Vol. 19, No. 1 (2020) 263-274

Revista Mexicana de Ingeniería Química

A BIOTECHNOLOGICAL INSIGHT TO RECYCLE WASTE: ANALYZING THE SPONTANEOUS FERMENTATION OF SHRIMP WASTE TO DESIGN THE HYDROLYSIS PROCESS OF CHITIN INTO N-ACETYLGLUCOSAMINE

UNA PERSPECTIVA BIOTECNOLÓGICA PARA RECICLAR LOS DESECHOS: ANALIZANDO LA FERMENTACIÓN ESPONTÁNEA DE LOS DESECHOS DE CAMARÓN PARA DISEÑAR EL PROCESO DE HIDRÓLISIS DE LA QUITINA EN N-ACETILGLUCOSAMINA

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Received: February 11, 2019; Accepted: May 22, 2019

Abstract

Vibrio alginolyticus, a native microorganism of the shrimp wastes, was selected to synthesize β-N-acetylglucosaminidase and produce N-acetylglucosamine by chitin hydrolysis. Successful design was achieving when the culture medium ingredients and fermentation parameters were optimized to maximize the active enzyme content in raw extracts. The results showed that a starter inoculum containing 1.0×10^6 cells/mL mixed with a liquid culture medium supplemented with chitin (35.13 g/L), sodium chloride (27.53 g/L), magnesium chloride (42.71 g/L), sodium sulfate (1.71 g/L), calcium chloride (20.84 g/L), and yeast extract (5.1 g/L) in a stirred tank bioreactor under operating conditions held constant at 26.6 °C, 7.3 pH value, 96.8 rpm and 1.6 VVM allows for obtaining an enzyme preparation with an activity of 0.041 U/mL, which compares favorably against the yield achieved using the reference commercial culture medium and represent three-fold more units of enzyme as compared to those obtained at the shake flask level (0.026 U/mL). These results suggest *V. alginolyticus* is a strong candidate for producing active chitinases and confirm that the microbial ecology studies for specific microorganisms selection and the optimizing of both the culture medium and fermentation parameters (for the selected microorganism) allow evolve efficiently from the spontaneous to directed fermentations.

Keywords: *Vibrio alginolyticus*, chitinases synthesis, chitin hydrolysis, acetylglucosamine production.

Resumen

Vibrio alginolyticus, microorganismo nativo de desechos de camarón, fue seleccionado por sintetizar β-N-acetilglucosaminidasa y producir N-acetilglucosamina por hidrólisis de quitina. El diseño exitoso se logró cuando los ingredientes del medio de cultivo y los parámetros de fermentación se optimizaron para maximizar el contenido de enzima activa. Los resultados mostraron que un inóculo iniciador conteniendo 1.0×10^6 células/mL mezclado con un medio de cultivo formulado con quitina (35.13 g/L), cloruro de sodio (27.53 g/L), cloruro de magnesio (42.71 g/L), sulfato de sodio (1.71 g/L), cloruro de calcio (20.84 g / L) y extracto de levadura (5.1 g/L) en un biorreactor de tanque agitado operando en condiciones constantes de 26.6 °C, 7.3 de pH, 96.8 rpm y 1.6 VVM permite obtener una preparación enzimática con 0.041 U/mL de actividad, valor que se compara favorablemente al obtenido usando el medio de cultivo comercial de referencia y es tres veces mayor al obtenido en matraz agitado. Estos resultados sugieren a *V. alginolyticus* como fuerte candidato para producir quitinasas y confirma que estudiar la ecología microbiana para seleccionar microorganismos específicos y la optimización tanto del medio de cultivo como de los parámetros de fermentación permiten evolucionar eficientemente de la fermentación espontánea a la fermentación dirigida.

Palabras clave: *Vibrio alginolyticus*, síntesis de quitinasas, hidrólisis de quitina, producción de acetilglucosamina

1 Introduction

As the human population grows, so also grows the requirements for infrastructure, including sanitation, transport, housing, and food. This situation also leads to negative impacts on the environment such as cutting down of forests for habitation and farming, increasing waste water and several kinds of solid household and industrial waste that cause serious.

