PRODUCING CHITIN AND CHITIN-GLUCAN COMPLEXES FROM ASPERGILLUS NIGER BIOMASS USING SUBCRITICAL WATER

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Abstract. Chitin is the second most abundant biopolymer on earth, occurring in marine invertebrates, insects, and the cell walls of some fungi, among them *Aspergillus niger*, which is used in the industrial production of citric acid. Such biopolymer and its derivatives have a broad range of applications in many economically relevant areas (biomedical, food, agriculture, paper, wastewater treatment, pharmaceutical, etc.). However, the conventional process for obtaining chitin uses elevated amounts of sodium or potassium hydroxide, and thus has difficulties associated with its environmental performance.

In this work we explored a process for obtaining chitin and chitin-glucan complexes from mycelium of *Aspergillus niger* by treatment with subcritical water. A continuous apparatus designed for operation at temperatures up to 300 °C, pressures up to 29 MPa and residence times of mycelium from 2.0 to 50 s approximately was built. A central composite design was run to investigate the effect of temperature and reaction time over the composition and properties of the product, such as yield, molecular weight and chitin concentration.

With this process a variety of products can be obtained. For example, at 228 °C, 21 MPa and 7.5 s residence time, a chitin-glucan complex is obtained, with a yield of 50.8% (based on the dry mycelium fed), a chitin concentration of 30% and a molecular weight of 21.5 kDa. This product might be useful as a raw material for biomedical products of high value. The developed process is promising for industrial production since it only uses the most abundant, inexpensive and environmentally friendly solvent: water.

Keywords: Aspergillus, chitin, glucan, subcritical water.

1. Introduction

Aspergillus niger is a microscopic fungus that is used in the industrial production of citric acid by fermentation. The vegetative part of the fungus (i.e., the so-called mycelium) is an important source of chitin, a substance that is known as the second most abundant biopolymer on earth after cellulose. Chitin and its chemical derivatives have an elevated number of applications in several economically important areas, including industries such as biomedical, food, agriculture, papermaking, wastewater treatment, pharmaceutical, etc. For example, it is well known the use of chitin as industrial adsorbent for environmental control [17], as support for enzyme inmobilization [16] and most importantly, as raw material for the manufacture of precursors for bioabsorbable fibers and films, which are used as surgery sutures, as encapsulating agents for controlled release of pharmaceutical products, as bioabsorbable films which also accelerate cicatrization processes, and act as templates for regenerating skin [1].

Chitin and its derivatives are also obtained from crustaceans such as shrimp, crab and lobster, but these sources have some deficiencies regarding their supply, which is seasonal, and their environmental impact during collection [3,4]. In contrast, mycelium from *Aspergillus niger* is an industrial byproduct and thus has a stable supply and low price. Its cellular wall is mainly composed of chitin (15 to 18%), glucans (37%), lipids (19%) and several sugars (8 to 15%) [12,13].

Since several years ago several researchers have been developing processes for isolation of chitin or the production of chitosan (its deacetylated derivative), and more recently chitin-glucan complexes from *A. niger* biomass. These processes are mostly chemical or enzymatic [3-7] but have several deficiencies. In fact, the chemical processes have environmental problems because they use large amounts of caustic and acid solutions, and the enzymatic processes have low yields and usually require long residence times.

In this work we report on the use of hot, compressed water (i.e., subcritical water) for destroying the mycelium structures and producing chitin-glucan complexes in a continuous process. Although to the best of our knowledge not such a process has been discussed in the literature, it is well known that there are several reports on the use of sub and supercritical water to break down chemical bonds for producing useful substances from biomass. Some of them relate to the hydrolysis of lignocellulose [8,9,10] with water at conditions close to those of the critical point of this solvent. Glucose, fructose and glycoaldehydes are obtained in reaction times from 0.0 to 10 s. Another example was reported by Sasaki [11], regarding the use of subcritical water at temperatures from 200 to 230 °C to break the lignin bonds present in sugar-cane bagasse to produce microcrystalline cellulose. These and other studies suggest that by subjecting *A. niger* biomass to treatment in subcritical water at operating conditions that are less aggressive than those reported for vegetable biomass, it might be possible to break the chemical bonds that attach the chitin biopolymers to the fungal cell wall, producing useful materials.

