β-amylase production using packaging-industry wastewater by a novel strain *Paenibacillus chitinolyticus* CKS 1

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Abstract

Factory of transport packaging generate a large amount of wastewater that contains residuals of starch glue. These residuals could be used as substrates for microorganism growth and enzyme production. In this study, β-amylase production by a new strain *Paenibacillus chitinolyticus*CKS1 was optimized using a wastewater from a Serbian factory of transport packaging. Optimization of the β-amylase production was carried out using Response Surface Methodology (RSM).The Central Composite Design under the RSM with four interacting parameters (incubation time, inoculum concentration, casein hydrolysate concentration and yeast extract concentration) was employed to identify optimal conditions the maximum β-amylase activity (334.20 U L^{-1}) and valued 62 h of incubation with 2.40 % inoculum, 2.02 g L^{-1} casein hydrolysate and 3.98 g L^{-1} yeast extract. A high performance liquid chromatography showed that the *P. chitinolyticus* CKS1 strain hydrolyzed starch to form maltose as a major product. Due to the application of wastewater as an inexpensive material for the enzyme and maltose production it may be considered that the economic and eco-friendly aspect of this method is very promising.

Keywords: wastewater; eco-friendly process; *Paenibacillus chitinolyticus* CKS1; β-amylase production; maltose; Response surface methodology

1. Introduction

Wastewaters, as well as the waste itself, represent a significant source of environmental pollution. The experience of Serbian factories for the production of transport packaging in terms of protection of environment is not very representative, since wastewater with a high COD and BOD are currently released untreated. This wastewater contains significant concentrations of biodegradable organic matter, which consists of the remains of starch glue and cellulose fibers from paper residues that are used in the production of cardboard. The organic matter in wastewater from packaging industry, could, therefore, be used as substrate for microbial growth, similar to that applied in treatment of wastewaters from food industry[1, 2]. Application of such biotechnological treatment of industrial wastewaters facilitates natural recycling process and typically results in production of valuable products together with effluent purification[1, 3,2].

The increasing concerns of environmental pollution have forced us to seek for cleaner industrial production and to employ some specific enzymes which can significantly reduce a pollution [4].In that line, nowadays, cellulases are used for improved cellulose hydrolysis of lignocellulosic biomass[5, 6], proteases have been used for the dehairing process [7], while laccase have a capability for dye decolouration [8] and pollutants degradation[4, 9].

Similar to other enzymes, amylases could be used in wastewater treatment for diminishing starch residues. Amylases hydrolyze starch molecules to give diverse products including glucose, maltose and specific or mixed malto-oligosaccharides[10,11]. Amylases can be divided into two categories, endoamylases and exoamylases[12]. Endoamylases or α-amylase catalyze hydrolysis of α-1,4 glycosidic linkages in a random manner in the interior of the starch macromolecule with the formation of oligosaccharides with varying length and α-limit dextrins, which constitute branched oligosaccharides. Exoamylases, either exclusively cleave α-1,4 glycosidic bonds such as β-amylase and produce maltose or cleave both α-1,4 and α-1,6 glycosidic bonds like amyloglucosidase or glucoamylase and α-glucosidase and produce glucose. To hydrolyze starch completely a combined action of various enzymes is required[12, 13].

β-amylase is used for starch processing and its mainapplication is for producing maltose syrup[14], a product that is widely applied in the food industry[15].

Most industrial α-amylases are produced by various *Bacillus* spp. during growth in starch medium[16-18]. Optimization of media components for amylase production using *Bacillus* spp. was studied thouroughly[10, 19]. *Paenibacillus* spp. are also amylases producers, and it has been shown that amylase production can be obtained with *Paenibacillus* spp. using commercial substrates [20] and agro industrial wastes [21]. Furthermore, there are two reports showing expression of amylase gene from *Paenibacillus* spp. [22, 23].

In the literature, there is no report of amylase production by *P. chitinolyticus,* but in our study we show, for the first time that this species can be used for amylase production using wastewater of transport packaging by strain CKS1.The aim of this study was to optimize conditions of wastewater from transport packaging utilization for amylase production by *P. chitinolyticus* CKS1. The wastewater from transport packaging was used for model solution. Response surface methodology (RSM) using a Central Composite Design (CCD) was used for optimization of fermentation parameters: incubation time, inoculum concentration, casein hydrolysate concentration and yeast extract concentration for obtaining maximum β-amylase activity. Analysis of the end products of fermentation by high performance liquid chromatography (HPLC) showed that treatment of wastewater by the strain CKS1 yields another valuable end product- maltose.

2. Experimental Methods

2.1. Microorganisms

The strain CKS1 was isolated from a soil sample taken from a coniferous forest, from a foot of the Alps and identified as *P.chitinolyticus* based on the almost full-length 16S rRNA gene sequence (KP 715850)[24]. A reference strain was *P.chitinolyticus* DSM11030. Both microorganisms were cultured on ISP1 liquid medium which consisted of casein hydrolysate 5.0 g L^{-1} and yeast extract 3.0 g L^{-1}.

