Citric Acid Production by Cellulose Decomposing Black Aspergillus Species Isolated from Sugarcane Bagasse

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Abstract

In the current investigation, the potentiality of black *Aspergillus* species isolated from sugarcane bagasse to produce cellulolytic enzymes, and citric acid was evaluated. Furthermore, the cellulolytic activity and citric acid production by the active producers were assayed on a carboxy methylcellulose medium. The study was extended to improve citric acid production from sugarcane bagasse using mixed cultures of the highest endo-cellulases (CMCase) and citric acid-producing isolates. All the tested fungal isolates exhibited varying amounts of acid production on Czapek's dextrose (Cz-Dox) solid medium containing $CaCO_3$. The highest concentration of citric acid produced using a Cz-Dox liquid medium was 8.49 ±2 g/L as recorded by *A. brasiliensis* Am 27. This amount of citric acid is equivalent to 29.22% of initial sugar. *A.niger* Am 270 showed the highest endo-cellulases production (0.73±0.2 IU/mL) whereas *A. niger* Am 258 exhibited the highest citric acid yield (14.74%). These isolates were chosen for the maximization of citric acid production from sugarcane bagasse. The highest amount of citric acid from sugarcane bagasse medium by *A. niger* Am 258 was obtained after 10 days of incubation, recording 34 g/L citric acid and by increasing the incubation period, the obtained amount of citric acid decreased. Whereas the mixed cultures of Am 270 and Am 258 strains revealed a slight enhancement of citric acid production recording 35.1 g/L citric acid after 8 days of the total incubation period. So the presented results may

provide a suitable strategy for citric acid production using sugarcane bagasse.

Keywords: Aspergillus niger; Sugarcane bagasse, Cellulolytic activity; Citric acid; Solid-state fermentation.

Introduction

Citric acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid) is a weak organic acid with the chemical formula $C_6H_8O_7.H_2O$ (molecular mass, 210.14 g/mol) that is naturally found in all citrus fruits, with pH 0.2 (Makut and Ekeleme 2018). Citric acid is characterized by its solubility in water and colorless appearance (Angumeenalet al. 2003) as well as it has a melting point of 153°C and solid at room temperature. Citric acid fermentation is one of the conventional fermentation, however, its consumption is increasing over time. About 70% of the produced citric acid is used in the food and beverage industry for several purposes, including pharmaceuticals (12%) and other industrial uses (18%) (Yigitoglu 1992). The worldwide citric acid market requirement was reached 2.39 million tons in 2020 and is expected to reach 2.91 million tons by 2026(Chatterjee and Mohan 2022). Although citric acid can be produced through chemical methods, it is not cost-effective. So, most citric acid production is produced by *Aspergillus niger* fermentation (Sawantet al. 2021). As well as, about less than 1% of the worldwide citric acid production is produced from citrus fruits. Industrially, the citric acid fermentation process is performed through batch fermentation using different strategies, including the Japanese koji, the submerged fermentation and the liquid surface culture processes (Yigitoglu1992; Behera 2020). Nowadays approximately most of the produced citric acid is produced using submerged fermentation but the profitability is quite low, and consequently the economics of the production processes are very constricted (Bu'lock1990). So, the utilization of sustainable cheap raw materials such as sugarcane molasses and bagasse may enhance the profitability of citric acid production technologies. The conventional substrates used for the citric acid fermentation process are beet or cane molasses (Pazoukiet al. 2000). Sugarcane bagasse is the residue obtained after the crushing of sugarcane

during sugar production. It is composed of fiber, pith, non-soluble solids and water. Fibers represent the major components and include cellulose, hemicelluloses and lignin of low molecular mass. The chemical composition of the Egyptian sugarcane bagasse was reported by Faris (2011) as 36.5-37.1% cellulose, 29.8-30.5% pentosan, 19.89-20.26% lignin, 1.86-1.93% ash and others.

Many microorganisms have the ability to produce citric acid such as fungi and bacteria but *A. niger* is still the potential organism for citric acid production due to its genetic stability, high yields, the capability of utilizing cheaper raw materials, absence of undesirable by-products (El-Holi and Al-Delaimy 2003). As well as, *A. niger* exhibited a high potentiality for the production of many industrial enzymes e.g., glucoamylase, pectinases, alpha-galactosidase and cellulases. So, the search for native *A. niger* isolates with high enzymatic activities may have unique characteristics within the biotechnology industry for the production of citric acid from different raw materials (Hess et al. 2000). This study aimed to investigate the potentiality of various strains of *A. niger* isolated from sugarcane bagasse for the production of citric acid and endo-cellulases (CMCase). Furthermore, to assay the capability of endo-cellulases producing isolates to produce citric acid from sugarcane bagasse.

Materials and Methods

Collection of sugarcane bagasse samples

Fifty samples (500 g each) of sugarcane bagasse were collected from sugarcane juice extracting shops in Assiut City, Egypt. Samples were transferred to the laboratory in sterile polyethylene bags and preserved at $4^{o}C$ until further investigations.

Isolation of fungal species

Fungi associated with sugarcane bagasse were isolated by direct plating and serial dilution techniques(Wainrightetal.1993; Nester et al.1995) using potato dextrose agar(PDA) and Czapek's

cellulose media. Cultures were incubated at 28±2^{O}C for 7days and examined daily until the presence of fungal growth. Black Aspergilli belonging to Section Nigri (*A. niger* group) were transferred to fresh potato dextrose agar (PDA) plates for purification and identification. Each pure fungal culture was identified based on cultural and microscopic characteristics using identification keys (Moubasher 1993; Domschet al. 2007). These recovered fungal cultures were recorded and kept on PDA slants and maintained at 4^{O}C for further studies.

