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Utilisation of date wastes as substrate for the production of α-amylase

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Abstract

Submerged fermentation of *Candida guilliermondii* CGL-A10 was carried out for enhanced production of α-amylase using date wastes as a substrate. The process parameters influencing the production of α-amylase were optimized. Effects of process variables, namely incubation time, incubation temperature, initial pH, starch concentration, supplementary nitrogen source, nitrogen and phosphorus concentrations on production of α-amylase have been studied, and accordingly optimum conditions have been determined. The result obtained show that the presence of starch induces strongly the production of α-amylase with a maximum at 5.0 g/L. Among the various nitrogen sources tested, yeast extract and urea at 5.0 g/L gave the maximum dry biomass and α-amylase estimated to 5.16 - 5.76 g/L and 2056.33 - 2304.19 µmol/L/min, respectively, 72 h after inoculation at 30°C, with an initial pH of 6.0 and potassium phosphate content of 6.0 g/L.

Introduction

In Algeria, the production of dates is estimated to be 530.000 tons of which 85.000 to 105.000 tons are of very low commercial value, constituted of common dates and date wastes of Deglet-Nour and Ghars varieties (Anonymous, 2010). Due to their low value, date wastes are an economical source of carbohydrates for conversion to industrial enzymes.

Several extra cellular enzymes are commercially available and widely used in industry. Among them, amylases are involved in the assimilation of starch based matter, respectively, provided as substrates. Fungal amylases are used for hydrolyzing carbohydrate and other constitutes of soy beans and wheat into simple sugar constituents. These enzymes find potential application in a number of industrial processes such as food processing, fermentation, textile, paper industries and in biotechnology (Lagzouli *et al*., 2007). The spectrum of amylase applications has expanded into many new fields such as clinical, medicinal and analytical chemistry (Pandey *et al*., 1999). The production of α-amylase by submerged fermentation using synthetic media has been reported by many workers (Hernandez *et al*., 2006; Kathiresan and Manivannan, 2006; Lagzouli *et al*., 2007; Wang *et al*., 2008). The contents of synthetic media are very expensive and uneconomical. Therefore, it needs to be replaced by the more economically available substrates to reduce the cost. In this regard, agricultural residues and food-industrial residues are generally considered as the best substrate for amylases production. Amylases are produced by a variety of micro-organisms such as *Bacillus subtilis*, *Rhizopus oryzae*, *Aspergillus niger*, *A. awamori*, *A. oryzae* (Ramesh *et al.*, 1987; Pandey *et al*., 1999; Hernandez *et al*., 2006; Ravi *et al*., 2009). *B. subtilis* and *R. oryzae* have been considered the most important for industrial application (Guillen-Moreira *et al*., 1999; Pandey *et al.*, 2000; Djekrif-Dakhmouche *et al*., 2006). The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are achieved. The thermal stability is an important feature of most of the enzymes sold in bulk for industrial application. So the selection of thermophilic micro-organisms is particular interest for the production of thermophilic α-amylases (Guillen-Moreira *et al*., 1999). Algeria imports enzymes from industrially advanced countries for its industrial needs and it involves a huge amount of foreign exchange. The production process for enzymes has not been developed in Algeria. This will lead to the development of an indigenous technology for the production of the industrially enzymes.

The aim of the present study was to investigate the potential of date wastes as substrate for the production of α-amylase using strains of *C. guilliermondii* isolated from leavening dough and the determination of optimized production conditions. To the best of our knowledge this is the first report on α-amylase

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production using date wastes.

Material and Methods

Material

The vegetable material used was date wastes produced by Deglet-Nour variety.

Isolation, identification and screening of C. guilliermondii

Several samples of leavening dough traditional were collected from different regions of eastern south of Algeria. One gram of each sample is placed directly into 250 ml Erlenmeyer flasks containing 50 ml of culture medium semi synthetic. To inhibit growth of bacterial contaminants, the chloramphenicol at 50 mg/L was added and sterilized by filtration in millipore filter. So, ten strains of *C. guilliermondii* were isolated from leavening dough. After 72 h of incubation at 30°C, the isolates were purified after five successive subcultures in the medium solid culture, containing starch as the sole carbon source. These isolates are identified by using all cultural, morphological, sexual, and physiological characteristics as reported by Guiraud and Galzy (1980). The identification was based on different biochemical tests including the fermentation of sugars, assimilation of carbon compounds and growth at 37°C.

