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Optimization of medium composition for enhanced chitin extraction from *Parapenaeus longirostris* by *Lactobacillus helveticus* using response surface methodology

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ABSTRACT

Chitin extraction by biological way, using the lactobacilli *Lactobacillus helveticus*, is a non-polluting method and offers the opportunity to preserve the exceptional qualities of chitin and its derivatives. However, the major disadvantage of the fermentative way is the low efficiency of demineralization and deproteinization. The aim of our study is to improve the yield of extraction.

Many factors, such as the initial concentration of carbon source, fermentation time, incubation temperature, inoculum size, shell size, volume and medium composition have been reported to influence the fermentation process and consequently demineralization and deproteinization efficiency. Based on the use of central composite design and response surface methodology ten factors with three levels each were examined to determine the optimal operational conditions of demineralization and deproteinization.

The analysis of the obtained results showed that the optimal conditions of 98% of demineralization and 78% of deproteinisation are 171.4 g L^{-1} of reducing sugars, 2.03 g of nitrogen source [$(NH_4)_2Fe(SO_4)_2$] and 1.29 g of calcium source ($CaCl_2$), used to ferment 4.84 g of shells, of 1.053 mm size heat treated at 120 °C, with 10 mL of inoculum (*L. helveticus*) incubated at 32.1 °C in 100 mL of juice date for 254.38 h (15 days).
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1. Introduction

Chitin is the second most abundant structural biopolymer found in nature (Ji, Wolf, Rodriguez, & Bowlin, 2012; Khuoshab, Jaruseranee, Tanthanuch, & Yamabhai, 2012). It occurs in a multitude of organisms from bacteria and fungi to molluscs and others, but is certainly most prominent in the largest and most diverse group of the animal kingdom particularly, arthropod group (Fabritius et al., 2011).

In arthropods, chitin is used together with various proteins and inorganic salts such as calcium carbonate, to form the exoskeleton. The actual chitin content varies depending on physiological stage of the organism (Benhabiles et al., 2012), harvesting season (Nitar, Tetsuya, & Hiroshi, 2011), health of the animals and geographical location (Kjartansson, Zivanovic, Kristberg, & Weiss, 2006). To date, the major source of industrial chitin comes from wastes of marine food production mainly crustacean shells, *e.g.* shrimp and crab shells or krill (Jayakumar et al., 2010; Mojarrad, Nemati, Valizadeh, Ansarin, & Bourbour, 2007; Xia, Liu, Zhang, & Chen, 2010; Xu, Gallert, & Winter, 2008).

The traditional processes of chitin production consist of the use of strong acids and bases under high temperature for demineralization and deproteinization, respectively. These processes, however, may cause pollution (Zakaria, Hall, & Shama, 1998) and significantly lower intrinsic viscosities of chitin (Rødde, Einbu, & Vårum, 2008). An alternative way to solve these problems is the use of biotechnological methods. The calcium and the protein in the shell waste were dissolved mainly by organic acids and proteases produced by microorganisms, respectively. Many factors, such as inoculum level (Shirai et al., 2001), shell content in medium, shell size (Oh, Kim, Nguyen, Jung, & Park, 2008), carbon source such as glucose (Rao, Muñoz, & Stevens, 2000; Shirai et al., 2001), sucrose (Choorit, Patthanamanee, & Manurakchinakorn, 2008), cassava flour (Rao and Stevens, 2006), molasses (Seda, Sebnam, Yekta, & Ali Fazil Yenidunya, 2004) and date juice (Adour, Arbia, Amrane, & Mameri, 2008), initial pH and its evolution during fermentation

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(Rao et al., 2000), temperature (Pacheco et al., 2009), carbon concentration and carbon on nitrogen ratio, have been reported to influence the fermentation process and consequently the demineralization efficiency (Choorit et al., 2008; Jo, Park, & Jung, 2011; Shirai et al., 2001).

It is to be noted that the major part of the reported studies (Adour et al., 2008; Rao et al., 2000) did not implement mathematical and especially statistical methods for process optimization, and hence only involve a separate optimization of each considered parameter with all others kept unchanged. Such empirical procedure needs several experimental runs, and hence is time-consuming, ignores interaction effects between the operating parameters and leads to low optimization efficiency. These limitations can be avoided by applying the response surface methodology that involves statistical design of experimentation in which all factors are varied together over a set of experimental runs. In fact, Response Surface Methodology is a collection of mathematical and statistical techniques useful for developing, improving and optimizing processes, and can be used to evaluate the relative significance of several affecting factors even in the presence of complex interactions (Khayet, Zahrim, & Hilal, 2011).

