). For this reason, its isolation on an industrial scale is affected equally by the seasonal nature and by the limited availability of chitin supplies in a select group of countries. Additionally, this method has been found to have a negative environmental impact due to the large amounts of waste generated during its processing. It is also important to emphasize that the conversion of chitin to chitosan by means of strong base solutions at high temperatures leads to variability in the product's properties, affecting the quality of the chitosan and increasing production costs (9).

An alternative source of chitosan is the cell wall of certain fungi, particularly of the *Zygomycetes* class (9). The most

important advantage of this source is that the cell wall of *Zygomycetes* fungi contains significant quantities of chitosan. In addition, the physico-chemical properties and the yield of chitosan can be manipulated and standardized by controlling the cultivation parameters and the processing conditions (13).

Large amounts of solid wastes are generated during the processing of agro-industrial products, such as cassava and apple. Apple pomace is an important by-product of the apple processing industry. It consists of the press cake remaining after juice extraction from the apples, and contains pulp, seeds and peel. In Brazil, about 800,000 tonnes of apple pomace are produced per year (1,11) and it is mostly used as animal feed. The State of Santa Catarina is the biggest producer of apple in Brazil, with an annually production of 400,000 tonnes (1). From this production, 55,000 tonnes of apple are transformed in the food industry, generating 25% of apple pomace (15). Due to its low protein and vitamin contents, meaning a low nutritional value, this residue has limited applications. It is traditionally disposed of on the land or used as animal feed (17). If it is used for animal feed without a previous biological treatment, it can cause a problem related to the alcohol production due to the apple pomace fermentation in the animal rumen (15).

Furthermore, it is a well known fact that the accumulation of these agro-industrial residues causes a major environmental pollution problem. Due to the high content of starch and sugars,

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these residues can be potentially used for the growth of many microorganisms (10,18).

The main goal of the present work was to verify the possibility of producing chitosan through liquid cultivation, reusing the apple pomace as a substrate for fungi growth.

MATERIAL AND METHODS

Microorganism and culture medium

The filamentous fungus *Gongronella butleri* CCT 4274 was obtained from the Tropical Culture Collection of “André Toselo” Foundation (Campinas - SP) and it was maintained at 4°C on MA2 agar plates (2% malt extract, 2% agar). The spore suspension was prepared according to the method described by Nagel *et al.* (8).

The culture medium used was the aqueous extract obtained from the apple pomace (AEAP), a residue kindly supplied by a local factory (Yakult S.A., Lages, SC, Brazil). The apple pomace, obtained from the juice extraction of apples from the variety Gala, was dried at 60°C for 3 days and then triturated. The soluble material present in the dry pomace was extracted in Erlenmeyer flasks using a proportion of 15 g of dry and triturated apple pomace to 135 mL of distilled water. After the extraction (60°C, 2 h, 150 rpm), the material was centrifuged (1,300g, 5 min) and the liquid was filtered on a qualitative filter (0.45 µm) to completely remove the suspended particles. The liquid extract was stored at -20°C for subsequent characterization.

Culture Conditions

Experimental design

In the first experiment a 2² factorial design with central point was elaborated to evaluate the influence of reducing sugars (RS) and of sodium nitrate (SN) concentrations upon the chitosan production by the fungus *Gongronella butleri* CCT 4274. The culture media studied are described in Table 2.

The AEAP was diluted with citrate phosphate buffer (pH 4.5) to obtain 20, 30 or 40 g/L of reducing sugar, after which sodium nitrate was added as indicated in Table 2. Fungal growth was also analyzed in a culture medium containing 20 g/L of reducing sugars without sodium nitrate (control). The culture was performed in 1 L Erlenmeyer flasks containing 350 mL of medium. Following sterilization (121°C, 15 min) each flask was inoculated with 1.5x10⁵ spores/mL of medium and was maintained in a shaking incubator (150 rpm, 30°C) for 130 h. Every twelve hours a sample was collected for the determination of cell growth.