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https://doi.org/10.24275/rmiq/Bio544
issn-e: 2395-8472

To mitigate these negative impacts of waste, several mechanical, chemical, biological and combined processes have been proposed for their recycling to produce new material, products or energy. Biological recycling uses biodegradable or organic waste as raw material for conversion with an objective two-fold: to develop an efficient and affordable treatment to reduce the carbon biogeochemical cycle time or to produce a new compound with high value added (Poulicek and Jeuniaux, 1991; Kumar and Sai-Gopal, 2015; Castañeda-Casasola *et al*., 2018). Guidelines about how the native microbial community associated with biogeochemical cycles can be used to design efficient processes to degrade specific wastes are necessary. Several organic wastes can be used as study models, but chitin was selected because their degradation can generate products with high commercial value and their biogeochemical cycle time is slow (Patil *et al*., 2000; Souza *et al*., 2011). Chitin is the second most abundant biopolymer in nature after cellulose. It consists of β-1,4-linked N-acetyl-Glucosamine (GlcNAc) residues that are arranged in antiparallel (α), parallel (β), or mixed (γ) strands that form nanofibers, which are frequently strengthened by crosslinking with proteins and incrustation of minerals and waxes, giving rise to a wide range of shapes and functions such as the cuticle and exoskeleton of insects, the shells of crustaceans, fungal and bacterial cell walls and part of hyaluronic acid (Haynes *et al*., 1999; Il'ina *et al*., 2004; Suginta, 2007; Liu *et al*., 2011). In nature, the annual generation of chitin is about 1.0×10^{10} tons, which is a rich source of carbon and energy for growth and reproduction of chitin-degrading microorganisms, mainly fungi that utilize environmental GlcNAc to support growth and induce cellular development, a property important for their survival in various host niches (Marguerite, 2006; Revathi *et al*., 2012). On the other hand, and according to the FAO (2014), every year, from 6.0×10^{6} to 8.0×10^{6} tons of waste crab, shrimp and lobster shells are produced globally as a result of human activities and often just are discharged in landfills or the sea, creating an serious environmental problem because the rate of waste generation is much more bigger than the rate of biological degradation or natural recycling. Based on the chemical structure, chitin has been used as a feedstock to produce GlcNAc by either chemical or enzymatic hydrolysis because it is a precursor of the disaccharide units in glycosaminoglycans (such as hyaluronic acid chondroitin sulfate and keratan sulfate), which are necessary to repair and maintain healthy cartilage and joint function, and are widely used as raw material for valuable pharmacological agents and functional food additives (Deng *et al*., 2005; Chen *et al*., 2010). There are several disadvantages associated with chemical hydrolysis such as high cost, low yield (below 65%), bitter taste, environmentally unfriendly and unsuitability for people with shellfish allergies (Deng *et al*., 2005; 2008; Liu *et al*., 2013). Hence, much attention has been focused on hydrolysis using chitinases, specifically β-N-acetylglucosaminidase (EC 3.2.1.30), which cleaves glycosidic linkages from the non-reducing end to give GlcNAc (Dahiya *et al*., 2006; Akhir *et al*., 2009; Liu *et al*., 2013). However, the feasibility to GlcNAc commercial production from enzymatic hydrolysis has not been achieved because the process yield is low and the enzyme production cost is high, perhaps due to lack of the appropriate microorganism and expression conditions that allow the production of active forms and large quantities of these enzymes (Deng *et al*., 2008; Navarrete-Bolaños, 2012). Several researchers have found a wide range of terrestrial and marine microorganisms that can produce chitinolytic enzymes and have shown that the marine microorganisms synthetizes more stable and active enzymes than the corresponding enzymes derived from others sources. The genus *Vibrionaceae* as the marine bacteria has more enzymatic activity to degrade chitin (Svitil *et al*., 1997; Lam, 2006; Suginta, 2007; Hunt *et al*., 2008; Bai *et al*., 2014). We studied the partial microbial ecology of crustacean shell wastes, a specific ecological niche, to select the native microorganism of the genus *Vibrionaceae* to produce efficiently in situ β-N-acetylglucosaminidase. We then designed an efficient culture medium and the process variables optimization associated to the bioreactor to maximize enzyme production yield. The goal is to design a viable process of β-N-acetylglucosaminidase production for chitin hydrolysis into N-Acetylglucosamine.

