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Optimization of ethanol, citric acid, and α-amylase production from date wastes by strains of *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Candida guilliermondii*

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Abstract The present study deals with submerged ethanol, citric acid, and α-amylase fermentation by *Saccharomyces cerevisiae* SDB, *Aspergillus niger* ANSS-B5, and *Candida guilliermondii* CGL-A10, using date wastes as the basal fermentation medium. The physical and chemical parameters influencing the production of these metabolites were optimized. As for the ethanol production, the optimum yield obtained was 136.00 ± 0.66 g/l under optimum conditions of an incubation period of 72 h, inoculum content of 4% (w/v), sugars concentration of 180.0 g/l, and ammonium phosphate concentration of 1.0 g/l. Concerning citric acid production, the cumulative effect of temperature (30°C), sugars concentration of 150.0 g/l, methanol concentration of 3.0%, initial pH of 3.5, ammonium nitrate concentration of 2.5 g/l, and potassium phosphate concentration of 2.5 g/l during the fermentation process of date wastes syrup did increase the citric acid production to 98.42 ± 1.41 g/l. For the production of α-amylase, the obtained result shows that the presence of starch strongly induces the production of α-amylase with a maximum at 5.0 g/l. Among the various nitrogen sources tested, urea at 5.0 g/l gave the maximum biomass and α-amylase estimated at 5.76 ± 0.56 g/l and 2,304.19 ± 31.08 µmol/l/min, respectively after 72 h incubation at 30°C, with an initial pH of 6.0 and potassium phosphate concentration of 6.0 g/l.

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Introduction

In Algeria, the production of dates is estimated to be 492,188 tons of which 80,000–95,000 tons are of very low commercial value, constituted of common dates and date wastes of Deglet-Nour variety [2]. Date wastes are an economical source of carbohydrates for conversion to ethanol, citric acid, and α-amylase because it is readily available and relatively low priced. Ethanol is normally produced from molasses by fermentation using common distillers yeast *Saccharomyces cerevisiae*. Algeria imports all its needs into ethanol, estimated at 50,000 tons [39]. The production of ethanol from dates will be an attractive option. Ethanol is used in a wide range of industrial products (cosmetics, perfumes, pharmaceuticals, solvents, detergents, disinfectants, and organic acids) [39]. Among the organic acids industrially produced, citric acid is the most important in quantitative terms, with an estimated annual production of about 1.4 million tons [1]. Citric acid is the major organic acid produced by fermentation with *Aspergillus niger* and is widely used in the food, beverage, chemical, pharmaceutical, and other industries. The production of citric acid by *A. niger* is one of the most commercially utilized examples of fungal overflow metabolism. Many microorganisms such as fungi and bacteria can produce citric acid. The various fungi, which have been found to accumulate citric acid in their culture medium, include strains of *A. niger*, *Aspergillus awamori*, *Penicillium restrictum*, *Pencillium simplicissimum*, *Trichoderma viride*, *Saccharomycopsis lipolytica*, and *Yarrowia lipolytica* [33]. 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. These enzymes find potential application in a number of industrial processes such as food processing, fermentation, textile, paper industries, and with the advent of new frontiers in biotechnology [26, 30]. Gluco amylases and alpha amylases are produced by a variety of microorganisms such as *Bacillus subtilis*, *Fusarium solani*, *Candida guilliermondii*, *Rhizopus oryzae*, *A. niger*, *Aspergillus awamori*, and *Aspergillus oryzae* [22, 26, 29, 30, 32, 41]. *A. niger* is an acidifying mould; thanks to its important hydrolytic capacities in the α-amylase production and its tolerance of acidity (pH < 3), it allows the avoidance of bacterial contamination [30]. 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 microorganisms is of particular interest for the production of thermophilic α-amylases [30]. Algeria imports ethanol, citric acid, and enzymes from industrially advanced countries and it involves a huge amount of foreign exchange.

The aim of the present study was to investigate the potential of date wastes as substrate for the production of ethanol, citric acid, and α-amylase using strains of *S. cerevisiae*, *A. niger*, and *C. guilliermondii* isolated locally and the determination of optimized production conditions.