2. Experimental

2.1 *Aspergillus niger* biomass

Mycelium from *A. niger* was obtained from a local industrial producer of citric acid. This material was obtained directly from the fermentation process, and was washed several times with distilled water to remove soluble impurities. The material was then dried at 45 °C and was sieved. The material passing mesh 16 (Tyler, average particle size 1 mm) was selected as raw material for this work. A suspension of this material in distilled water was then prepared by using 2-(2-(4-nonylphenoxy)etoxy)ethanol (Arkopal) as emulsifier. This suspension was stable for several days and was used in experiments of reaction in subcritical water.

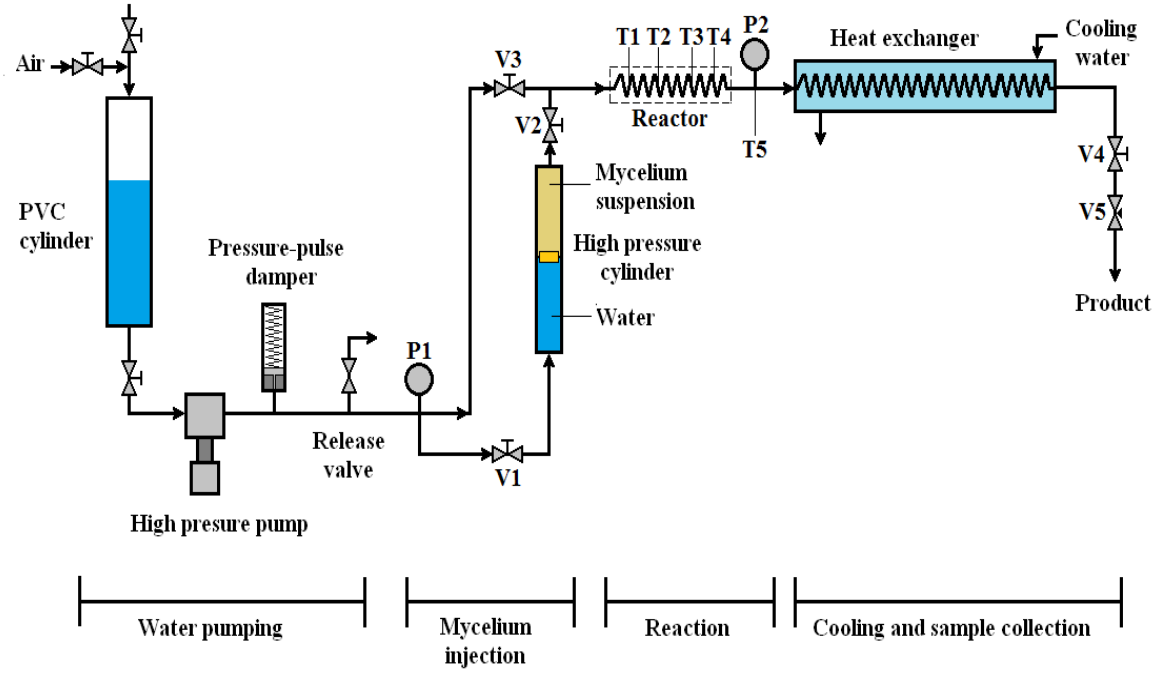

Figure 1. Schematic of the experimental apparatus.

2.2 Experimental apparatus

Figure 1 shows a schematic of the experimental apparatus that was used in this research. This apparatus allows one to operate continuously at temperatures up to 300 °C, pressures up to 29 MPa, and residence times up to 50 s. Distilled water contained in a PVC cylinder is pressurized using air at 10 psig, and is delivered by a high pressure pump either to the reactor at the beginning of an experimental run until steady state is reached, or to a high pressure cylinder with a moving piston in which the biomass suspension has been previously loaded. This high pressure cylinder acts as a syringe during an experimental run. Water is pumped to the lower part of the cylinder and displaces the moving piston injecting the biomass suspension into the reactor.