The strain CKS1 and the reference strain were screened for amylase production on starch agar plate containing 0.1 g L^{-1} starch and 0.1 g L^{-1} agar in ISP1 liquid medium. Five microlitres of tested bacterial strains, which had previously been grown in the liquid ISP1 medium, were spot plated on starch agar plates. After incubation for 24-48 h at 30 °C, plates were flooded with Gram's iodine (2g KI and 1g iodine in 300 mL distilled water) for 3 to 5 minutes and observed for starch hydrolysis. Zone of clearance observed around the colonies indicated amylase activity.

2.2. Inoculum and medium preparation for amylase production

P.chitinolyticus CKS1 was grown in ISP liquid medium in a rotary shaker with mixing speed of 150 rpm at 30 °C for 24h.

Wastewater, which was used for the amylase production medium, was obtained from Serbian factory of transport packaging. The composition and characteristics of the wastewater were provided by the supplier Table S1 (Supplementary file 1).

BOD and COD analyses of wastewater were carried out using Merck-Spectroquant BOD test 1.00687 and Merck- Spectroquant COD test 1.09773, respectively. Nitrates, nitrites and iron were analysed according to Merck-Spectroquant Nitrate test 1.14773, Merck-Spectroquant Nitrite test 1.14776 and Merck-Spectroquant Iron test 1.00796, respectively. Gravimetric method was used for determination total dissolved solids in wastewater and electrometric method for determination of pH value of wastewater. Standard methods for determination of metals in wastewater were described previously[25].

The production medium contained the same ingredients (yeast extract 3g L^{-1} and casein hydrolysate 5g L^{-1}) as ISP medium, with exception that wastewater was used instead of distilled water for medium preparation. After sterilization at 121 °C for 20 min, an overnight bacterial culture was inoculated into fresh medium in a rotary shaker with mixing speed of 150 rpm at 30 °C. All fermentations were carried out in a 300 ml Erlenmeyer flasks with 30 ml of production medium in an orbital shaker (150 rpm) at 30 °C. The culture medium was centrifuged at 6000rpm for 15 min to remove the cells. The crude cell-free supernatant was analysed for β-amylase activity. The effect of culturepassaging on β-amylase production was examined by transffering the inoculum of 3% culture every 24h into fresh medium (passaging).Each passage was monitored for β-amylase activity for 4 days.

2.3. Enzyme test for amylase

The activity of the amylase was measured by modifiedBernfeld method[26].

2.3.1. Determination of amylase activity

The reaction mixture consisted of0.50 mL of 1% (w/v) soluble starch solution made in 0.02Macetate buffer (pH 6.90) or 0.016 M sodium acetate buffer (pH 4.80) and 0.50 mL enzyme solution (the crude cell-free bacterial supernatant) incubated at 50°C for 15min. The reaction was stopped by the addition of 1mLDNS reagent. The reaction mixture was then boiled for 5 min in a water bath. After cooling at room temperature, 5 mL of distilled waterwas added to each tube and absorbance of the solution was measured at 540 nm on spectrophotometer (Ultrospec 3300 *pro*Amersham Bioscience). One unit of the enzyme was defined as the amount of enzyme producing reducing sugars corresponding to 1μmol of maltose from the soluble starch per minute under the assay condition and per milliliter of the enzyme.

2.3.2. Effect of pH on activity of the crude amylase

To determine the optimum pH, the crude enzyme was incubated for 15 min at 50 ºC with 1%starch prepared in the following buffer solutions: 0.02M citrate buffer (pH 3.0, 4.0, 4.8 and

5.0), 0.02M sodium phosphate buffer (pH 6.0, 6.9 and 7.0), 0.02M Tris–HCl (pH 8.0 and 9.0), and 0.02M glycine–NaOH (pH 10.0). The amylase activity was measured as described above.

2.4. Experimental design

Based on preliminary single factor experiments(data not shown) a CCD was chosento examine the effect of four independent variables: incubation period (A), inoculum concentration (B), casein hydrolysate concentration (C) and yeast extract concentration (D) within the defined ranges that favored optimal feedback of the β-amylase production response. Each factor in this design was studied at five different levels (Table 1).

The data from CCD were analysed by multiple regression to fit to a second-order polynomial regression model containing the coefficient of linear, quadratic, and two factor interaction effects.

The model equation of response (Y) of the four independent variables (A, B, C and D) is given in the following equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (1)$$

where Y (β-amylase activity, UL^{-1}) is the dependent variable or predicted response associated with each factor level combination; A (incubation time, h), B (inoculum concentration, %), C (casein hydrolysate concentration, g L^{-1}), D (yeast extract concentration concentration, g L^{-1}); β_0 is the intercept term; β_1, β_2 and β_3 are the linear effects (main effect);β_{11}, β_{22} and$\beta_{33}$are the quadratic effects; and β_{12}, β_{13} and $\beta_{23}$are the interaction effects.

The RSM was applied using a statistical package, Design-Expert(Version 8, Stat-Ease, Inc., Minneapolis, United States).