Materials and methods

Biological strains

Isolation, identification, and screening of Saccharomyces cerevisiae

Fifty strains of *S. cerevisiae* were isolated from dates produced by several varieties of date palm. Dates were cut in small pieces and placed on agar plates. The plates were incubated at 30°C for 48 h. The colonies produced were purified and then identified by using cultural, morphological, sexual, and physiological characteristics as reported by [19].

Isolation, identification, and screening of Candida guilliermondii

Thirty 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 [19].

Isolation, identification, and screening of Aspergillus niger

Thirty strains of *A. niger* were isolated from the saline soils collected from five localities of arid regions of Algeria. The strains were then seeded into Petri plates containing in (g/l): sucrose 30, Na_2NO_3 2.0, KH_2PO_4 1.0, $MgSO_4$ 0.5, KCl 0.5, $FeSO_4$ 0.01, and agar 20.0. After incubation at 30°C for 3–5 days, the Mycelial colony was sub-cultured in water agar (agar 2.0%) for purification [9]. The identification of strains of *A. niger* is based on the observations of the fungal mycelium: color and diameter of the colony, the presence of the thallus, the presence or the absence of the septum, the nature of the production and characteristics of fruiting bodies and spores [21]. Finally, the strains were screened qualitatively in Petri plates containing Czapek-Dox agar medium with Bromocresol Green as an indicator [9].

Experimental protocol

(1) Preparation of culture medium or date wastes syrup

The syrup 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 12.67% glucose, 10.15% fructose, and 32.00% sucrose in dry weight.

(2) Production of ethanol

The strain of SDB maintained on agar slope was reactivated in Carlsberg medium, composed by (g/l): yeast extract 20, sucrose 100, $MgSO_4$ 1.0, $(NH_4)_2$ PO_4 1.0 at pH 4.5. Thus, in 250-ml Erlenmeyer flasks, we put 20 ml of sterilized Carlsberg medium and then inoculated from the agar tube. The Carlsberg medium was incubated at 30°C for 24 h under continuous agitation. After incubation, the inoculum was transferred to a bioreactor (New Brunswick Scientific) of 3-l capacity filled to 2/3 of its volume of date wastes syrup enriched with ammonium phosphate at 0.5–2.5 g/l. The fermentation was conducted anaerobically at a temperature of 30°C for 72 h. All the experiments were run parallel in triplicates.

(3) Production of citric acid

A loop full of *A. niger* ANSS-B5 was spread on a sterilized potato dextrose agar plate and incubated at 30°C for 120 h.

After growth and sporulation, 40 ml of sterilized distilled water was added to each Petri dish. About 10^6 CFU/ml were produced. The fermentation was performed in a bioreactor (New Brunswick Scientific) of 3-l capacity containing 1.0 l of date wastes syrup. Agitation intensity was also maintained at 200 rpm/min. All the experiments were run parallel in triplicates.

(4) Production of α-amylase

The strain CGL-A10 of *C. guilliermondii* was 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 [26]. The cultures were developed in 250-ml Erlenmeyer flasks containing 50 ml of date wastes syrup inoculated with 2% (by volume per mass) inoculum level and incubated at 30°C for 96 h at 200 rpm/min. All the experiments were run parallel in triplicates.

Analytical methods

Mycelial was harvested by paper filtration using a pre-dried and pre-weighted Whatman filter paper N°1, washed with distilled water and dried to constant weight at 70°C [1, 22]. Ethanol was assayed by gas chromatography (Shimadzu, Kyoto, Japan). Thus, we prepared a series of mixtures containing, per 10 ml of ethanol content solution ranging from 3 to 21%. In each mixture, we added 1.0 ml of propanol. Then, we injected 0.3 µl of each solution implemented. We drew a calibration curve to determine the ethanol content [34]. Yield was determined by the ratio between the amount of ethanol produced and the amount of sugar consumed (%). Sucrose, glucose, and fructose of the date wastes, residual sugars of the culture medium, and citric acid were determined by a Shimadzu HPLC instrument (Japan) equipped with UV detector model SPD-10Avp, column Oven model CTO-10Avp [13]. Amylase activity 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 a UV spectrophotometer. A calibration curve of absorbance and concentration of D-glucose was established with a known amount of glucose [7]. 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