At the end of the cultivation, the cells were recovered by centrifugation (1,300g, 15 min) and stored at 4°C for subsequent quantification of their chitosan content. All the experiments were performed in duplicate.

Kinetics of fungi growth and chitosan production

Once the best concentration of reducing sugars and sodium nitrate for the fungal chitosan production had been determined,

an experiment was performed to elaborate the curve of chitosan production as a function of the time of cultivation.

Ten 1 L Erlenmeyer flasks, containing 350 mL of the medium, were sterilized (121°C, 15 min) and inoculated with 1.5x10⁵ spores/mL. The flasks were maintained in a shaking incubator (150 rpm, 30°C) for 110 h.

Every twelve hours, one sample was collected from each Erlenmeyer flask in order to determine cell growth. At the same time, one flask was removed from the cultivation, the cells were recovered by centrifugation (1,300g, 15 min) and stored at 4°C for subsequent quantification of the chitosan content.

Analytical Methods

The AEAP was characterized in relation to the content of reducing sugars, soluble protein, phosphate and pH, using the methods of 3,5-dinitrosalicylic acid (7), Bradford (3), colorimetric kit (Dolles Reagentes, Goiânia, GO, Brazil) and pH-meter TEC2 (Tecnal, Piracicaba, SP, Brazil), respectively. The colorimetric analysis of phosphate is based on its reaction with Molybdenum in an acid media, follow by the addition of an alkali solution, thus originating ascorbic acid that is responsible for the blue color. The absorbance at 600nm is measured and a calibration curve is used to determine the phosphate concentration on the sample.

The microbial growth was monitored through the direct method of gravimetric (dry weight) (4). 1.5 mL of the culture media were centrifuged (10,060g, 8 min) in an eppendorf previously dried (95°C for 24 h) and weighed. The supernatant was recovered and stored for further analysis of reducing sugars by the 3,5-dinitrosalicylic acid method. Cells were washed in distilled water (1 mL), centrifuged (10,060g, 8 min) and dried at 95°C until constant weight.

The extraction of the chitosan from the cells was performed following the protocol of Synowiecki and Al-Khateeb (12). The biomass was treated with 2% NaOH (1:30 p/v) and maintained in a rotating shaker (200 rpm) at 90°C for 2 h. The alkali insoluble fraction (AIF) was collected by centrifugation (1,300g, 15 min) and washed with distilled water (1/10 p/v) and 95% ethanol (1/10 p/v). The AIF was then treated with 10% acetic acid (1:10 p/v) at 60°C for 6 h at 150 rpm. The material was centrifuged (1,300g, 15 min), the pH of the supernatants was adjusted to 11 and the precipitated chitosan was collected by centrifugation (1,300g, 15 min). Finally, this was washed with distilled water (1/10 p/v), 95% ethanol (1/10 p/v) and dried at 90°C until constant weight.

RESULTS AND DISCUSSION

Table 1 presents the results of the aqueous extract of the apple pomace (AEAP) characterization concerning reducing sugar, protein and phosphate contents, aswell its pH.

Through the analysis of AEAP (Table 1), it can be seen that this constitutes a suitable source of carbon for the development

Table 1. Characterization of the aqueous extract of the apple pomace (AEAP) obtained from 15 g of dry and triturated apple pomace mixed with 135 mL of distilled water, maintained at 60°C and 150 rpm for 2 h before centrifugation (1,300g, 5 min) and filtration (0.45 µm).

| Parameter | Concentration |
|----------------|---------------|
| Reducing sugar | 60 g/L |
| Protein | 0.20 g/L |
| Phosphate | 0.024 g/L |
| pH | 4.00 |

of microorganisms, in view of its high content of reducing sugars. Another positive feature of the AEAP is that its low pH is very close to the optimal value for fungal growth (pH 4.5). On the other hand, its low protein and phosphate content (indispensable for the growth of microorganisms) indicates the need for supplementation with nitrogen and phosphate sources.