2.5. HPLC analyses of starch hydrolyses

The starch hydrolysis product, obtained from CCD with maximum β-amylase activity, was analyzed by high performance liquid chromatography (HPLC). 5.0 mL of enzyme solution (crude bacterial supernatant) was incubated at 50 °C with 5.0 mL of 1% (w/v) soluble starch solution made in 0.016M sodium acetate buffer (pH 4.8). After different time intervals (15, 30, 60 and 120 min), samples were withdrawn and hydrolysis was stopped by boiling the samples for 5 minutes. The samples were then filtered through a 0.22 μm membrane filter.

For quantitative analysis of obtained samples, the Dionex Ultimate 3000 Thermo Scientific (Waltham, USA) HPLC system was used. A carbohydrate column (Hyper REZ XP Carbohydrate Ca^{2+}, 300 mm × 7.7 mm, 8μm) on 80°C was employed. Water (HPLC grade, JT Baker (USA)) was used as sole mobile phase with an elution rate 0.6 mL min^{-1} during the analysis. Detection was performed by RI detector (RefractoMax 520, ERC, Germany). All data acquisition and processing was done using Chromeleon Software. The separated hydrolysis products were identified by comparison with standard glucose, maltose, raffinose and dextrin and with literature data of used HPLC system for oligosaccharides. The soluble starch (Merck) solution was included as a control.

3. Results and discusssion

3.1. Screening for amylolytic activity

Amylase production was indicated by the appearance of a halo around the bacterial colony, indicative of areas of hydrolysis. *P.chitinolyticus*CKS1 produced clear zones of 4.00±0.29 mm diameter. Reference strain *P.chitinolyticus* DSM 11030 showed modest amylolytic activity with 0.50±0.01 mm area of hydrolysis. The strain CKS1 was used in further investigations as it was identified as the potent amylolytic strain of the *P. chitinolyticus* species.

3.2. pH influence on *P.chitinolyticus* CKS1 amylolytic activity

Testing the influence of pH on the amylolytic activity of the crude enzyme showed the presence of two peaks indicative of presence of two amylolytic enzymes produced by *P. chitinolyticus* CKS1 (Fig. 1).The data indicated that one enzyme had an optimum of activity at pH 4.8 and the other at pH 6.90. To determine if the type of enzymes produced by the tested strains, the products of hydrolysis obtained with crude enzyme at pH 4.80 and 6.90 were analysed using HPLC. The results indicated the predominant presence of maltose in hydrolysate obtained in solution with pH 4.80 with traces of other carbohydrates including glucose and longer oligosaccharides (Fig. S1). Based on the literature data, and the hydrolysis products it was preliminary concluded that when accessing the activity of the crude enzyme at pH 4.80, the enzymatic activity could predominantly attributed to β amylase.

3.3. Amylase production

Amylase production by *P. chitinolyticus*CKS1 was followed in media prepared with wastewater from transport packaging supplemented with organic sources of nitrogen, yeast extract and casein hydrolysate. In order to obtain achieve maximal hydrolysis until maltose, the hydrolisates obtained after 24 and 48 h of incubation were measured (Fig. 2). β-amylases showed activity after 24h of incubation of the strain CKS1, that further incrased with the incrase of the incubation time until 48h and valued 185.25± 1.89 U L^{-1}. Compared to the activity of amylases produced by other bacteria, this is a dramatically lower value. However, one should keep in mind that the obtained amylase activity was also almost 10 fold lower than obtained using starch as inducer of amylases synthesis (Fig. 1). Amylases are inducible enzymes and for its production a source of carbon is required. In this study, wastewater was used a s a substrate for microorganism growth and enzyme production. This wastewater contains only 0.1% suspended solids (Table S1) which contains mainly of starch glue residues which serves as a source of carbon for microorganism growthand amylase production. Low values of amylase activity can be explained by the low concentration of starch in wastewater. In addition to the limmited substrate amount, the wastewater could have contained various inhibitors of microbial growth (not measured), a variety of toxic waste matter which may affect the growth of bacteria and the enzyme production.

Hernandez et al.[1] studied the influence of the initial concentration of starch 10-40g L^{-1} in a brewery and meat processing wasteaters on amylase production. This wastewaters were supplemented with different starch concentartions and the highest amylase production of 70.29 EU/ml and 60.12 EU/ml was obtained in brewery and meat processing wastewaters supplemented with 40 g L^{-1} starch indicating the great influence of carbon (starch concentration) on enzyme production. However, since the goal of this study was to purfy the wastewater in line with amylase production, no additional carbon sources were added in the wastewater.

Subculturing (passaging) of an microorganism in a medium of essentially the same composition as that employed for the final culture has been an effective tool of enhancing a desired property [27]. This indicates that a certain adaptation of microorganism is required for the desired characteristic. In order to define if adaptation of the microorganism in the medium for β-amylase production has an impact on amylase activity, the influence of passaging of culture microorganism was examinated, and proved positive. β-amylase activity increased with culture passaging and with the incubation time (Fig.3). The highest β-amylase activity was detected for the third passage and on the third day of incubation with values of 212.11±2.44U L^{-1}. A slight decrease in β-amylase activity was observed in the fourth passage. Therefore, the second passage was used as the inoculum for further investigation ofβ-amylase production as this design enabled to perform other tests with the third passage of the bacterial culture.

3.4. Fitting the process variables

A total of 30 randomized experiments, including six replicates as the centre points were assigned to evaluate the pure error (Table 2).