(1) Production of ethanol

Fifty strains of *S. cerevisiae* were tested to evaluate their potential production of ethanol and all tested strains in screening experiment were capable of producing ethanol. However, strains SDB, SHW, STISB, and SELK were selected as the best producer of ethanol and the production conditions of SDB were optimized. The ethanol content evolves gradually during the fermentation, reaching a maximum of 126.00 ± 1.81 g/l at 72 h. Therefore, the optimum fermentation period for maximum production of ethanol was 72 h. Our finding is in agreement with those obtained by [27, 39]. However, [11] obtained optimum ethanol content for 48 h using a strain of *Zymomonas mobilis*. In contrast, [35] obtained lower levels of ethanol during a fermentation period of 96–105 h. In the fermentation reactions, the quantity of inoculum is very important in obtaining maximum production of ethanol in the shortest amount of time. So, the ethanol content and metabolism yield increase gradually as the amount of inoculum used increases to stabilize beyond 4% (w/v). Thus, the ethanol content and metabolism yield obtained reached maximum values of 136.00 ± 0.66 g/l and 78.61 ± 0.75%, respectively, using an inoculum greater than or equal to 4% (w/v) (Fig. 1). The weakest ethanol content and metabolism yield, i.e., 57.00 ± 2.11 g/l and 49.56 ± 1.71%, respectively, were obtained with an inoculum level of 1%. This is probably due to the decrease in the number of yeast cells in the culture medium. So, the optimal inoculum for maximum ethanol production was 4% (w/v). This result is in agreement with that obtained by [10]. In addition, the ethanol content and metabolism yield increase with an

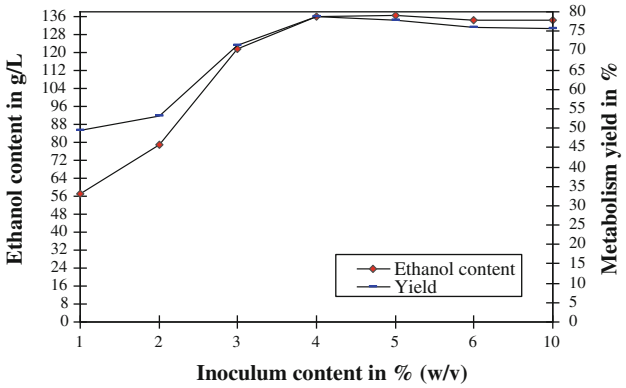

Fig. 1 Evolution of ethanol and metabolism yield following inoculum content. Process conditions: fermentation period of 72 h, temperature of 30°C, pH = 4.5, sugar concentration of 180 g/l, and ammonium phosphate concentration of 2.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

increase in initial sugar concentration up to 180 g/l, but decrease beyond this value. So, the ethanol content and metabolism yield spend to 55.50 ± 1.96 g/l and 56.63 ± 1.66% with a sugar concentration of 100.0–136.00 ± 0.66 g/l and 78.61 ± 0.75% with a sugar concentration of 180.0 g/l to drop to 100.00 ± 1.86 g/l and 44.64 ± 1.31% with a sugar concentration of 300.0 g/l. So, the optimal sugar concentration for maximum ethanol production is 180 g/l. These results are similar to those obtained by [11, 27]. The sugar concentration (180 g/l) above which both the ethanol production and metabolism yield decrease are due to the combined effect of decreased water activity and plasmolysis of cell [11]. On the other hand, the obtained results show an improvement of the ethanol content and metabolism yield with increasing ammonium phosphate concentration to stabilize at a maximum of 136.00 ± 0.66 g/l and 78.61 ± 0.75%, respectively, with a concentration greater than or equal to 1.0 g/l. So, the optimum ammonium phosphate concentration is 1.0 g/l, while several authors [8, 11, 27, 39] advocate 2.0–2.5 g/l.