Experimental design

Table 2 presents the coded and the real values for the two factors analysed in the factorial design, as well as the results obtained for the biomass productivity (P_x) and the yield of chitosan by biomass ($Y_{p/x}$). All the experiments were conducted in duplicate. Fig. 1 presents the kinetics of *G. butleri* growth for all the culture medium studied.

The low growth of the microorganism in the medium without sodium nitrate (control) confirms the need for the addition of a nitrogen source to the culture medium (Fig. 1). Yokoj *et al.* (16), working with the liquid residue generated during the production

Table 2. Experimental design used to study the effect of reducing sugar and sodium nitrate concentrations on the biomass productivity (P_x) and the yield factor of chitosan by biomass ($Y_{p/x}$) of *Gongronella butleri* CCT4274.

| Culture Medium | Coded Factors | | Real Factors | | P_x (g/(L.h)) | $Y_{p/x}$ (g/g) |
|----------------|-----------------|-----------------|--------------|------|-----------------|-----------------|
| | RS _c | SN _c | RS | SN | | |
| 1 | -1 | -1 | 20 | 2.5 | 0.069 | 0.079 |
| 3 | -1 | 1 | 20 | 12.5 | 0.038 | 0.182 |
| 5 | 1 | -1 | 40 | 2.5 | 0.091 | 0.178 |
| 7 | 1 | 1 | 40 | 12.5 | 0.078 | 0.049 |
| 9 | 0 | 0 | 30 | 7.5 | 0.046 | 0.104 |

RS_c: coded value of reducing sugars; SN_c: coded value of sodium nitrate; RS: reducing sugars (in g/L); SN: sodium nitrate (in g/L).

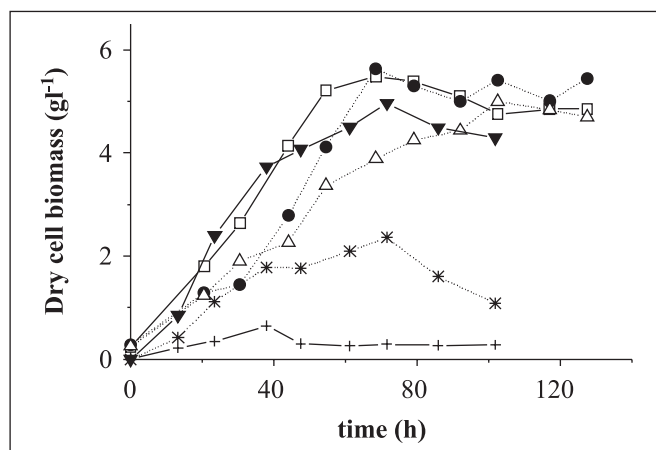


Figure 1. *G. butleri* growth curves for the six media presented in Table 2: ▼: 20 g/L of RS and 2.5 g/L of SN; *: 20 g/L of RS and 12.5 g/L of SN; □: 40 g/L of RS and 2.5 g/L of SN; ●: 40 g/L of RS and 12.5 g/L of SN; △: 30 g/L of RS and 7.5 g/L of SN; +: control (20 g/L of RS). Cultivations were conducted at 30°C in a shaking incubator (150 rpm).

of a distillation of barley, wheat and sweet potato (*shochu*), obtained approximately 7 g/L of biomass for the fungus *Gongronella butleri* IFO 8081. In that case, addition of minerals to the medium was found to be unnecessary, indicating a medium rich in nitrogen and/or proteins.

Table 3 presents the results of the statistical analysis (probability levels and effects estimation) performed with the aid of the software Statistica 5.11 with a confidence level of 95%.