For the four examined factors the CCD model efficiently designed a second order response fit for the surface. The quadratic model was found to be the most suitable model. TheANOVA test of significance of the regression model for the one response was evaluated (Table 3).

The second order equation was used to predict the maximum β-amylase production:

$$Y= 203.29 + 25.29A - 6.72B – 5.14C + 8.53D - 6.55AC + 42.52BC – 27.84BD + 9.79A^2 – 3.13B^2 – 11.71C^2 – 16.45D^2 \quad (1)$$

A positive sign in equation represents a synergistic effect of the variables, while a negative sign indicates an antagonistic effect of the variables.

The significant factors (p-value<0.05) that influenced the response were A, B, C,D, the quadratic coefficients of A, B, C and D as well as interaction AC, BC and BD. The analysis of variance (ANOVA) for the experimental results (Table 3) showed small probability value ($P < 0.001$) indicating the individual terms in the model are significant on the effect. The non-significant F-value for the lack of fit (1.63) compared with the pure error indicates that the model was adequate for predicting β-amylase production. The fit of the model was checked by calculating the determination coefficient (R-squared, adjusted R-squared, predicted R-squared). The value of R-squared is close to 1 for the model, which is very high and indicates a good correlation between the observed and the predicted values and good fitness with a low dispersion (Fig. 4)[28, 29]. Actual values were the measured response data for a particular run, and the predicted values were evaluated from the model. The Adequate precision value 42.683 was greater than 4 which indicate the signal was adequate. The coefficient of variance (CV) defines reproducibility of the model and is the ratio of the standard error of estimate to the mean value of the observed response. If CV of the model is not greater than 10%, model can be considered reproducible. The value of the coefficient of variation 3.85 suggested that the model was reliable and reproducible[29, 30].

3.5. Effects of process variables

Regression analysis revealed that influence of casein hydrolysate concentration(C) and yeast extract concentration (D) on β-amylase production was statistically significant ($p<0.05$) but their interactions CD was non-statistically significant (Table 3). Similar applied for the incubation time (A) and the inoculum concentration (B) and their interaction. Interactions AC, BC, and BD were statistically significant as well as quadratic parameters A^2, B^2, C^2 and D^2. Equation (1) shows that time of incubation (A) and yeast extract concentration (D) have linear positive influence on β-amylase production while inoculum concentration (B) and casein hydrolysate concentration (C) have significant negative linear effect. Among four quadratic parameters only A^2(incubation time) had a positive influence on β-amylase production. The influence of different variables on the β-amylase production was in following order: incubation time (A)>yeast extract concentration (D)>inoculum concentration (B) >casein hydrolysate concentration (C).

The incubation time of *P.chitinolyticus* CKS1, which showed the most prominent influence,varied from 18 – 74h (Table 2) and the maximum β-amylase production was obtained after 60 h (Run 19, Table 2, Fig. 5). The decrease in enzyme yield after the optimum incubation period (60 h) might be the consequence of the denaturation or decomposition of amylase, due to interaction with other components in the culture medium[17].

In general, the optimal incubation period depends on the culture characteristics and growth rate [17]. *P. amylolyticus* produced maximum α-amylase activity (80 U/g/min) after 72 h of solid state fermentation while growing on wheat bran[21]. An incubation period of 60 h for solid state fermentation using cassava fibrous residue by *Streptomyces erumpens* MTCC 7317 was also reported to yield a maximum amylase activity(3457.67 U/gds)[31]. For solid state fermentation of agro-industrial residues by *Bacillus megaterium* B69 maximum amylase production (1034 U/g) was achieved after 84 h of incubation [32]. Shorter time of incubation of 42 h,with maximum amylase activity (965.9 U/ml) was achieved when *Bacillus amyloliquefaciens* was incubated on a combination of wheat bran and groundnut oil cake (1:1) as the substrate in submerged fermentation [17]. In contrast, the longest reported optimal incubation time for a amylase production was 180h for α-amylase production by *Streptomyces rimosus* during growth on sweet potato residue as the substrate in SSF[33].

The contour plots are not perfectly elliptical which indicates that there may be less interaction occurring among the independent variables corresponding to the response surfaces [34].

The literature data for amylase production on wastewaters or waste materials by *Paenibacillus* spp. is very limited and results are difficult to compare with each other due to different growing conditions of different microorganisms[31,32,17], different substrates or waste materials[1, 33], and different procedures and units used for expressing the enzymatic activity[17, 35,36]. Nevertheless, it should be noted that the other studies typically report higher enzymatic activity than this in our study. While relatively low amylolytic activity might be to some extent a characteristic of *P. chitinolyticus* species, that is depicted as non-amylolytic in the Bergey's manual[37] , one should keep in mind that the substrates concentrations in the waste material treated in this study are much lower than in other wastes typically used for amylases production.