The reactor is made of 1/8" high-pressure tubing and is electrically heated. Four K-type thermocouples (T1 to T4) allow one to determine the external surface temperature in different points of the reactor. Another thermocouple (T5) was inserted inline to measure the temperature of the reactive mass at the reactor exit. After the reactor, the product enters a heat exchanger to quickly reduce its temperature up to 28°C.

A pressure pulse damper that was designed and built in our laboratory was inserted at the exit of the high pressure pump to decrease the pressure pulses that are present when pumping an incompressible fluid such as water at room temperature. In this way, the pressure oscillation during pumping was decreased from ±1000 psi to ±100 psi. Flow rates were determined from calibration curves previously prepared for the pump. Operating pressures were measured by using two pressure gages (P1 and P2) as indicated in the figure. After leaving the heat exchanger, the product was collected and maintained at 5 °C until analysis.

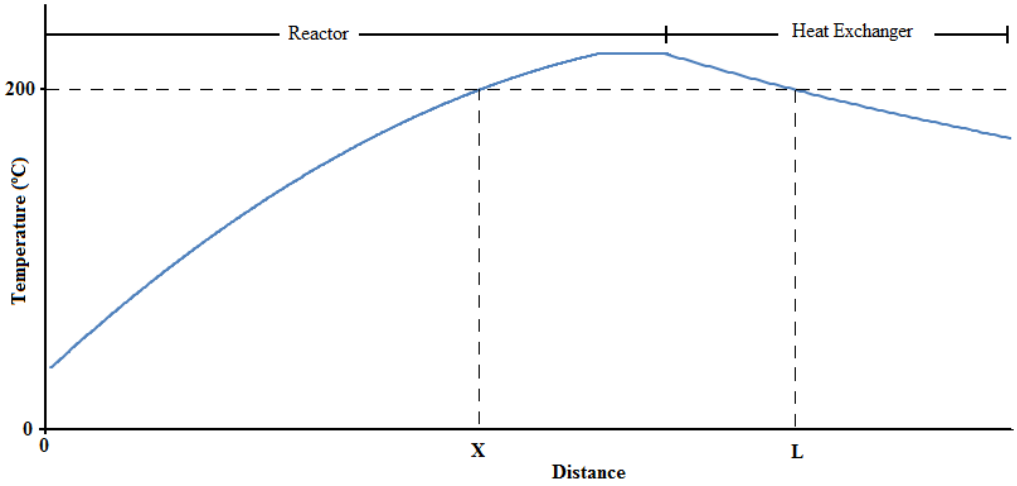

Figure 2. Schematic of the temperature of the fluid inside the reactor and the heat exchanger.

2.3 Reaction temperature and time

Figure 2 shows a schematic of the temperature of the fluid inside the reactor and the heat exchanger. The average reaction temperature of the fluid was determined by using the equation:

$$\bar{T} = \frac{1}{L - X}\int_{X}^{L} T(X)dX \qquad (1)$$

Where X is the distance from the entrance of the reactor where the fluid reaches 200 °C (we assumed that at this temperature the rupture of the chemical bonds turns out relevant), L is the distance from the reactor entrance to a point inside the heat exchanger where the fluid decreases its temperature again up to 200ºC, and thus the reaction stops. The temperature profile of the fluid inside the reactor was determined by using the data of external surface temperature of the reactor measured by the four thermocouples mentioned above, together with an energy balance and the equation for heat transfer in a pipe, such that the computed temperature of the fluid at the exit of the reactor is exactly the one measured at this point. The temperature

profile of the fluid inside the heat exchanger was determined measuring the refrigerant temperature changes and the product temperature changes.

The reaction time was determined as that between the moment in which the fluid reaches 200 °C in the reactor, and the moment in which it reaches 200 ºC again in the heat exchanger. It was computed by using the equation:

$$t = \frac{(L - X)A}{Q} \tag{2}$$

where A is the tubing cross-sectional area and Q is the volumetric flow rate of the fluid.