Only the RS and SN presented a significant effect on biomass productivity. Increasing the concentration of RS in the medium led to an improvement of 6.23% in the productivity, while increasing the concentration of SN caused a reduction of 4.63%. The positive effect of RS is related to the larger availability of substrate for the development of the fungus. On the other hand,

Table 3. Statistical results for the 2² factorial designs with a central point (probability and effects estimation) for the biomass productivity (P_x) and the yield of chitosan by biomass ($Y_{p/x}$).

| Factor | P_x | | $Y_{p/x}$ | |
|---------|-------------|--------|-------------|--------|
| | Probability | Effect | Probability | Effect |
| RS | 0.0015* | 6.23 | 0.0130* | -3.77 |
| SN | 0.0056* | -4.63 | 0.0329* | -2.92 |
| RS x SN | 0.0789 | 2.20 | 0.000002* | -25.92 |

* Significant at the 5% level ($P < 0.05$).

RS: reducing sugars; SN: sodium nitrate; RS x SN: interaction between reducing sugars and sodium nitrate concentrations.

a reduction in the productivity caused by the increase of SN concentration can be related to changes in the culture medium (pH, for example) that hindered the development of the microorganism. This opposite behaviour of the factors explains the absence of significant effect of their interaction on the biomass productivity.

RS and SN concentrations had a significant effect on the yield of chitosan by biomass ($Y_{P/X}$), their interaction presenting the most significant effect (25.9%). Analyzing the factor RS, a reduction on the yield of chitosan on the cells was observed when a higher concentration of reducing sugars was added to the medium. As a high concentration of RS was responsible for a high productivity, which means a higher biomass concentration, the amount of nitrogen in the media was not enough to permit the cells to increase their chitosan content in the same proportion. Increasing the concentration of reducing sugars permits the production of more biomass but with a less chitosan content. In the same way, a negative effect of a high concentration of SN was observed on the yield of chitosan. Once this same factor had a negative effect on the biomass productivity, probably due to changes on cultivation environment, cells were in a not favourable physiological state, thus affecting directly their chitosan yield. How both factors presented the same behaviour, which means, a negative effect, their interaction led to a reduction of 25.9% in the chitosan content of the cells.

These results can be easily visualized through the response surfaces constructed for each of the scenarios discussed above (Figs. 2 and 3). Analyzing Fig. 2, it is possible to observe the

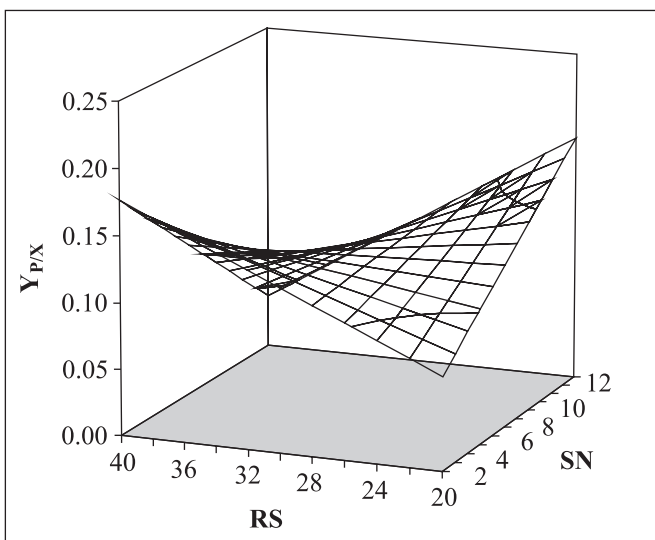


Figure 2. Estimated response surface representing the effects of reducing sugar (RS, in g/L) and sodium nitrate (SN, in g/L) concentrations on the yield of chitosan by biomass ($Y_{P/X}$) of *G. butleri* CCT4274.