In addition to the incubation time, the concentration of yeast extract had a profound effect and stimulated the β-amylase production. In our experiment yeast extract concentrations varied from 0.50 to 6.5 g $L^{-1}$and the maximum β-amylase activity 322.52 U L^{-1} was obtained with 5 g L^{-1}

yeast extract (Run 19, Table 2).Generally, yeast extract is the main nutritional supplement which serves as a rich source of amino acids, vitamins, nitrogen and carbon for bacterial growth and thus on the enzyme production. The concentration of 5 g L^{-1} yeast extract was also reported to yield maximum β-amylase production by a *Streptomyces* sp. [36]. The maximum α- amylase activity from *Aspergillusoryzae* was achieved using 4.5 g L^{-1} yeast extract[38], while 20.0 g L^{-1} of yeast extract was needed for the maximum amylase production by *Bacillus circulans* GRS 313 [19].It is interesting to note that a relatively low yeast extract (0-1.0 g L^{-1}) result in maximum amylase activity of a highly potent *Bacillus* sp. α-amylase producer[35].

The effect of casein hydrolyasate, as another source of nitrogen,was tested and showed a negative effect both as a linear factor and ininteraction with incubation time (AC) (Fig.5).Only when incrased along with inoculum size, casein hydrolysate concentration had positive effect on β-amylase production (Fig. 6). The maximum amylase production was obtained using 2g L^{-1} casein hydrolysate (Run 19, Table 2). Casein hydrolysate is an excellent source of free amino acids and short peptide fragments, which are required by microorganisms for growth. Also, it contains trace of minerals and ions that could enhance the enzyme secretion [39].While amylase activity in some fungal strains could be increased by using more N-sources like urea, casein acid hydrolysate, soybean meal hydrolysate and $(NH_4)_2SO_4$[39, 40], *P.chitinolyticus* CKS1 prefered yeast extract in combination with smaller proportion of casein hydroysate as nitrogen sources.

Another factor that significantly affected the β-amylase production was the amount of inoculum that had a negative influence on β-amylase production. This factor had additional negative effect on β-amylase production, if the increase of inoculum size was accompaniedwith increased yeast concentration (Fig. 7).

It has been fairly well established that extracellular amylase secretion in microorganisms is substantially influenced not only by medium components including carbon source and nitrogen source, but also by culture conditions including pH, temperature, dissolved oxygen, and inoculum density. The importance of inoculum size with regard to microbial fermentation processes is generally accepted[34]. As it is shown in Fig.7, β-amylase production increased with decreases in the inoculum size from 5 to 3%, until reaching a certain percentage of inoculum at which enzyme productivity achieved maximum levels. This demonstrates that inoculum density does not exert an unlimited effect on fermentation processes. There is an optimum value to be achieved, and this appears to be dependent on the microbial species and fermentation system being utilized [34]. The inoculum density is particularly important in the growth of sporulating bacteria [41] such as *Paenibacillus* spp. and consequently can influence the production yield of β-amylase production. The optimization of inoculum density is quite important, as high inoculum density can reduce enzyme production due to competition for available nutrients. In a similar manner, low density can result in a reduce of enzyme secretion, owing to a drop in cell numbers [34].

3.6. Validation of the model

Model validation was also performed. For optimization β-amylase production the desirability function approach was employed. The desirability function in ideal case should be equal to 1 but in practical situation should be close to 1. Design Expert provides five options– none, maximum, minimum, target and within range– for choosing the desired goal for each variable and response[29]. Desired goal for β-amylase production was set on maximize.

It was done for two points selected from the numerical optimization results (Table 4).The obtained value of predicted and validated response shows that the estimated function may represent the experimental model and desired conditions indicating that the model was reliable.

3.7. Hydrolysis products of β-amylase

P. chitinolyticus CKS1 β-amylase hydrolyzed starch to form maltose as major product (Supplementary file 2). This product was readily apparent even during the early stages of the reaction (15 min) and increased in concentration along with the timecourse of the reaction. Maltooligosaccharides (i.e., limit-dextrins), maltotriose, rafinose with minor amounts of glucose were also produced. Hyun et al. [42] reported that a main product of starch hydrolysis by β-amylases of *C. thermosulfurogenes* was maltose. The appearance of maltose as the major hydrolysis product and the relatively small amount of glucose with*Clostridium thermosulfurogenes* SV2 implies that the amylase produced by this microorganismis is of the β type [43]. The amylase from *Halobacillus* sp. LY9 hydrolyzed soluble starch to form maltose as the main product with trace amounts of longer oligosaccharides [44]. According to Hensley et al. [45], linear amylose chains (soluble starch) with odd numbers of glucose units are responsible for the small amounts of glucose and maltotriose formed when amylose is digested with β-amylase. Hence, the amylase from *P. chitinolyticus* CKS1 may preferentially cleave at the α-1,4 linkage from non-reducing ends of starch molecule, releasing maltose which indicated a β-amylase activity. Given that all experiments were performed with crude, not purified enzyme, the traces of other carbohydrates in the HPLC profile could be explained by residual activity of α-amylase under pH conditions not favorable by this enzyme.