Preparation of culture medium or date wastes juice

The juice is produced by heating the date wastes in water at 85°C for 45 min with continuous stirring. The extract is filtered, decanted, further clarified through and sterilized at 120°C during 20 min. The carbohydrate composition of the date wastes was found to be 12.85% glucose, 11.11% fructose, and 32.23% sucrose in dry weight.

Fermentation process

The strains of *C. guilliermondii* were inoculated in sterile 100 ml Erlenmeyer flasks containing 20 ml of culture medium composed by (g/L): glucose, 20.0, yeast extract, 5.0, KH_2PO_4, 5.0, at pH 5.0 (Lagzouli *et al*., 2007). The cultures were developed in 250 ml Erlenmeyer flasks containing 50 ml of date wastes syrup inoculated with 2% (w/v) inoculum level and incubated at 30°C for 72 h at 200 rpm/min.

Analytical methods

Mycelial was harvested by paper filtration using a pre-dried and pre-weighted Whatman filter paper, washed with distilled water and dried to constant weight at 70°C (Hernandez *et al*., 2006).

Amylase was assayed by adding 0.1 ml of enzyme fermented broth supernatant to 0.2 ml of 0.5% soluble starch and incubated for 30 min at 37°C. The reaction was stopped by adding 0.4 ml of 3.5 dinitro-salicylic acid, followed by boiling for 10 min. The final volume was made to

10 ml with distilled water and the absorbency measured at 540 nm with U.V spectrophotometer. A calibration curve of absorbance and concentration of D-glucose was established with known amount of glucose (Bernfeld *et al*., 1955). One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as D-glucose per min under the assay conditions. The results are presented as specific activity (µmol/L/min).

Results and Discussion

Ten strains of *C. guilliermondii* were isolated from leavening dough and the colonies of these strains are white to cream colored, smooth, glabrous and yeast-like in appearance. The microscopic morphology shows spherical budding yeast-like cells or blast conidia, and 2.2 - 3.6 x 2.88 - 5.85 µm in size. For the physiological tests, these strains were not hydrolysis urea, but they growth on cycloheximide medium, and at 37°C. They assimilate the glucose, sucrose, melibioze, galactose, maltose, glycerol, raffinose, trehalose, rhamnose, arabinose, mannitol, sorbose, succinic acid and citric acid. In contrast, they not assimilate the potassium nitrate, lactose and soluble starch.

These strains were tested to evaluate their potential in α-amylase production and all tested strains in the screening experiment were able to grow on date wastes juice and were capable of producing α-amylase, i.e., 2.28 - 6.12 g/L and 225.33 -1519.23 µmol/L/min (Table 1). However, CGL-A10 was selected as the best producer of α-amylase and its production conditions were optimized. The results obtained indicate that after 24 h of incubation, biomass and α-amylase production were 2.14 g/L and 762.33 µmol/L/min, then 3.22 g/L and 1222.66 µmol/L/min at 48 h and increased to 3.92 g/L and 1519.23 µmol/L/min at 72 h. So, the optimal incubation period was 72 h.

Table 1. Dry biomass and α-amylase activity following different strains

Strains of *C. guilliermondii*	Dry biomass in g/L	Enzymatic activity in µmol/L/min
CGL-A2	2.56	225.33
CGL-A3	2.28	228.56
CGL-A10	**3.92**	**1519.23**
CGL-C12	4.26	625.35
CGL-C20	5.33	712.36
CGL-D4	4.26	425.33
CGL-D6	5.22	412.45
CGL-E9	6.12	812.56
CGL-F13	3.56	324.44
CGL-F16	3.66	658.78

Similar results were reported by various research groups (Selvakumar *et al.*, 1998; Kathiresan and Manivannan, 2006; Lagzouli *et al*., 2007; Wang *et al*., 2008). In contrast, Kunameni *et al.* (2005) reported that Thermomyces lanuginosus strain produced high titters of enzyme at 120 h. At the later stage, when nutrients were depleted, it reached its stationary phase and resulting in a lower α-amylase activity, i.e., 1168.33 µmol/L/min at 96 h. Similar results were obtained by Guillen-Moreira *et al.* (1999) with *A. tamarii*. The decrease in enzyme production after the optimum level may be in denaturation or decomposition of α-amylase due to interaction with other components in the medium as it is reported by Ramesh and Lonsane (1987).