2 Materials and methods

2.1 Strain isolation

Fresh shrimp samples were collected from a local market of Celaya, Guanajuato, Mexico. Shrimp heads and skins were separated, washed with tap water, crushed with a mortar and pestle, placed in polyethylene bags and incubated at 28 (±1) °C, until partial autolysis associated with spontaneous

fermentation was observed. Fermented samples were mixed with distilled water, milled in a blender, and filtered to obtain a solution containing the native microorganisms associated with spontaneous fermentation. Aliquots of 1.0 mL were placed into Petri dishes containing thiosulfate-citrate bile salt-sucrose (TCBS) agar (Difco, Detroit, Michigan, USA), a selective medium used for cultivating heterotrophic marine bacteria, mainly microorganisms of *Vibrionaceae* genus. Petri dishes were incubated at 28, 32, and 37 (±1) °C for 24, 48, and 72 h. Developed colonies having different morphologies were collected and transferred to a fresh agar of TCBS medium and incubated again. The procedure was repeated until pure cultures were obtained. To assure the purity of the isolated colonies, several biochemical tests were performed (oxidase, sucrose fermentation, string, lysine decarboxylase, and salt tolerance) as well as microscopic analysis on cells stained according to the Gram method (Harris *et al*., 1996; Rodríguez-Lerma *et al*., 2011).

2.2 *Enzymatic extracts production*

A sample from each isolated strain was transferred to 250 mL Erlenmeyer flasks containing 100 mL of marine liquid culture medium (Zobell, 1941), mixed, and incubated at 30 °C for 48 h at 100 rpm on a rotary shaker (Forma Scientific, Model 4520, Marietta, Ohio, USA) for biomass propagation. The product of the propagation was centrifuged at 10000g (Hermle, model Z383, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was considered raw enzymatic extract and used to assay β-N-acetylglucosaminidase activity (Rattanakit *et al*., 2003).

2.3 *Strain selection*

The strain selection was based on concentration chitin-degrading enzymes contained in each raw enzymatic extract, specifically β-N-acetylglucosaminidase, which was quantified by the release of p-nitrophenol (pNPh) from p-nitrophenyl-N-acetyl-β-D-glucosaminide based on modified method described for Tronsmo and Harman (1993). It involves the addition of 200 μl of p-nitrophenyl-N-acetyl-glucosamine (1.0 mg/ml) (Sigma Chemical Co., St. Louis, Missouri, USA) into 200 μl citrate/phosphate buffer (0.02M, pH 5.6), mixed with 200 μl of enzyme extract and incubated at 37 °C and 100 rmp on a rotary shaker for 60 minutes. After incubation, 1.5 ml of 0.02 M NaOH was added to stop the reaction, and the A400 was read in a spectrophotometer UV-Vis (Perkin Elmer, lambda 20, Waltham, Massachusetts, USA). The absorbance values are directly correlated to the released p-nitrophenol based on the calibration curve of absorbance versus p-nitrophenol concentration. The enzymatic activity in each extract was defined based on p-nitrophenol quantity released and was expressed as one enzyme unit. One unit of chitinases was defined as the amount of enzyme releasing 1μmol p-nitrophenol equivalent per minute under the given assay conditions.

2.4 *Pure culture propagation and starter inoculums*

The selected strain was cultured in agar slants at 37 °C for 24 h. For the propagation step, biomass samples were taken from each slant, transferred to 250 mL Erlenmeyer flasks containing 100 mL of marine liquid culture medium (Zobell broth), and incubated at 37 °C for 48 h at 120 rpm on a rotary shaker ((Forma Scientific, Model 4520). The product of the propagation step was adjusted to 1.0×10^6 cells/mL using a Neubauer hemocytometer. This "stock" suspension was used to inoculate different liquid culture media.

2.5 *Strain identification*

Once the best chitinolytic strain was selected, the identification was performed using molecular biology tools. In this study, the ribosomal sequence analysis of the 16s rRNA gene and two universal bacterial primers GM3F and GM4R was amplified by Polymerase Chain Reaction (PCR), followed by restriction analysis using endonucleases. The method is based on genomic DNA extraction from selected strains according to the protocol proposed by Ausubel *et al*. (2002). For rRNA gene amplification, the extracted DNA was mixed with PCR super mix (Invitrogen, Thermo Fisher Scientific. Carlsbad, California, USA) containing high-fidelity DNA polymerase (Invitrogen). PCR was performed at the following conditions: initial denaturalization at 94 °C by 5 min, 30 cycles with 1 min of denaturation at 94 °C, 2 min of annealing at 55 °C, 10 min of extension at 72 °C, and a final extension for 10 min at 72 °C. Agarose gel electrophoresis and ethidium bromide staining were used to check the quality and quantity of PCR product and purified using the QIAquick PCR purification kit EX II kit (Qiagen NV, Venlo, Netherland), and nucleotide

sequencing occurred using the Sanger technique on an Applied Biosystems Automated 3730xl DNA Analyzer (Applied Biosystems, Foster, California. USA) and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen) at Langebio Cinvestav, Mexico. The sequence obtained was compared with those reported in the NCBI (National Center for Biotechnology Information) database using the "Blastx" algorithm for strain identification.