(2) Production of citric acid

Thirty strains of *A. niger* were tested to evaluate their potential in terms of production of citric acid and all tested strains in the screening experiment were able to grow on date waste syrup and were capable of producing citric acid. However, *A. niger* ANSS-B5 was selected as the best producer of citric acid and its optimized production conditions were determined. The optimal incubation period for maximum citric acid production varies both with the strain and fermentation conditions. The obtained results show that the consumed sugars, mycelial dry weight, and citric acid production increases gradually during the incubation period and reaches its maximum values, i.e., 142.00 ± 3.04, 6.45 ± 0.91 and 58.12 ± 2.13 g/l, after 144 h. So, the optimum incubation period was 144 h. Our finding is in agreement with that obtained by [1, 37]. However, [28] have obtained high citric acid yield 192 h after inoculation. On the other hand, when the temperature was kept at 20 and 25°C, consumed sugars, mycelial dry weight, and citric acid production were 92.40 ± 2.81–98.25 ± 4.71 g/l, 3.70 ± 0.43–4.45 ± 0.46 g/l, and 20.36 ± 2.81–29.44 ± 3.92 g/l, respectively. The highest amount of citric acid, i.e., 58.12 ± 2.13 g/l, was obtained at 30°C. So, the optimum citric acid production was 30°C. This result was in agreement with that obtained by [1, 4, 5]. The decrease in citric acid production observed at higher temperature (>40°C) may be due to that the high temperature can cause denaturation of citrate synthase and accumulation of by-products such as oxalic acid [1]. Moreover, the maximum consumed sugars, mycelial dry weight, and citric acid production, i.e., 142.00 ± 3.04, 6.45 ± 0.91, and 58.12 ± 2.13 g/l, respectively, were obtained at 150.0 g/l of sugars. These results were in agreement with those obtained by [1, 37]. However, [14] reported that the maximum citric acid production was obtained at 140.0 g/l of sugars. When the sugar concentration was higher than 200.0 g/l, a reduction in citric acid production, i.e., 56.45 ± 2.44 g/l, was observed. This may be due to the overgrowth of the mycelium, which resulted in increased viscosity of the medium and polyalcohol formation [14, 31]. Finally, citric acid production decreased, i.e., 50.35 ± 2.45 g/l, at 120.0 g/l of sugars. This is probably due to the accumulation of oxalic acid in the culture medium [14, 31]. The addition of light alcohol concentration such as methanol in the fermentation medium increases the tolerance of *A. niger* to the toxicity of certain metals such as zinc, copper, and iron [23]. The obtained results show that the citric acid production increased significantly with an increase in methanol concentration up to 3.0%. So, the addition of 3.0% (w/v) methanol in the fermentation medium increases the citric acid production from 58.12 ± 2.13 to 75.28 ± 3.22 g/l. The consumed sugar was 142.26 ± 2.77 g/l and mycelial dry weight was observed to be 6.84 ± 0.48 g/l. So, the optimum methanol concentration for maximum citric acid production was 3.0% (w/v). According to [23, 28], the higher citric acid yield was obtained at 1.5% of methanol concentration. On the other hand, [14] reported that the addition of 4% (w/v) of methanol to the fermentation medium induced citric acid production. Methanol is not assimilated by *A. niger* and its exact role in stimulation of citric acid production is still not clear and the effects of methanol are at the cell permeability level, allowing metabolites to be excreted from the cell [14]. An important factor that affects the performance of citric fermentation is the initial pH of the medium. The obtained results show that the maximum amount of citric acid, i.e., 84.25 ± 2.61 g/l, was achieved when the initial pH of fermentation medium was kept at 3.5. The consumed sugar was 140.15 ± 3.34 g/l, while the mycelial dry weight was 6.88 ± 1.05 g/l (Fig. 2). So, the optimum citric acid production was 3.5. This result was consistent with findings of [40]. A low pH also inhibits the production of unwanted organic acids (gluconic acid, oxalic acid) and this makes the recovery of citric acid from the broth simpler [31]. In addition, low pH inhibits lightly the growth of *A. niger* and reduces the risk of contamination of the fermentation medium. An increase in the initial pH caused a reduction in citric acid production. When the pH was further increased from 4.5, the production of citric acid decreased gradually, and the amounts of citric acid obtained at pH 4.5, 5.0, and 6.0 were 69.14 ± 2.21, 66.32 ± 2.49, and 60.14 ± 2.34 g/l, respectively. The higher initial pH leads to the accumulation of oxalic acid as reported by [31]. However, [1] have obtained a maximum