The effectiveness of modeling chitin extraction using Response Surface Methodology was recently shown (Bhaskar, Suresh, Sakhar, & Sachindra, 2007; Pacheco et al., 2009). Chitin recovery from a mixture of cephalothoraxes of shrimp species *Litopenaeus vannamei*, *Litopenaeus stylrostris* and *Litopenaeus setiferus* using Lactobacillus *plantarum* showed the highest demineralization and deproteinization yields in the range 27–36 °C and 30–40 °C respectively, using Response Surface Methodology (Pacheco et al., 2009). Culture conditions were also previously optimized by means of Response Surface Methodology, leading to the following optimal conditions, pH 4.3 ± 0.1, 5% (v/w) inoculum, 15% (w/w) glucose and 72 h fermentation time at 37 ± 1 °C, resulting in 97.9 ± 0.3% deproteinization and 72.5 ± 1.5% demineralization yields of shrimp biowaste using *Pediococcus acidolactici* (Bhaskar et al., 2007).

In the present study, attempts were made to improve demineralization and deproteinization obtained in our previous study by fermenting shrimp shell waste of *Parapenaeus longirostris* using *Lactobacillus helveticus*. Culture medium and conditions were optimized by means of Response Surface Methodology, namely the concentrations of carbon, nitrogen and calcium sources, the temperature, the inoculum level, the medium volume (date juice), shell size and its amount and the heat treatment.

2. Methodology

2.1. Materials

All chemicals used in this study were analytical grade and purchased from Sigma Chemical Co. (St. LouisMo). Shrimp shells were obtained from a seafood restaurant. It was confirmed that all shells were from a single species of shrimp *P. longirostris*. *L. helveticus* strain *milano* was supplied by Dr A. Fur (Even Ltd, Ploudaniel, France). Stock cultures were maintained at −18 °C in skimmed milk.

2.2. Preparation of the shells

Before use, the flesh, antennas and legs were removed from the shrimp shells. They were then boiled in water for 1 h to remove the maximum amount of flesh. Thereafter, they were dried at 163 °C for 1 h in a drying oven (Prolabo, model Volca MC18, French). After cooling, the shells were subjected to a thermal shock and facilitate crushing. Using a standard grinder (Model KU-2, PredomMesko, Skarzyskokam., Poland), the shells were ground into a particle grain size ranging between 0.31 and 1 mm for studying the effect of shell size on demineralization and deproteinization.

2.3. Date juice preparation

Dates were carefully washed, then pitted and 2 L of water per kilogram of pulp added, and the solution heated at 80 °C for 2 h. The extract obtained was then centrifuged for 30 min at 5000 rpm to remove any remaining cellulosic material (Boudjelal & Nancib, 2001). The supernatant was then used to prepare solutions of date juice at different concentrations of reducing sugars (100 g L^{-1}, 150 g L^{-1} and 200 g L^{-1}) as a carbon and energy source for lactic acid fermentation.

2.4. Cultures

Stock cultures of *L. helveticus* were reactivated on Man Rogosa Sharp (MRS) agar medium incubated for 24 h at 30 °C. Two successive pre-cultures on liquid MRS medium incubated for 24 h at 30 °C were carried out, and then culture Erlenmeyer flasks were inoculated with the final pre-culture. Batch cultures were carried in Erlenmeyer flasks, whose the volume varies with experiments conditions. The agitation speed was 200 rpm, the initial pH was fixed at 8.5 and the temperature was maintained constant at specified values in the range 20–40 °C. Culture media and all other materials used for this study were sterilized (20 min at 121 °C).

2.5. Optimization of fermentation conditions: experimental design and statistical analysis

In statistics-based approaches, response surface methodology has been extensively used in fermentation medium optimization. Response Surface Methodology is a useful model for studying the effect of several factors influencing the response by varying them simultaneously leading to a limited number of experiments (Popov, Rankovik, Dodić, Dodić, & Jokić, 2010). In addition, RSM was applied to understand the interaction of various variables and then used to find the optimal conditions that affect the response (De Lima, Coelho, & Contiero, 2010).

Optimization of the parameters (reducing sugars, nitrogen and calcium source concentrations, temperature and fermentation time) for demineralization and deproteinization of shrimp shell was done by a central composite design (CCD), which required three levels coded as (+1) (0) and (−1) ($N = 16$ experiments and ten factors at three levels). Table 1 shows the different levels of each parameter. The basic points (reducing sugars, nitrogen and calcium source concentrations, temperature and fermentation time) for the design were selected from a preliminary study (Adour et al., 2008).

Table 1
Design of experiment-levels of the various process parameters.

	Factor	Basic level	Variation interval	Factor value (−)	0	+
Reducing sugars (g L^{-1})	X_1	150	50	100	150	200
Temperature (°C)	X_2	30	10	20	30	40
Weight of shell powder (g)	X_3	7	3	4	7	10
Volume of culture medium (mL)	X_4	300	200	100	300	500
Inoculum volume (mL)	X_5	15	5	10	15	20
Heat treatment (°C)	X_6	60	60	0	60	120
Fermentation time (h)	X_7	162	138	24	162	300
Nitrogen source (g)	X_8	1.4	1.4	0	1.4	2.8
Calcium source (g)	X_9	0.65	0.65	0	0.65	1.3
Shrimp shell size (mm)	X_{10}	0.655	0.655	0.31	0.655	1

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