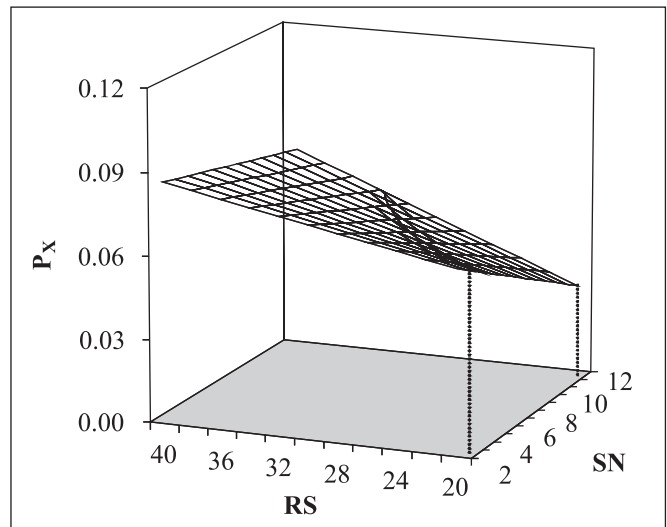


Figure 3. Estimated response surface representing the effects of reducing sugar (RS, in g/L) and sodium nitrate (SN, in g/L) concentrations on the biomass productivity (P_x) in liquid cultivation.

strong interaction between RS and SN. Two conditions permitted to obtain a high yield of chitosan on the biomass: high concentration of RS and low concentration of SN and the opposite one, low concentration of RS and high concentration of SN. In contrast, from Fig. 3, it is possible to visualize that just one condition permitted to obtain the higher biomass productivity: high concentration of RS (40 g/L) and low concentration of SN (2.5 g/L). With this cultivation conditions, a biomass productivity of 0.091 g cell/(L.h) was obtained, the same containing 18% of chitosan.

Kinetics of chitosan production

The objective of this experiment was to verify if the chitosan content in the cell was related to the growth phase of the microorganism.

Fig. 4 presents the kinetics of growth and chitosan production for the fungus *G. butleri* grown in the culture medium with 40 g/L of reducing sugars and 2.5 g/L of sodium nitrate.

The curve for chitosan production exhibits the same profile as the growth curve of the microorganism. This is due to the fact that chitosan is a component of the cell wall of filamentous fungi, so the greater the growth, the greater will be the amount of chitosan produced.

After 72.5 hours of cultivation it was possible to obtain 1.19 g/L of chitosan, representing an important improvement on chitosan productivity when compared to the work of Yokoj *et al.* (16). Working with *Gongronella butleri* IFO8081 in SPS culture medium (originating from the residue of the production

of *Shochu*) these authors obtained 0.73 g/L of chitosan after 120 hours of cultivation.

Table 4 presents the yield of chitosan by biomass obtained during the different phases of the culture growth.

Analyzing the amount of chitosan obtained over the whole period of cultivation, it can be seen that it was extracted easily towards the end of the exponential phase and at the beginning of the stationary growth phase (49.5 - 63 h), with a chitosan content of around 21%.

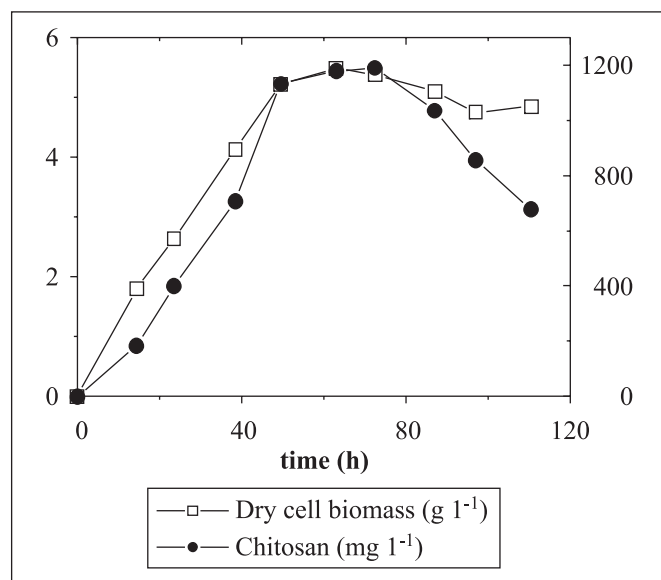


Figure 4. Kinetics of growth and chitosan production for *G. butleri* grown at 30°C in the culture medium containing 40 g/L of reducing sugars and 2.5 g/L of sodium nitrate.