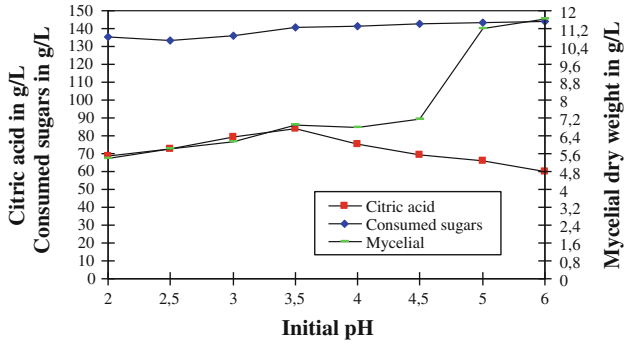

Fig. 2 Evolution of quantity of citric acid, consumed sugars, and mycelial dry weight following initial pH. Process conditions: Fermentation period of 144 h, temperature of 30°C, aeration rate of 1.0 vvm, sugars concentration of 150 g/l, methanol concentration of 3.0%, ammonium nitrate concentration of 2.0 g/l, and potassium phosphate concentration of 2.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

citric acid yield at pH 5.5. Finally, [40] recommends an initial pH of fermentation medium to 4.0 in order to optimize the citric acid production. Nitrogen has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but is also the basic part of cell proteins. Also, the concentrations of nitrogen affect fungal growth and the synthesis of citric acid. As shown in Fig. 3, the maximum citric acid production, i.e., 91.15 ± 1.64 g/l, was obtained at an ammonium nitrate concentration of 2.5 g/l. The consumed sugar was 138.45 ± 2.63 g/l while the mycelial dry weight was 7.78 ± 1.39 g/l. These results are in agreement with those obtained by [31, 37]. An advantage of using ammonium salts is that the pH declines as the salts are consumed and a low pH is a requirement for citric acid fermentation [31]. On the other hand, we noted an increase of fungal growth, i.e., 10.33 ± 1.52 g/l and a

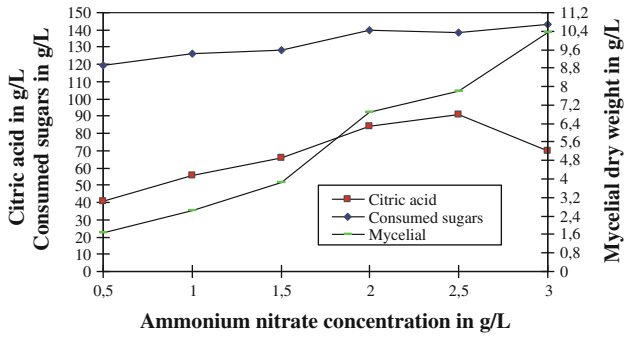

Fig. 3 Evolution of quantity of citric acid, consumed sugars, and mycelial dry weight following ammonium nitrate concentration. Process conditions: Fermentation period of 144 h, temperature of 30°C, aeration rate of 1.0 vvm, sugars concentration of 150 g/l, methanol concentration of 3.0%, pH = 3.5 and potassium phosphate concentration of 2.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

diminution in citric acid production, i.e., 70.12 ± 2.06 g/l at nitrogen concentration superior or equal to 3.0 g/l. The same results were obtained by [14, 37]. In contrast, [36] reported that ammonium nitrate at a concentration of 3.0 g/l gave the maximum citric acid production. According to these facts, the amount of citric acid obtained in this study would decrease at both lower and higher nitrogen levels. Moreover, a maximum citric acid production, mycelial dry weight, and consumed sugars, i.e., 98.42 ± 1.41, 7.92 ± 0.96, and 146.12 ± 0.66 g/l, respectively, were obtained at a potassium phosphate concentration of 2.5 g/l. Our findings are not in agreement with those of [6], who reported that the higher citric acid yield was obtained at a potassium phosphate concentration of 1.5 to1.8 g/l. In contrast, [37] have shown that the accumulation of citric acid is higher when the potassium phosphate concentration is kept between 2.0 and 2.2 g/l. However, [6] noted that a high potassium phosphate concentration (≥2.0 g/l) promotes the growth of *A. niger* at the expense of citric acid accumulation. The citric acid production decreases either below or above a potassium phosphate concentration of 2.5 g/l.