2.4 Product characterization

The treated biomass was washed several times with distilled water and centrifuged at 3000 rpm for 15 min, to remove soluble products, such as proteins and sugars. The resulting solid phase was then lyophilized and weighed to determine the product yield according to:

$$R = \frac{W_C}{W_F}\ 100 \tag{3}$$

where W_C is the mass of the final solid product and W_F is the mass of the treated biomass, on a dry basis.

Samples of the product were subjected to elemental analysis. The chitin concentration of the product (Q) was then determined as a mass percentage by using the nitrogen percentage obtained in the elemental analysis (N), and an equation that relates these two variables for the chemical structure of chitin:

$$Q = 14.199\ N \tag{4}$$

Finally, the molecular weight of the product was determined by using the Mark-Houking equation [21], which relates molecular weight to intrinsic viscosity of aqueous solutions of chitin, sodium hydroxide and urea:

$$\eta = 0.26(M_W)^{0.56} \tag{5}$$

In this equation, M_W is the molecular weight. η represents the intrinsic viscosity, which was determined following the procedure reported by Weska [18].

2.5 Preliminary experiments and experimental design

Several preliminary experiments were conducted to explore the general characteristics of the process and to refine the experimental technique. In these experiments, pressure was maintained constant at 21 MPa (3000 psi), and temperature and reaction time were changed for 214 to 274 °C as shown in Table 1. Based on the results of these preliminary runs, an experimental program was planned to determine the effect of temperature and reaction time on product yield and molecular weight and chitin content of the product. Such experimental program corresponds to a central composite design, with four replications in the central point. Temperature was studied between 214 and 231 °C, and reaction time between 5.4 and 10 s. Pressure was maintained at 21 MPa in all the runs. This pressure is above the vapor pressure of water and allows one to maintain the reacting mixture in a liquid phase.

3. Experimental results

3.1 Preliminary runs

Table 1 shows the operating conditions that were chosen for the preliminary experiments. The product that was obtained at the two higher temperatures (i.e., tests 1 and 2) exhibited a dark color as well as a smaller

amount of solids. In addition, a strong smell like sugar was recognizable, and was most notorious in the product obtained at the highest temperature (274 °C). This indicates that at the highest temperatures considered there is an excessive rupture of chemical bonds freeing the sugars that are present in the chemical structures of chitin and other biopolymers that are present in the mycelium. In contrast, no dark color was observed in the product obtained in tests that were run at lower temperatures (i.e., tests 4 and 5).

Table 2 shows a chemical characterization of the products obtained in tests 4 and 5. Notice that changing the operating conditions of the process results in products with different chemical characteristics, as molecular weight and chitin concentration. In particular, when chitin concentration increases the product yield decreases. This suggest that chitin hydrolysis occurs at more aggressive conditions (i.e., higher temperatures and longer reaction times) and that at less aggressive conditions it might not be a sufficient separation of chitin from the mycelium cell wall. To investigate the variety of products that can be obtained in the range of conditions specified for tests 4 and 5, a experimental design was prepared as described above.

Table 1. Operating conditions for preliminary experiments.

Test	Mean temperature (°C)	Reaction time (s)
1	274	34.8
2	257	24.4
3	234	17.2
4	231	13.5
5	214	5.0

Table 2. Characterization of products obtained in preliminary tests 4 and 5.

Characteristic		Test	
		4	5
Elemental analysis (wt%)	C	43.9	43.6
	N	5.7	3.0
	H	6.5	6.8
Chitin concentration (wt%)		80.9	42.9
Molecular weight (kDa)		30.0	43.0
Product yield (wt%)		13.9	25.8

3.2 Experimental design

Table 3 shows the chemical characterization of the products obtained from the experimental program. First, consider the results that were obtained in the central runs of the experimental design (i.e., at 222 °C, rows 5 to 8 in the table). These runs were performed at identical operating conditions and allow one to analyze the reproducibility of the experimental techniques that were used. For these runs, the obtained product yield varied between 51.1 and 57.9%, and the chitin concentration between 22.7 and 27.3%, an acceptably high reproducibility. However, molecular weight varied between 24.9 and 61.0 kDa, which indicates that the technique that was used for determination of molecular weight has much higher uncertainty.