2.6 *Specific culture medium design for vibrio strain selected*

Eight different culture media were evaluated for β-N-acetylglucosaminidase production (Table 1). Volumes of 100 ml of each culture media were autoclaved in Erlenmeyer flasks (250 ml capacity). Each flask was inoculated with *Vibrio* strains at a concentration of 1.0×10^6 cells /ml. The inoculated flask cultures were incubated at 37 °C and 200 rpm for 120 h on a rotary shaker (Forma Scientific, Model 4520). After incubation, the product obtained from the Erlenmeyer flask propagation was centrifuged at 10000 g (Hermle, model Z383), and the supernatant was collected and used to assay β-N-acetylglucosaminidase activity. Based on the screening results and seeking to obtain a more efficient culture medium, a new formulation was defined and used as a starter culture medium to analyze and evaluate the ingredient effects and their concentration on the β-N-acetylglucosaminidase production (Table 2). A sequential approach of five steps in two different phases based on response surface methodology was used to find the optimal and specific formulation for the *Vibrio* strain selected according to Navarrete-Bolaños *et al*. (2017). The inoculation and incubation of the new culture media formulated were similar to those described above.

Table 1. Composition (g/L distilled water) of the culture media used to produce β-N-acetylglucosaminidase by *V. alginolyticus*.

Ingredient	Culture media composition (g/L)							
	M1	**M2**	**M3**	**M4**	**M1+**	**M2+**	**M3+**	**M4+**
Chitin	0	0	0	0	24	24	24	24
$(NH_4)_2SO_4$	7	0.0016	0	2	7	0.0016	0	2
KH_2PO_4	1	0	0	0.7	1	0	0	0.7
NaCl	10	19.4	20	0	10	19.4	20	0
$MgSO_4$	0.1	0	0	0	0.1	0	0	0
Yeast extract	0.5	1	5	0	0.5	1	5	0
$MgCl_2$	0	18.77	0	0	0	18.77	0	0
Peptone	0	5	0	0	0	5	0	0
Na_2SO_4	0	3.24	0	0	0	3.24	0	0
$CaCl_2$	0	2.38	0	0	0	2.38	0	0
KCl	0	0.55	0	0	0	0.55	0	0
H_3BO_3	0	0.022	0	0	0	0.022	0	0
Na_2HPO_4	0	0.0151	0	0.2	0	0.0151	0	0.2
NaF	0	0.0024	0	0	0	0.0024	0	0
KBr	0	0.08	0	0	0	0.08	0	0
$EsCl_2$	0	0.034	0	0	0	0.034	0	0
Na_2O_3Si	0	0.004	0	0	0	0.004	0	0
Tryptone	0	0	10	0	0	0	10	0
$FeSO_4$	0	0.1	0	0.001	0	0.1	0	0.001
$MnSO_4$	0	0	0	0.001	0	0	0	0.001
Artificial seawater	0	0	0	500	0	0	0	500
Y= U/mL	3.40E-05	9.80E-04	1.10E-03	3.80E-05	3.30E-03	8.60E-03	4.30E-03	2.30E-03

M1: Experience of the researchers; M2: Based on Zobell (1941); M3: Based on Park *et al*. (2000); M4: Based on Stivil *et al*. (1997), Y is the enzymatic activity of β-N acetylglucosaminidase expressed as units of enzyme by milliliter (U/mL).

Table 2. New culture medium and boundary values for constructing the first experimental design of screening.