4. Conclusions

In this study, a cleaner and environmentally friendly enzyme production using wastewater was demonstrated. The results shows that the wastewater from the factory of transport packaging could be used as a substrate for microorganism growth and amylase production. This is the first application of a *P.chitinolyticus* strain for production of amylases, which makes reported results fundamental. The novel strain *P. chitinolyticus* CKS1 could produce α and β-amylase while growing on wastewater supplemented with yeast extract and casein hydrolysate. Considering that the major product of the β-amylase hydrolysis of the starch is maltose a β-amylase production was studied in more detail. Conditions for β-amylase production were optimized using the CCD under RSM. This approach indicated that β-amylase activity was mostly affected by the incubation time followed by yeast extract concentration, and negative effects of inoculum size and casein hydrolysate concentration. The optimized conditions for obtaining the maximal β-amylase activity 334.20 U L^{-1} were defined to be 62 h of incubation, 2.40 % of inoculum, 2.02 g L^{-1} casein hydrolysate and 3.98 g L^{-1} yeast extract. This study shows that the use of wastewater for the production of β-amylase is a procedure that when applied would have a positive economic and environmental effects as it generates cleaner water, β-amylase and maltose as the major product of starch hydrolysis.

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Table captions

Table 1. Experimental ranges of the independent variables in the experimental design

Table 2. The design matrix and the corresponding response

Table 3. The analysis of variance (ANOVA) for quadratic model

Table 4. Numerical optimization solutions

Figure captions

Fig. 1 Effect of pH on the amylolytic activity of the crude enzyme obtained on starch substrate (a) and ISP medium with wastewater (b)

Fig. 2 The influence of the time of incubation on β-amylase activity

Fig. 3 The influence of culture passaging on the β-amylolytic activity

Fig. 4 Plot of depicting the correlation between the measured and the model predicted values of the β-amylolytic activity

Fig. 5 Surface plot of interactive effects of incubation time and casein hydrolysate concentration and (AC) on the β-amylolytic activity

Fig. 6 Surface plot of interactive effects of inoculum concentration and casein hydrolysate concentration (BC) on the β-amylolytic activity

Fig. 7 Surface plot of interactive effects of inoculum concentration and yeast extract concentration (BD) on the β-amylolytic activity

Table 1. Experimental ranges of the independent variables in the experimental design

Factors	-1	0	+1	Axial (-α)	Axial (+α)
A: incubation period, h	32	46	60	18	74
B: inoculum, %	3	4	5	2	6
C: casein hydrolysate,gL^{-1}	2	3.5	5	0.5	6.5
D: yeast extract, $g\ L^{-1}$	2	3.5	5	0.5	6.5

513 Table 2. The design matrix and the corresponding response

Run	Independent variable				Response
	A (h)	B (%)	C (g L^{-1})	D(g L^{-1})	Y (U L^{-1})
1	60	5	5	5	231.086
2	60	5	5	2	281.263
3	32	3	5	2	117.211
4	60	3	5	5	223.194
5	60	5	2	2	205.684
6	60	5	2	5	177.076
7	46	4	3.5	3.5	226.154
8	32	3	5	5	171.921
9	60	3	2	2	258.215
10	32	3	2	2	182.191
11	32	5	2	2	155.978
12	46	4	3.5	3.5	218.262
13	60	3	5	2	141.241
14	32	3	2	5	265.121
15	46	4	3.5	3.5	231.351
16	32	5	5	5	202.725
17	46	4	3.5	3.5	226.647
18	32	5	5	2	235.526
19	60	3	2	5	322.520
20	32	5	2	5	106.048
21	46	4	3.5	6.5	134.903
22	46	4	6.5	3.5	119.612
23	46	4	3.5	0.5	93.7172
24	46	2	3.5	3.5	186.380
25	74	4	3.5	3.5	270.132
26	46	6	3.5	3.5	148.846
27	46	4	3.5	3.5	187.242

28	46	4	3.5	3.5	175.843
29	18	4	3.5	3.5	168.458
30	46	4	0.5	3.5	146.988

A: incubation period; B: inoculum concentration; C: casein hydrolysate concentration; D: yeast extract concentration; Y: β-amylase activity.

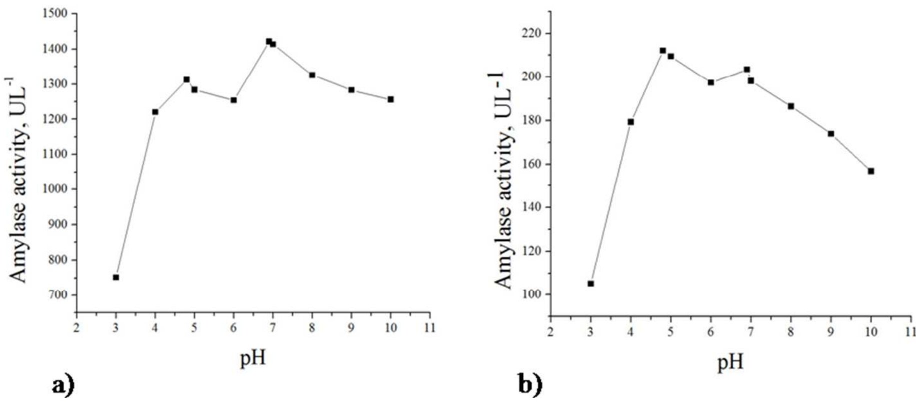
Table 3. The analysis of variance (ANOVA) for quadratic model