The data shown in Table 3 indicates that a great variety of products can be obtained at the experimental conditions considered in the experimental design. Chitin concentrations from 19.7 to 54.2% and molecular weights from 1.7 and 155.2 kDa were observed, with product yields from 21.7 to 57.9%.

3.3 Statistical models

The data presented in Table 3 were correlated using statistical models which relate each response variable in the experimental design (i.e., product yield, chitin concentration and molecular weight) to the operating

variables of the process (temperature and reaction time). For each response variable a second order model was proposed according to:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_{12} + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 \quad (6)$$

Where Y is the response variable (product yield, chitin concentration or molecular weight), X_1 is temperature and X_2 is reaction time, both normalized to the [-1 +1] interval, and β are the adjustable parameters of the model.

Table 3. Chemical characterization of the products obtained in the experimental program.

Operating conditions		**Characteristics of the product**					
Temperature (°C)	**Reaction Time (s)**	**Yield (wt%)**	**Elemental analysis (%)**			**Chitin concentration (wt%)**	**Molecular weight (kDa)**
			C	**N**	**H**		
214	5.4	44.8	41.9	2.3	6.4	32.5	44.2
214	10.0	45.2	42.4	2.2	6.4	31.4	18.8
231	5.4	35.7	44.3	2.8	7.0	39.7	16.4
231	10.0	24.9	42.5	3.8	6.8	54.2	1.7
222	7.5	57.9	42.1	1.6	6.5	22.7	58.2
222	7.5	57.9	42.0	1.7	6.7	23.9	37.1
222	7.5	51.1	42.5	1.7	6.2	24.6	24.9
222	7.5	56.7	42.1	1.9	6.9	27.3	61.0
255	8.1	21.7	43.9	3.6	6.8	50.8	15.2
227	4.7	55.9	36.9	1.7	7.0	24.1	30.1
222	10.8	39.9	40.1	2.5	6.7	35.3	16.9
207	6.0	68.0	41.6	1.4	7.2	19.7	155.2

Table 4 shows the coefficients of the statistical model that were found for each response variable, together with the correlation coefficient (R^2) for each model. Because the two independent variables are scaled to the same interval, the sign and magnitude of the coefficients allow one to quickly observe that in all three cases temperature is the variable that affects the most the response variables.

Table 4. Coefficients of statistical model for each response variable.

Variable	**β_0**	**β_1**	**β_2**	**β_{12}**	**β_{11}**	**β_{22}**	**R^2**
Product yield (wt%)	55.0	-5.91	-2.79	-3.30	-0.9	-12.7	0.9
Chitin concentration (wt%)	26.0	6.17	3.07	4.23	-0.1	7.35	0.8
Molecular weight (kDa)	37.3	-28.3	-15.5	9.12	6.09	-8.29	0.8

The statistical models allow one to estimate the characteristics of the products that can be obtained at a particular set of operating conditions. For example, at 228 °C, 7.5 s of reaction time and 21 MPa, a chitin-glucan complex would be obtained, which would have a chitin concentration of 30 wt% and a molecular weight of 21.5 kDa, with a product yield of 50.8 wt%. This product has characteristics that are well appreciated as a raw material for the manufacture of high-value biomedical products.

4. Conclusions

A process based on reaction in subcritical water was developed for obtaining chitin and chitin-glucan complexes from *Aspergillus niger* biomass. It was shown that at the operating conditions that were considered

a great variety of chitin-glucan complexes are produced. The fact that the percentage of chitin can be controlled indicates that these products might be suitable for a wide range of applications.

Reaction of *A. niger* biomass in subcritical water produced complexes with chitin concentrations from 19.7 to 54.2 wt%, molecular weights from 1.7 to 155.2 kDa and product yields from 22 to 68 wt%. The exploratory preliminary tests suggest that it might be possible to isolate products with chitin concentrations superior to 80 wt%, by running the process at temperatures above 230 °C and reaction times above 13.5 s but below 35 s. In this case, however, the product yield would be below 20 wt%.

The developed process not only has the flexibility to produce a diverse range of products, but it is also environmentally friendly because it does not use caustic or acid solutions. It only uses the most abundant and inexpensive solvent: water.

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