(3) Production of α-amylase

Thirty strains of *C. guilliermondii* 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 syrup and were capable of producing α-amylase. However, CGL-A10 was selected as the best producer of α-amylase and its production conditions were optimized. The results obtained indicate that after 48 h of incubation, 762.33 ± 21.55 µmol/l/min of α-amylase was produced, this exponentially increased to 1,519.23 ± 34.46 µmol/l/min at 72 h. So, the optimal incubation period was 72 h. A similar result was reported by [26, 41]. In contrast, [25] reported that *Thermomyces lanuginosus* strain produced high titters of enzymes at 120 h. On the other hand, a significant level of α-amylase was produced by *C. guilliermondii* over a temperature range of 20–40°C, with an optimum at 30°C, i.e., 1,519.23 ± 34.46 µmol/l/min. The optimum temperature for the maximum α-amylase activity was 30.0°C, and [16, 26, 41] have also reported similar results. A further increase in temperature resulted in a decrease in α-amylase activity. Above a temperature of 35°C, amylase activity decreased and it lost approximately 30.4% of its activity at this temperature, and at 40°C, it kept approximately 46.7% of residual activity. A temperature above 45°C results in moisture loss of the substrate, which affects metabolic activities of the microorganism and results in reduced growth and α-amylase production [38]. Among the physico-chemical parameters, the pH of the 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 medium. Therefore, the selection of optimum pH is essential for α-amylase production. According to [20], 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 ± 0.91 g/l and the lowest at pH 9.0 and 4.0, i.e., 2.56 ± 1.01 and 3.40 ± 1.11 g/l, respectively (Fig. 4). As for the enzymatic activity, the maximum value, i.e., 1,651.21 ± 32.68 µmol/l/min was obtained at an initial pH of 6.0. So, the optimum pH for maximum biomass and α-amylase activity was pH = 6.0. Our findings are comparable to previously reported results from the literature [16, 26]· [3] that reported maximum α-amylase production by *Aspergillus* sp. and *A. niger* at a pH varying between 5.0 and 6.0. In contrast, [32] reported pH = 4.0 to be the best for the production of α-amylase by *Aspergillus 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 9.0 and 4.0 are 666.66 ± 17.62 and 781.33 ± 21.22 µmol/l/min, respectively. According to [18], the growth and enzyme production were inhibited when the initial pH of the medium was above 10.0 or below 4.0. 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 enzymatic activity increases significantly with an increases in sugars concentration to reach to a maximum values at 20.0 g/l, i.e., 4.33 ± 0.91 g/l and 1,651.21 ± 32.68 µmol/l/min, respectively. The lowest yield, i.e., 666.66 ± 21.11 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 with the studies undertaken by [22] with *A. niger*. On the other hand, the obtained results show that the enzyme production gradually accumulates in the medium to achieve maximum α-amylase activity, i.e., 2,056.33 ± 38.22 µmol/l/min, by adding to the culture medium 5.0 g/l of starch. Beyond 5.0 g/l, α-amylase activity stabilizes around 2,000.00 ± 33.53 µmol/l/min. So, the optimum starch content for maximum α-amylase production is 5.0 g/l. Similar results were reported by [26] with *C. guilliermondii*. Studies on supplementation of inorganic and organic nitrogen sources at 5.0 g/l to the date wastes syrup showed a mixed trend on enzyme production. The results presented in Fig. 5 show that the highest biomass production was obtained with meat extract, i.e., 6.47 ± 0.98 g/l followed by urea, i.e., 5.70 ± 0.44 g/l, peptone, i.e., 5.08 ± 0.71 g/l, and ammonium phosphate, i.e., 4.66 ± 0.53 g/l. The lowest biomasses were obtained with ammonium nitrate, ammonium sulfate, and ammonium carbonate, i.e., 3.22 ± 0.34, 3.40 ± 0.41, and 3.45 ± 0.37 g/l, respectively. Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes including α-amylase in submerged fermentation [24]. Among the organic sources, supplementation of peptone, yeast extract, and urea showed an increased in α-amylase activities, i.e., 1,844.54 ± 30.29, 2,056.33 ± 38.22, and 2,100.22 ± 26.52 µmol/l/min, respectively. Ammonium phosphate also enhanced the amylase activity relatively, i.e., 1,765.12 ± 22.96 µmol/l/min but ammonium nitrate showed a negative influence, showing a steep decrease in α-amylase activity, i.e., 865.00 ± 24.55 µmol/l/min. So, we could say that the peptone, urea, and yeast extract are the best nitrogen sources. Similar observations were recorded by [15, 16, 22, 25, 26] with *A. niger, C. guilliermondii*, *Bacillus licheniformis* and