	F - value	p- value Prob >f
Model	97.48864	< 0.0001[a]
A	276.3014	< 0.0001[a]
B	19.5155	0.0006[a]
C	11.42568	0.0045[a]
D	31.44915	< 0.0001[a]
AB	0.217224	0.6483[b]
AC	12.34753	0.0034[a]
AD	0.177629	0.6798[b]
BC	520.7398	< 0.0001[a]
BD	223.2311	< 0.0001[a]
CD	0.253497	0.6225[b]
A^2	47.34463	< 0.0001[a]
B^2	4.833885	0.0452[a]
C^2	67.67623	< 0.0001[a]
D^2	133.6956	< 0.0001[a]
Lack of Fit	1.63	0.3376[b]
R-squared	0.9898	
Adjusted R-squared	0.9797	
Predicted R-squared	0.9435	
C.V. %	3.85	
Adequateprecision	42.683	

[a]Significant coefficient ($P < 0.05$)
[b]Non-significan coefficient

Table 4.Numerical optimization solutions

Sample	Incubation time, h	Inoculum, %	Casein hydrolysate, g L^{-1}	Yeast extract, g L^{-1}	β-amylase activity, U L^{-1}	
					Predicted	Validated
1	62.00	2.40	2.02	3.98	333.145	334.201
2	18.00	2.14	3.05	6.50	262.89	260.674



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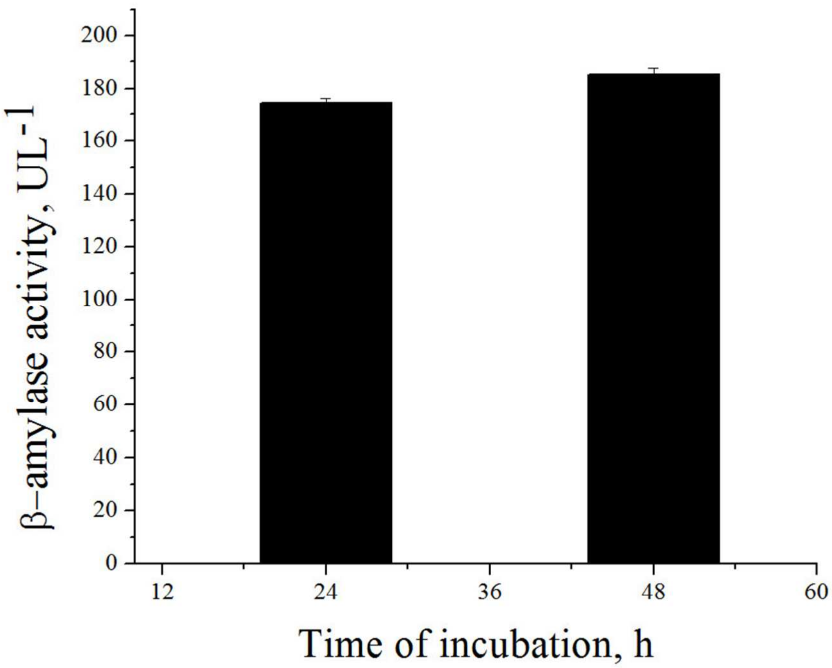

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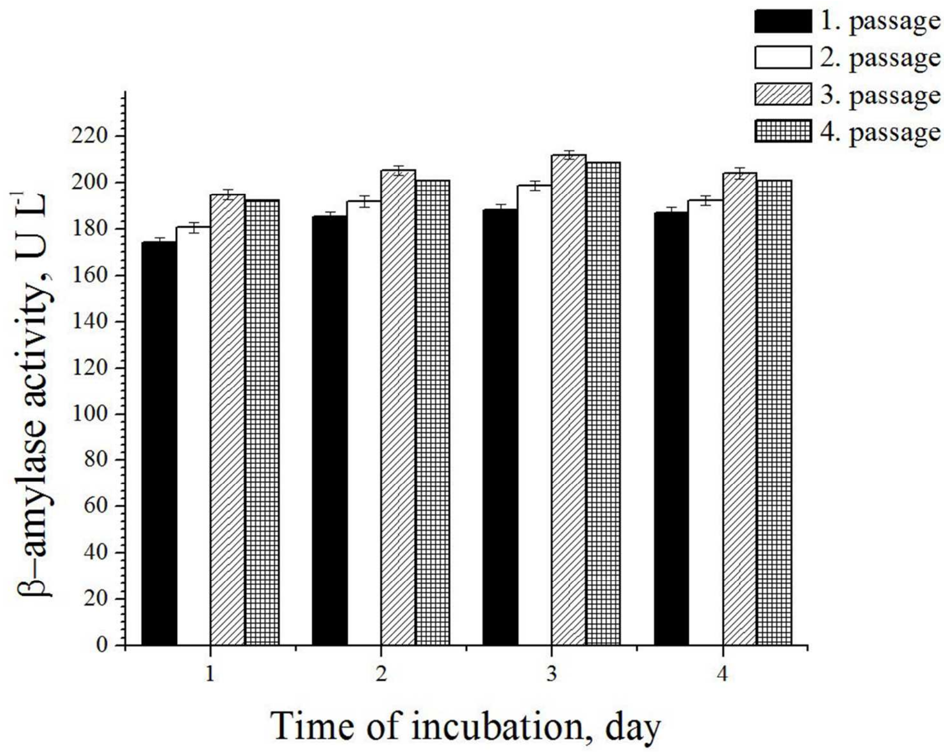