Ingredient	Content (g/L)	Variable	Boundary values (g/L)	
Chitin	25	X_1	18	30
Sodium chloride	20	X_2	15	25
Magnesium chloride	19	X_3	14	24
Peptone	10	X_4	6	14
Sodium sulfate	3	X_5	1	5
Calcium chloride	3	X_6	1	5
Yeast extract	3	X_7	1	5
Potassium chloride	0.55	X_8	0.1	1
Ferric citrate	0.1	X_9	0.05	0.15
Potassium bromide	0.08	X_{10}	0.04	0.12
Strontium chloride	0.035	X_{11}	0.01	0.06
Boric acid	0.03	X_{12}	0.01	0.05
Disodium Phosphate	0.02	X_{13}	0.01	0.03
Sodium silicate	0.004	X_{14}	0	0.008
Sodium fluoride	0.0025	X_{15}	0	0.005
Sodium bicarbonate	0.0015	X_{16}	0	0.003

2.7 Experimental design to maximize chitinolytic enzyme production at the bioreactor level

Once the composition of the liquid culture medium was defined, an experimental strategy for bioreactor process variable optimization was developed (Felix *et al*., 2018; Shakir *et al*., 2019). The process variables involved were temperature, acidity (expressed as pH), agitation and aeration. These variables were singled out on the basis of their relationship with the biomass growth and metabolic activities (temperature and pH) and with the heat and mass transfer limitations in the batch reactor (agitation and aeration). Agitation and aeration are essentially collinear variables affecting the oxygen transfer rate, which implicitly affects microbial growth, substrate transformation, and synthesis of product. The feasible ranges for these variables were defined on the basis of a preliminary screening design (not shown) as 23-30 °C for temperature (V_1), 6-8 for acidity (V_2), 50-250 rpm for agitation (V_3), and 0-2 VVM for aeration (V_4). Values that were used as boundaries of the search region to construct a central composite experimental design (Table 9). For this study, a stirred tank bioreactor (AZ control bioreactor, Applikon Dependable Instruments, Schiedam, Netherlands) of 3 L capacity was used. In all experimental fermentations, the starter inoculum concentration (15 ml of the suspension of 1.0×10^6 cells/ml), volume medium (1.5 L), and composition medium were kept constant. The output function was yield of chitinolytic enzyme measure as g/L of pNPh dissociated (Y).

2.8 Analysis of the chitin hydrolysis using the raw enzymatic extract

The hydrolysis of chitin was carried out after addition of the enzymatic extract into a 100 mM sodium acetate buffer (pH 6) containing chitin in a 1:1 ratio among the enzyme and substrate (HYTC: Organic chitin. Agrinos, Davis, California, USA). The reaction mixture was incubated at 28 °C for 24 h and then incubated in boiling water for 5 min to stop the reaction. The hydrolyzed extracts were filtered on Millipore membranes of 45 μm and analyzed by HPLC as described by Chen *et al*. (2012) with slight modifications. Briefly, GlcNAc concentration was determined on HPLC (Agilent 1200 series, Santa Clara, California, USA) equipped with UV-Vis, refractive index and Evaporative light scattering detectors, and a Shodex Asahipak NH2P-50 4E column (Showa Denko K.K., Tokyo, Japan). The solvent elution was operated at 1.0 mL/min of a mixture containing 70% acetonitrile and 30% water. The separation was performed while maintaining the column temperature at 25 °C, using the RI detector. The identification was made by matching the recorded chromatograms with those obtained using external standards of GlcNAc (Sigma-Aldrich, Saint Louis, Missouri, USA).

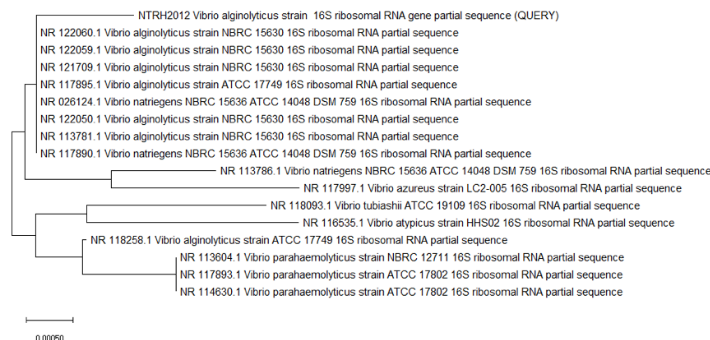

Fig. 1. Molecular Phylogenetic analysis by Neighbor-Joining method based on 16S ribosomal RNA sequences from bacteria and archea databases, showing the phylogenetic relationship between strain KX389670.1 (QUERY) and other members of the genus *Vibrio*.