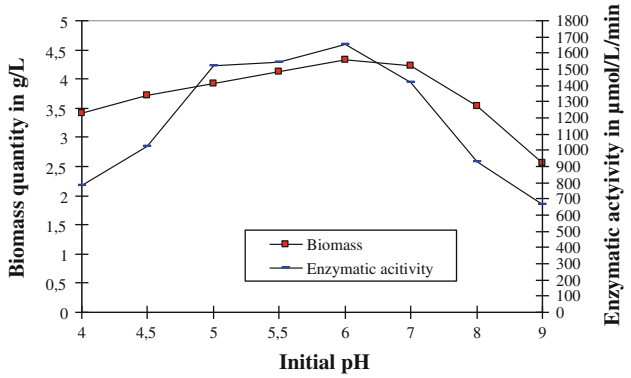

Fig. 4 Evolution of biomass and enzymatic activity following initial pH. Process conditions: Incubation period of 72 h, temperature of 30°C, sugars concentration of 20.0 g/l, starch concentration of 5.0 g/l, yeast extract at 5.0 g/l as a source of nitrogen, and phosphate concentration of 4.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

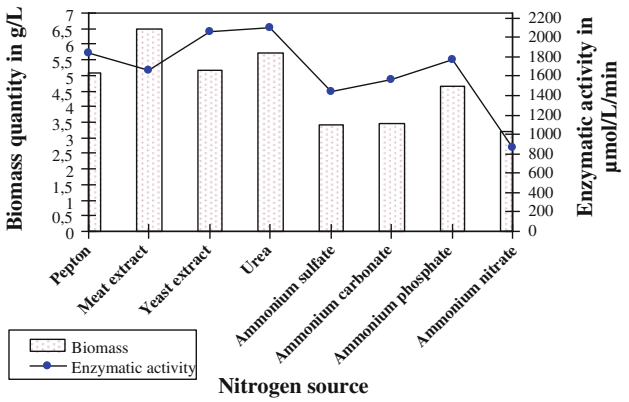

Fig. 5 Evolution of biomass and enzymatic activity following nitrogen source. Process conditions: Incubation period of 72 h, temperature of 30°C, pH = 6.0, sugars concentration of 20.0 g/l, starch concentration of 5.0 g/l, nitrogen concentration of 5.0 g/l, and phosphate concentration of 4.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