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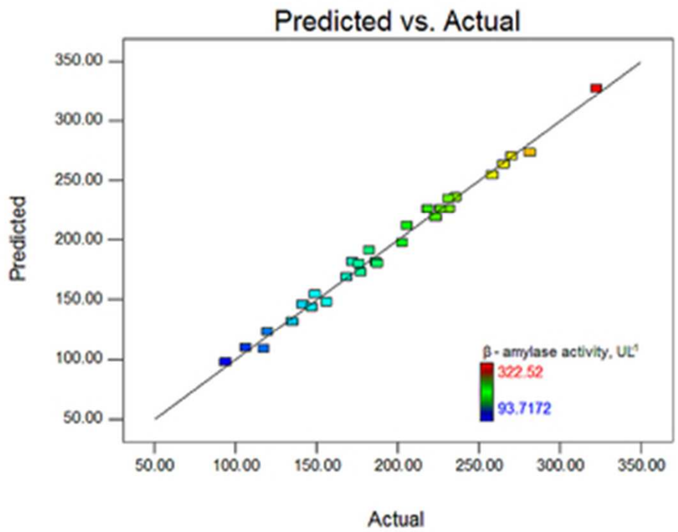

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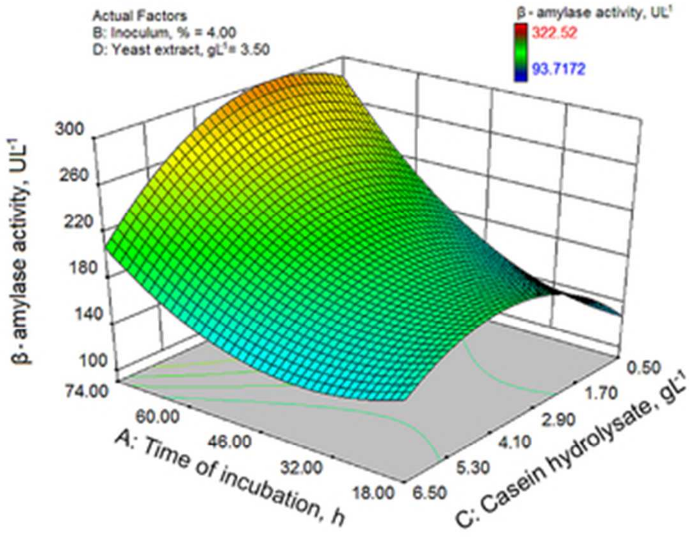

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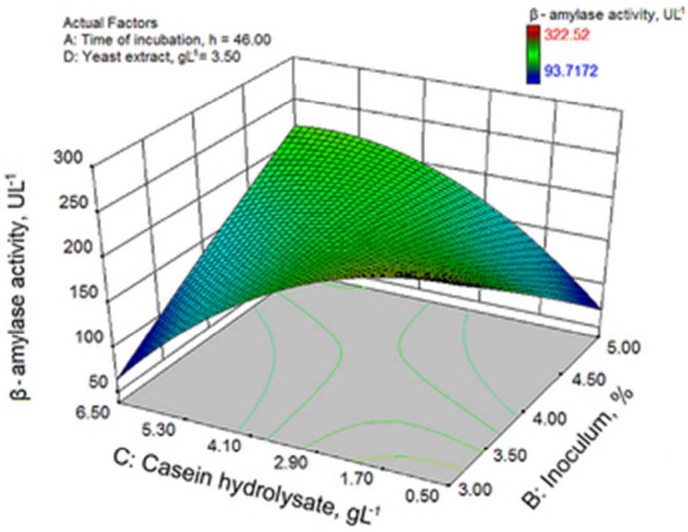

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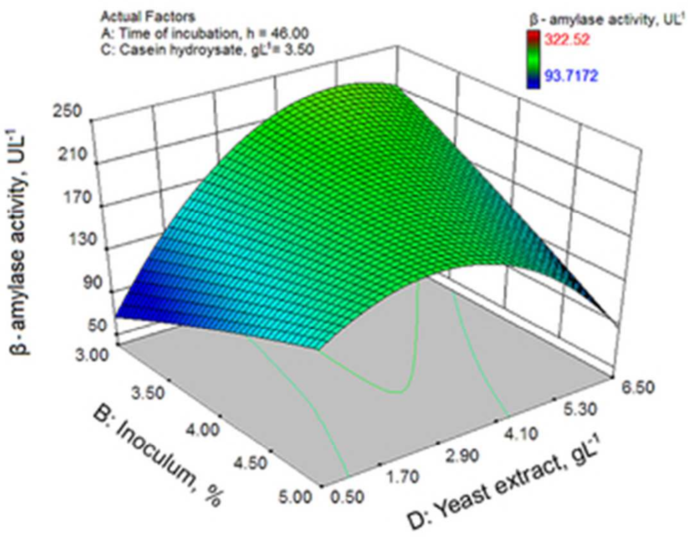

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