3 Results and discussion

3.1 *Sequence analysis and phylogenetic analysis*

The sequences obtain were aligned with 16S ribosomal RNA sequences in the Genbank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify the bacteria isolate. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 1). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004)) and are in the units of the number of base substitutions per site. This analysis involved 17 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 950 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018). As a result, *Vibrio alginolyticus* (for a 99.9% identity score; 0% expectancy) is the strain selected to synthesize chitin-degrading enzymes. The sequence was deposited in GenBank under accession KX389670.

3.2 *Liquid culture media composition in chitinolytic enzyme production train selected*

The eight culture media evaluated could be used to produce chitinolytic enzymes of *V. alginolyticus* (last row of Table 1). However, the chitinolytic enzyme yields indicated that the strain responded differently depending on the culture media composition ($F_{(7,24)} = 10238.2$; $p < 0.001$), being the culture media containing chitin, and the common salts found in seawater (Samuelsson and Kirchman, 1990) and specifically high sodium chloride concentration that had higher chitinases activity (M2+ and M3+), whereas the remaining media yielded low chitinolytic enzyme production. Based on this, a new and more efficient culture medium was obtained (Table 2) and was used to find the optimal formulation for *V. alginolyticus* strain according to the strategy developed by Navarrete *et al*. (2017). The results shown that a culture medium composed of 35.13 g/L of chitin, 27.53 g/L of NaCl, 42.71 g/L of MgCl, 51.0 g/L of peptone, 1.71 g/L of Na_2SO_4, 20.84 g/L of $CaCl_2$, and 5.1 g/L of yeast extract allows obtaining an enzyme preparation with an activity of 0.026 U/mL of chitinases, which represents three-fold more units of enzyme compared with those values obtained in the flask that contain the best culture medium without optimizing (0.0086 U/mL). These results show that the chitinases can be produced at basal levels as constitutive enzymes by *V. alginolyticus* strain because it is the main carbon source used for their cell growth, but its synthesis level or production is increased by the chitin presence in the culture

medium. This finding suggests that the chitinases are inducible enzymes for *V. alginolyticus*. Moreover, the optimized culture medium contains the dissolved ions most abundant in seawater (sodium, chloride, magnesium, sulfate and calcium), which is consistent with the ecological niche where the chitinases are habitually present and emphasizes the importance of considering the chemical effectors of the ecosystem where the strain was isolated. However, the value concentrations for the salts are different, which shows the difference among the base requirements that are the average values of nature and optimum requirements desirable for bioprocesses' design. Likewise, the culture medium optimized and designed specifically for *Vibrio* growth and chitinases synthesis must be supplemented with peptone and yeast, which besides being the main sources of nitrogen also contribute vitamins, especially of B complex, amino acids and other compounds that help *V. alginolyticus*' growth and nutrition. Finally, the culture medium obtained as a result of the optimizing strategy developed is simpler in composition than the other culture media used for the same goal, which is an important prerequisite for the design of cost-efficient fermentation processes.

3.3 Variable optimization on bioreactor

Once the best culture medium for *V. alginolyticus* was identified, the experimental assays for the variable process optimization were performed. The results (see the last column of Table 3) revealed that based on the analysis of variance (Table 4), none of the variables exhibited a significant effect within their defined limits ($p < 0.05$), suggesting the presence of curvature and optimum proximity. Therefore, the results were used to construct a second-order polynomial model (Ec. 1) using least squares method to identify the relationships between the independent variables (process variables) and dependent variables (β-N-acetylglucosaminidase synthesis), obtaining the following expression:

$$\begin{aligned} Y = &-1.003 + 0.036v_1 + 0.147v_2 + 5.5\times10^{-4}v_3 \\ &+ 4.4\times10^{-3}v_4 - 8.0\times10^{-4}v_1v_1 + 8.0\times10^{-4}v_1v_2 \\ &- 1.9\times10^{-6}v_1v_3 + 5.2\times10^{-5}v_1v_4 - 0.012v_2v_2 \\ &- 4.3\times10^{-5}v_2v_3 + 3.32\times10^{-3}v_2v_4 - 6.4\times10^{-7}v_3v_3 \\ &- 3.8\times10^{-5}v_3v_4 - 8.3\times10^{-3}v_4v_4 \end{aligned} \quad (1)$$

Table 3. Central composite design (24+4α+2c) to optimize bioreactor process variables for maximize β-N-acetylglucosaminidase production by *V. alginolyticus*.