Table 4. Results of the yield factor of chitosan by biomass ($Y_{P/X}$) obtained during the growth of *G. butleri* cultivated at 30°C in the medium containing 40 g/L of reducing sugars and 2.5 g/L of sodium nitrate.

| Time of cultivation (h) | $Y_{P/X}$ (g/g) |
|-------------------------|-----------------|
| 0 | 0 |
| 14.5 | 0.102 |
| 23.5 | 0.152 |
| 38.5 | 0.171 |
| 49.5 | 0.217 |
| 63 | 0.215 |
| 72.5 | 0.179 |
| 87 | 0.203 |
| 97 | 0.180 |
| 110.5 | 0.139 |

According to Tan *et al.* (13), after the exponential phase of growth, it becomes more difficult to solubilize the chitosan and, as a consequence, to extract it. This can be explained by the fact that once the culture enters the stationary phase, a larger amount of chitosan is arrested in the cell wall of the *Zygomycetes* through links with chitin and other polymeric components, hindering its extraction. Amorim *et al.* (2) observed a reduction in the amount of chitosan extracted from the fungi *Mucor racemosus* IFM40781 and *Cunninghamella elegans* URM 46109 after 24 hours of cultivation in a synthetic medium containing yeast extract, peptone and dextrose (YPD medium). According to the authors, this reduction can be related to physiologic changes in the cell wall of the fungus during the cultivation period. In the work of Hang (5), it was possible to verify the influence of the culture medium on the amount of chitosan isolated from the microorganism. In the same cultivation conditions, the fungus *Rhizopus oryzae* NRRL 395 growing in a rice culture medium produced more chitosan than when it was grown in a corn based culture medium (0.7 g/L of chitosan instead of 0.4 g/L). The largest amount of extracted chitosan (0.7 g/L) represents more than 20% of the dry weight of the cells, which is in agreement with our results.

CONCLUSION

In this work we found that the aqueous extract of apple pomace is a good medium for the development of microorganisms, in view of its high content of reducing sugars. In this way, utilizing apple pomace in biotechnological processes offers an interesting alternative to the commercial medium used to obtain numerous products.

The chitosan production in liquid cultivation using the aqueous extract of apple pomace as culture medium and sodium nitrate as the source of nitrogen gave the maximum yield (21% of the cell weight) after approximately 50 hours of cultivation.

Results from the effect of reducing sugars and sodium nitrate concentration on biomass productivity and yield of chitosan on the biomass were interesting but, in the same way, complex. To better understand these effects, mainly the one of SN concentration, the study of different nitrogen sources seems to be necessary.

The characterization of the chitosan obtained by this biotechnological process will be the next step of this work, looking to establish applications where fungal chitosan could be more suitable than chitosan extracted from shrimp and crab shells.

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RESUMO

Produção de quitosana fúngica em cultivo líquido utilizando bagaço de maçã como substrato

Este trabalho propõe o reuso do bagaço de maçã como substrato para a produção de quitosana fúngica em cultivo líquido do fungo *Gongronella butleri* CCT4274. Diferentes concentrações de açúcares redutores e nitrato de sódio foram adicionadas ao extrato aquoso do bagaço de maçã. O melhor resultado foi obtido para concentrações de 40 g/L e 2,5 g/L de açúcares redutores e nitrato de sódio, respectivamente. Os resultados indicam a possibilidade de produzir 1,19 g/L de quitosana após 72,5 horas de cultivo, representando 21% da composição da biomassa.

Palavras-chave: bagaço de maçã, quitosana fúngica, *Gongronella butleri*, cultivo líquido

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