Temperature is one of the important factors, which strongly affect the submerged fermentation (Vidyalakhsmi *et al.*, 2009). Variation of the temperature brought about a change in metabolic pattern of the micro-organism; it exhibited its best amylase production in the mesophilic range (Vidyalakhsmi *et al*., 2009). The results obtained show that the dry biomass and α-amylase produced at 20°C are low, i.e., 1.18 g/L and 450.37 µmol/L/min, and increased to a maximum of 3.92 g/L and 1519.23 µmol/L/min, respectively at 30°C. The optimum temperature for the maximum α-amylase activity was 30.0°C as confirmed by other reports (Selvakumar *et al*., 1998; Lagzouli *et al*., 2004; Ramashadran *et al.*, 2004; Djekrif-Dakhmouche *et al*., 2006; Alva *et al*., 2007; Wang *et al*., 2008; Renato *et al*., 2009). A further increase in temperature resulted in a decrease in dry biomass and α-amylase production, i.e., 00.85 g/L and 2000.33 µmol/L/min, respectively at 45°C. At higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation (Nawaz-Bhatti *et al*., 2007). Temperature above 45°C results in moisture loss of the substrate, which affects metabolic activities of *Penicillium janthinellum*, and results in reduced growth and α-amylase production (Sindhu *et al*., 2009). However, Kunameni *et al.* (20005) and Ravi *et al*. (2009) reported that optimum temperature for amylase production by *T. lanuginosus* and *Humicola lanuginosa* is 50°C.

Among the physical parameters, the pH of medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. The production of α-amylase is very sensitive to initial pH of the fermentation medium. According to Gupta *et al.* (2003), the synthesis of extra-cellular α-amylase is affected by the pH, just like its secretion and the stability of the amylolytic system. Under experimental conditions, a maximum biomass was produced at pH=6.0, i.e. 4.33 g/L and the lowest at pH=9.0 and pH=4.0, i.e., 2.56 - 3.40 g/L, respectively (Figure 1). As for the enzymatic activity, the maximum value, i.e., 1651.21 µmol/L/min was obtained at an initial pH=6.0 (Figure 1). So, the maximum biomass and α-amylase activity were produced at pH=6.0. Our findings are comparable to previous reports (Lagzouli *et al*., 2004; Anto *et al.*, 2006; Djekrif-Dakhmouche *et al*., 2006; Hernandez *et al.*, 2006; Alva *et al.*, 2007; Renato and Nelson, 2009) with *Aspergillus* spp. and *A. niger* at pH varying between 5.0 and 6.0. In contrast, Gangadharan *et al*. (2006); Pavezzi *et al*. (2008) reported pH=3.5 and pH=4.0 to be the best for the production of α-amylase by *B. amyloliquefaciens* and *A. awamori*. When the pH is above or below 6.0, the α-amylase production is greatly reduced or becomes denatured and the enzyme activities obtained at pH pH=9.0 and pH=4.0 are 666.66 and 781.33 µmol/L/min, respectively. According to Guillen-Moreira *et al*. (1999), the growth and enzyme production were inhibited when the initial pH of the medium was above 10.0 or below 4.0.

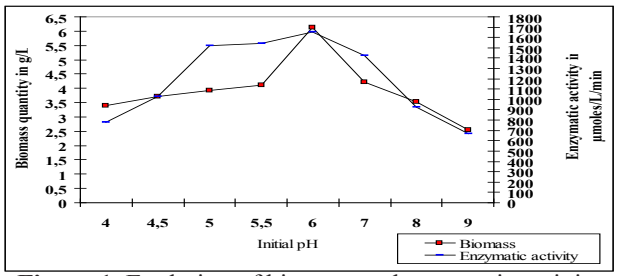

Figure 1. Evolution of biomass and enzymatic activity following initial pH

Variation in initial sugars content of substrate showed that the α-amylase synthesis was related to the availability of sugars content. The obtained results show that the growth and α-amylase activity increases gradually with an increases in sugars content to reach to a maximum values at 15.0 g/L i.e., 4.33 g/L and 1648.33 µmol/L/min. The lowest yield, i.e., 666.66 µmol/L/min was obtained at 2.5 g/L of sugars. So, the optimum sugars concentration for maximum α-amylase activity was 15.0 g/L. This result corroborates the studies undertaken by Duran-Paramo *et al*. (2000); Hernandez *et al.* (2006) with *A. niger* and *B. subtilis*.