Assays	V_1	V_2	V_3	V_4	Y
1	30	8	50	2	4.47E-02
2	19.5	7	150	1	1.41E-02
3	23	6	250	2	1.75E-02
4	33.5	7	150	1	5.04E-03
5	30	8	50	0	2.66E-03
6	26.5	7	-50	1	3.03E-02
7	30	8	250	2	1.21E-02
8	23	8	250	2	3.78E-03
9	23	6	50	0	2.14E-03
10	26.5	9	150	1	2.28E-03
11	23	8	50	2	3.27E-02
12	30	8	250	0	3.12E-03
13	26.5	7	150	1	4.72E-02
14	26.5	7	350	1	1.57E-02
15	30	6	50	2	5.41E-03
16	26.5	7	150	1	4.64E-02
17	23	8	50	0	1.79E-03
18	23	6	250	0	5.03E-03
19	30	6	250	0	2.08E-03
20	26.5	7	150	-1	3.22E-03
21	26.5	5	150	1	2.17E-03
22	30	6	50	0	1.53E-03
23	23	6	50	2	1.26E-02
24	23	8	250	0	2.35E-03
25	26.5	7	150	3	2.78E-02
26	30	6	250	2	5.37E-03

V_1, V_2, V_3 and V_4 are variables of temperature in °C, pH value, agitation in rpm, and aeration in VVM respectively. The table shows their influence on the yield (enzymatic activity of β-N-acetyl-glucosaminidase) expressed as units of enzyme by milliliter (U/mL).

The model was analyzed with statistical tools based on the analysis of variance (Table 5), which shows a statistically significant relationship between the chitinases synthesis and the process variables ($F_{(14,11)} = 8.85$; $P = 0.0004$).

Therefore, on the basis of this model, the process variables values that maximized the chitinases synthesis were accurately computed based on solving a set of linear equations (Ecs. 2-5) achieved by differentiating the second-order model:

$$\begin{aligned} \frac{\partial Y}{\partial v_1} = &\, 0.036 - 2 * 8.0\times10^{-4}v_1 + 8.0\times10^{-4}v_2 \\ &- 1.9\times10^{-6}v_3 + 5.2\times10^{-5}v_4 \end{aligned} \quad (2)$$

$$\frac{\partial Y}{\partial v_2} = 0.147 + 8.0\times10^{-4}v_1 - 2*0.012v_2 - 4.3\times10^{-5}v_3 + 3.32\times10^{-3}v_4 \quad (3)$$

$$\frac{\partial Y}{\partial v_3} = 5.5\times10^{-4} - 1.9\times10^{-6}v_1 - 4.3\times10^{-5}v_2 - 2*6.4\times10^{-7}v_3 - 3.8\times10^{-5}v_4 \quad (4)$$

$$\frac{\partial Y}{\partial v_4} = 4.4\times10^{-3} + 5.2\times10^{-5}v_1 + 3.32\times10^{-3}v_2 - 3.8\times10^{-5}v_3 - 2*8.3\times10^{-3}v_4 \quad (5)$$

The model solutions based on solving their sets of linear equations are V_1=26.6, V_2=7.3, V_3=96.8 and V_4=1.6 for maximizing Y (0.049). This procedure indicates that the optimum conditions for chitinases synthesis maximization are 26.6 °C of temperature, 7.3 of pH value, 96.8 rpm of agitation and 1.6 VVM of aeration level. Under these experimental conditions, confirmation assays were performed; the results yielded 0.044 U/mL, which compares favorably against the predicted model value of 0.049 U/mL and represents three-fold more units of enzyme compared with those obtained at level shake flask using the optimized culture medium (0.026 U/mL) and five-fold more units of enzyme compared with those obtained at level shake flask using the best not optimized culture medium (0.0086 U/mL). This result confirms the suitability of the optimization of culture media and production parameters for the design of cost-efficient fermentation processes, the larger obstacle to enzymatic hydrolysis of chitin and of all the fermentative processes. The optimized process variables (7.3 of pH and 27 °C of temperature) are consistent with the average values in the seawater of the coasts of Mexico, from where the shrimp was obtained, and confirm again the importance of the ecological considerations among the microorganisms, their environment and the requirements for their growth. Moreover, the operating conditions in bioreactor for efficient enzymatic preparations production are within the limits of normal conditions (near room temperature, near neutral pH, and low values of agitation and aeration to maintain a constant dissolved oxygen concentration because vibrio is a obligate aerobic bacteria), which contributes to production of highly active enzyme preparations at reasonable cost.