Thermomyces lanuginosus. On the other hand, the obtained results show that cell growth of *C. guilliermondii* increased significantly with an increase in urea content of medium to reach to a maximum value, i.e., 6.85 ± 0.76 g/l, at 6.0 g/l (Fig. 6). As for enzymatic activity, this latter increases significantly with the urea content of the medium to reach to a maximum value, i.e., 2,100.22 ± 26.52 µmol/l/min at 5.0 g/l. However, beyond 5.0 g/l, the α-amylase activity dropped to 1,465.42 ± 30.29 µmol/l/min at 6.0 g/l. So, the optimum value for the maximum enzyme activity was obtained at 5.0 g/l. Similarly, [16] obtained a maximum enzyme activity with *A. niger* at a nitrogen concentration of 5.0 g/l. Moreover, the maximum gluco-amylase produced was obtained with concentrations of yeast extract ranging between 3.0 and 5.0 g/l [25], whereas with *Aspergillus fumigatus*, the best concentration is 2.0 g/l [12]. Phosphate serves as the construction material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides, and coenzymes [17]. The obtained results show that the growth of *C. guiliermondii* and enzymatic activity increases gradually with an increase of potassium phosphate concentration to reach to maximum values of 5.76 ± 0.56 g/l and 2,304.19 ± 31.08 µmol/l/min, respectively, at 5.0 g/l. In contrast to, the lowest values were obtained at potassium phosphate concentrations ≤2.0 g/l, i.e., 3.20 ± 0.86–3.40 ± 0.93 g/l and 856.66 ± 18.52 µmol/l/min. So, the optimum phosphate concentration for the maximum growth and level of α-amylase activity was 5.0 g/l. Similar results were obtained by [17]. Moreover, [42] reported that the synthesis of α-amylase is stimulated by adding phosphorus to the culture medium and the concentration recommended is 7.0–10.0 g/l.

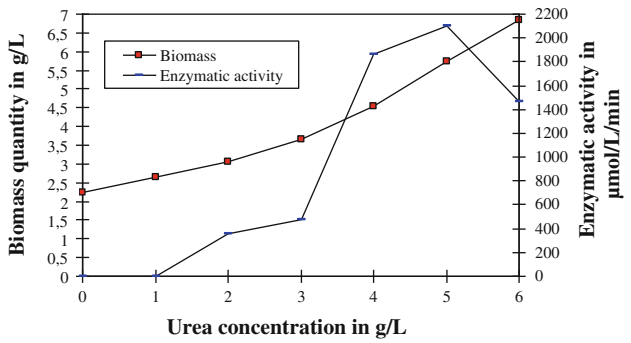

Fig. 6 Evolution of biomass and enzymatic activity following urea concentration. Process conditions: Incubation period of 72 h, temperature of 30°C, pH = 6.0, sugars concentration of 20.0 g/l, starch concentration of 5.0 g/l, urea concentration of 5.0 g/l, and phosphate concentration of 4.0 g/l. Each data point shows the average of three independent experiments and *error bars* represent standard deviation

Conclusions

The present study describes the optimization of the cultural conditions in a stirred bioreactor for the production of ethanol, citric acid, and α-amylase. In summary, we can say that the optimum production of ethanol with *S. cerevisiae* SDB was obtained at a fermentation period of 72 h, inoculum content of 4% (w/v), sugar concentration of 180 g/l, and ammonium phosphate concentration of 1.0 g/l. On the other hand, the optimum citric acid production with *A. niger* ANSS-B5 was obtained at a fermentation period of 144 h, temperature of 30°C, sugars concentration of 150.0 g/l, initial pH = 3.5, methanol concentration of 3.0%, and ammonium nitrate and potassium phosphate concentrations of 2.5 g/l. A maximum citric acid production, i.e., 98.42 ± 1.41 g/l, was obtained at these optimal conditions. By elsewhere, *C. guilliermondii* CGL-A10 isolated from leavening dough was observed to hyper produce of α-amylase under submerged fermentation indicating its potential for industrial-scale application in the future. The obtained results indicate that the composition of the medium is a major factor in the regulating of the synthesis of α-amylase. Under respective optimum conditions, fermentation period of 72 h, temperature of 30°C, initial pH = 6.0, sugar concentration of 15.0 g/l, starch content of 5.0 g/l, urea or yeast extract concentration of 5.0 g/l, and potassium phosphate concentration of 6.0 g/l, a maximum α-amylase of 2,304.19 ± 31.08 µmol/l/min could be produced, proving date wastes as a promising substrate for its production at industrial scale. It is concluded from the results of the present study that the date wastes could serve as a potential substrate for ethanol, citric acid, and α-amylase production by *S. cerevisiae* SDB, *A. niger* ANSS-B5, and *C. guilliermondii* CGL-A10 isolated locally.

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