On the other hand, the results shows that starch concentration have an important effect on dry biomass and α-amylase production. So, the growth and α-amylase activity increases gradually with an increases in starch content to reach to a maximum values at 5.0 g/L, i.e., 6.12, and 2056.33 µmol/L/min. Beyond 5.0 g/L, α-amylase activity stabilizes around

2000.00 µmol/L/min. So, the optimum starch content for maximum α-amylase production is 5.0 g/l. Similar results were reported by Lagzouli *et al.* (2007) with *C. guilliermondii*. In contrast, the optimum starch concentration for maximum α-amylase production with *Lipomyces kononenkoae* was 10.0 g/L (Spencer-Martins, 1982).

Nitrogen has a profound effect on enzymes production. According to Kaur *et al*. (2003), nitrogen sources have been reported to have an inducing effect on the production of various enzymes including α-amylase. The results presented in figure 2 show that the highest dry biomass was obtained with meat extract, i.e., 6.47 g/L followed by urea, i.e., 5.70 g/L, peptone, i.e., 5.08 g/L and ammonium phosphate, i.e., 4.66 g/L. The lowest biomasses were obtained with ammonium nitrate, ammonium sulphate and ammonium carbonate, i.e., 3.22, 3.40 and 3.45 g/L, respectively (Figure 2). Among the organic sources, supplementation of yeast extract and urea showed an increased in α-amylase activities, i.e., 2056.33 and 2100.22 µmol/L/min respectively (Figure 2). Ammonium phosphate also enhanced the α-amylase activity relatively, i.e., 1765.12 µmol/L/min but ammonium nitrate showed a negative influence, showing a steep decrease in α-amylase activity i.e., 865.00 µmol/L/min. We conclude that the urea and yeast extract were the best nitrogen sources. Similar results were recorded by other researchers (Djekrif-Dakhmouche *et al*., 2006; Gangadharan *et al*., 2006; Hernandez *et al.*, 2006; Lagzouli *et al*., 2007) with *A. niger*, *B. amyloliquefaciens* and *C. guilliermondii.* However, in contrast some studies shows that the peptone was the best nitrogen source with *T. lanuginosus*, *P. fellutanum* and *B. licheniformis* (Dharani-Aiyer, 2004; Kunameni *et al*., 2005; Kathiresan and Mnivannan, 2006).