3.4 Analysis of end products from colloidal chitin hydrolysis

Enzymatic extracts from both the shake flask and the bioreactor were evaluated related to chitin hydrolysis and specifically with the resulting end products of the reaction by HPLC.

Table 4. Analysis of variance for analyzing the process variables effect on β-N-acetylglucosaminidase synthesis based on central composite design results.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temp_A	0,0000151051	1	0,0000151051	0,36	0,5617
B:pH_B	0,000111629	1	0,000111629	2,65	0,1321
C:Agit_C	0,000276082	1	0,000276082	6,54	0,0266
D:Aire_D	0,00110189	1	0,00110189	26,12	0,0003
AA	0,00167306	1	0,00167306	39,66	0,0001
AB	0,000125664	1	0,000125664	2,98	0,1123
AC	0,00000764522	1	0,0000076452	0,18	0,6785
AD	5.33E-04	1	5.33E-04	0,01	0,9125
BB	0,00235949	1	0,00235949	55,93	0,0000
BC	0,00029584	1	0,00029584	7,01	0,0227
BD	0,000177289	1	0,000177289	4,20	0,0650
CC	0,000722311	1	0,000722311	17,12	0,0017
CD	0,000233478	1	0,000233478	5,53	0,0383
DD	0,00120401	1	0,00120401	28,54	0,0002
Total error	0,000464087	11	0,0000421897		

Table 5. Analysis of variance that shows the data fit level for the 2nd-order polynomial model constructed from central composite design results.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0,00522536	14	0,00037324	8,85	0,0004
Residual	0,000464087	11	0,00004219		
Total (Corr.)	0,00568945	25			

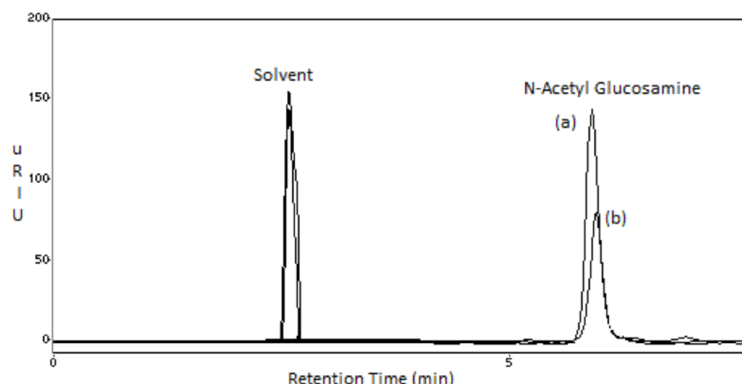

Fig. 2. HPLC chromatograms of the hydrolysate of chitin using the raw enzymatic extract produced by *Vibrio alginolyticus*., (a) standard and (b) hydrolysate.

The results show (Fig. 2) that both enzymatic extracts hydrolyzed the chitin yielding the N-acetyl glucosamine (GlcNAc) monomer, which suggests that *V. alginolyticus* synthesizes, under these conditions, the β-N-acetylglucosaminidase enzyme. These results suggest that *V. alginolyticus* can considered a potentially strong candidate for producing active chitinases for chitin hydrolysis to high added value products for their use in future applications.

Conclusions

The success of any enzymatic process depends on the production of highly active enzyme preparations at reasonable cost. In this work, a guideline to design a cost-efficient enzymatic preparation production was successfully undertaken. *V. alginolyticus*, a specific strain isolated and selected from spontaneous fermentation of shrimp wastes, can be used to produce enzymatic preparations with β-N-acetylglucosaminidase active in favor of the recycling chitin wastes and produces N-acetylglucosamine. The waste natural treatment to environmental control pollution is a service provided by the native microorganisms in a spontaneous process to recycle organic material, the initial phase of the biogeochemical cycles. However, human activities have leading to an imbalance of the recycling rate and the consequent pollution. Therefore, efficient fermentation processes, similar to those spontaneous processes, must be designed to mitigate the imbalance in the environment. Hence, the process design must start with microbial ecology studies of the spontaneous fermentation for the selection of the starter culture and continue with the optimizing physic-chemical effectors. The main idea evolves from spontaneous to directed fermentation to obtain a successful process based on the adaptability and affinity of the starter culture for the substrate and the innocuous of the process.

Disclosure statement

There is no known actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations associated with this work.

Acknowledgements

The authors acknowledge the Tecnológico Nacional de México for financing the research (5039.13-P).

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