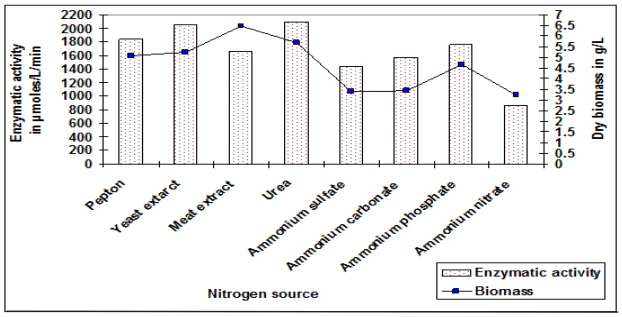

Figure 2. Evolution of biomass and enzymatic activity following nitrogen source

The concentrations of nitrogen affect yeast growth and the synthesis of enzymes. As shown in figure 3, the growth of *C. guilliermondii* CGL-A10 increased significantly with an increase in urea content to reach to a maximum value, i.e., 6.85 g/L at 6.0 g/L (Figure 3). As for enzymatic activity, this latter increases gradually with the urea content to reach to a maximum value, i.e., 2100.22 µmol/L/min at 5.0 g/L (Figure 3). However, beyond 5.0 g/L, the α-amylase activity dropped to 1465.42 µmol/L/min at 6.0 g/L. So, the optimum urea content for the maximum enzyme activity was obtained at 5.0 g/L. Similarly, Djekrif-Dakhmouche *et al.* (2006) obtained a maximum enzyme activity with *A. niger* at 5.0 g/L of nitrogen. Moreover the maximum gluco-amylase produced was obtained with concentrations of yeast extract ranging between 2.0 and 5.0 g/L (Kunameni *et al.*, 2005), whereas with *A. fumigatus*, the best concentration is 2.0 g/L (Cherry *et al*., 2004). In contrast, the maximum α-amylase activity was obtained at 20.0 g/L with *A. oryzae* (Zhu *et al*., 2004).

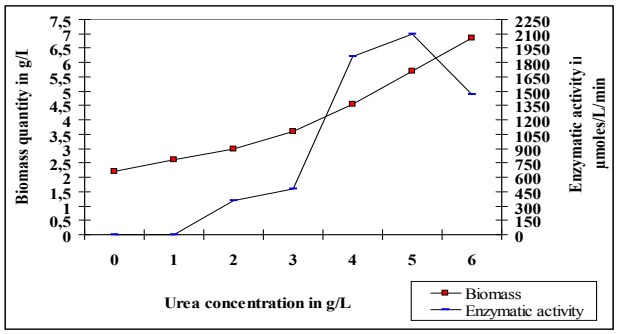

Figure 3. Evolution of biomass and enzymatic activity following urea content

Phosphate serves as the construction material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides and coenzymes. At this effect, α-amylase synthesis was found to be stimulated by phosphorus (Gangadharan *et al.*, 2006). The results obtained show that the growth and α-amylase production increases gradually with increases of potassium phosphate content to reach to a maximum values, i.e., 5.76 g/L and 2304.19 µmol/L/min at 6.0 g/L. In contrast, the lowest growth and α-amylase activity were obtained at potassium phosphate concentrations ≤ 2.0 g/L, i.e., 3.20 - 3.40 g/L and 856.66 µmol/L/min (Figure 4). So, the optimum phosphate concentration for the maximums growth and level of α-amylase activity was 5.0 g/L. Similar results were obtained by Gangadharan *et al.* (2006). Moreover, Zaldivar *et al*. (1997) reported that the synthesis of α-amylase is stimulated by adding phosphorus to the culture medium and the concentration recommended is 7.0 to 10.0 g/L. In contrast, Bertolin *et al.* (2003) did not obtain a significant improvement in gluco-amylase yields by adding phosphorus to the fermentation medium.

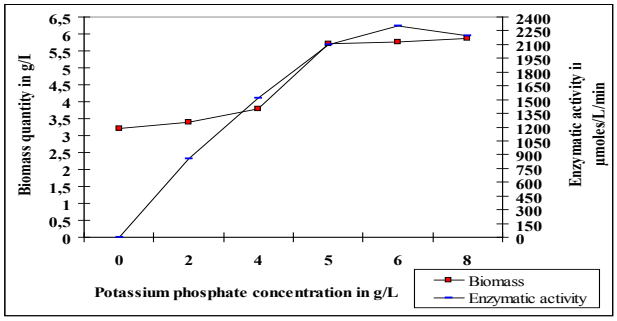

Figure 4. Evolution of biomass and enzymatic activity following potassium phosphate content

Conclusion

The research indicated that amylolytic yeasts such as *C. guilliermondii* CGL-A10 is very important for optimizing α-amylase production using date waste products that are rich in sugars. The obtained results show that date wastes serve as a good substrate, enabling the growth of *C. guilliermondii* CGL-A10, which produced a considerable amount of the α-amylase. Evidently date wastes provided necessary nutrients for this strain to grow and synthesize this enzyme. So, the composition of the medium is a major factor in regulating the synthesis of α-amylase. Under respective optimum conditions, fermentation period of 72 h, temperature of 30°C, pH=6.0, sugar concentration of 15 g/L, starch content of 5.0 g/L, urea concentration of 5.0 g/L and potassium phosphate concentrations of 6.0 g/L, a maximum α-amylase of 2304.19 µmol/L/min could be produced, proving date wastes as a promising substrate for its production. This strain can also be used in industry to produce amylase and other very important enzymes.

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