Screening for acid production on solid medium

One hundred twenty-five isolates of *A.niger*group recovered from sugarcane bagasse were screened for their abilities to produce acids by growing on Cz-Dox agar medium supplemented with 5 g/L $CaCO_3$. Fungal isolates were inoculated on the center of agar plates and incubated at 28^{O}C for 7days. After incubation, cultures were examined and the clear zone formed below each *A. niger* isolate (disappearance of white color of $CaCO_3$) was measured. The dissolution of $CaCO_3$ indicates the production of organic acids (Patil and Patil 2014).

Screening for citric acid production

Preparation of fungal culture and cultivation process

Twenty isolates of *A.niger* showing high acid production were selected to evaluate their capabilities to produce citric acid using submerged fermentation. Erlenmeyer flasks (250 mL capacity) containing 50 mL Cz-Dox liquid medium were used (Kim 2004). The culture medium contained (g/L): glucose, 30; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.1 and yeast extract, 0.5.After autoclaving, the medium was inoculated by 1 mL of spore suspension (10^6 conidia/mL) of the test fungal isolate. Experiments were carried out in three replicates and incubated at 28±2^{O}Cfor 7 days in an orbital shaking incubator (Stuart Model SSL1, China)at 120 rpm.

Citric acid determination

Citric acid (CA) was determined spectrophotometrically at 420 nm using double beam UV-Visible spectrophotometer (Model:

T80+, United Kingdom) after adding pyridine and acetic anhydride. One mL of culture filtrate and 1.3 mL pyridine were mixed briskly in a test tube, followed by the addition of 5.7 mL acetic anhydride. The assay mixture was placed in a water bath at 30±2^{O}C for 30 min till color development (Marier and Boulet 1958). The control tube was prepared by replacing 1.0 mL of the culture filtrate with distilled water. Citric acid concentration in each fungal filtrate was estimated from a standard curve prepared using pure citric acid. Fungal biomass and pH values were determined at the end of incubation periods for each fungal culture. To determine fungal biomass, the whole fungal growth in each culture was filtered through Whatman#1 filter paper, washed with distilled water and oven-dried at 105 ^{O}C until constant weight and the resultant weight was expressed as g/L (Haq and Daud 1995). Residual sugars were assayed using the 3,5dintrosalyslic acid method (Miller 1959). The pH value of fungal culture was measured by Genway pH meter.

Screening for extracellular endo-cellulases (CMCase) production

Culture medium and cultivation conditions

The highest citric acid producers of *A.niger* isolates were grown aerobically in 250 mL Erlenmeyer flask containing 50 mL of sterilized liquid medium containing (g/L): carboxy methyl cellulose (CMC), 10; $NaNO_3$,2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.1; yeast extract, 0.5 and chloramphenicol 0.25 dissolved in 1 liter of distilled H_2O. Cultures were incubated in an orbital shaking incubator (Stuart Model SSL1, China) for 7 days at 120 rpm and 28±2^{O}C. The culture broth was then centrifuged (Optima, BHG 500, Russia) at 4293 x g to remove fungal biomasses. The clear supernatant was collected for endo-cellulases (CMCase) assay.

Assay of endo-cellulases (CMCase) enzyme activity

Endo-cellulases (CMCase) activity was determined using the 3,5Dinitrosalicylic(DNS) method as described by Miller(1959). One mL of 2% CMC in 100 mM phosphate buffer (pH 7) was

mixed with 1 mL fungal filtrate(crude enzyme) and then incubated for 60 min at 30^{O}C. The liberated reducing sugars were measured as glucose by adding 2 mL DNS reagent to the reaction mixture, followed by incubation in a boiling water bath for 15 min and finally cooling under running tap water for 20 min. The absorbance was measured spectrophotometrically (double beam UV-Visible spectrophotometer (Model: T80+, United Kingdom)at 540 nm against control tubes (boiled culture filtrate). The standard curve was performed using glucose as reducing sugar. One endo-cellulase unit is defined as the amount of enzyme that liberated 1.0 µM reducing sugar per min under the assay standard conditions. Each experiment was conducted in three replicates. Endo-cellulases specific activity is defined as the number of endo-cellulases (CMCase) units per mg extracellular protein. The total extracellular protein was measured in extracellular crude enzyme as described by Bradford (1976). A standard curve was prepared using bovine serum albumin.

Assay of citric acid production from carboxy methyl cellulose:

The highest endo-cellulases (CMCase) producing fungal isolates were tested for their potentiality to produce citric acid from CMC. The selected *A. niger* isolates were grown on CMC liquid medium as described previously and incubated for 7 days at 120 rpm and 28±2^{O}C. At the end of the incubation period, the yield of citric acid was determined as described previously.

Citric acid production from sugarcane bagasse

The highest citric acid-producing isolate was selected for evaluation of its capability to produce citric acid from sugarcane bagasse (moisture content about 22.5%) as renewable raw material. *A. niger* isolate was grown on bagasse containing medium using solid-state fermentation. An amount of 5 g of ground bagasse (length about 1 cm) was mixed with yeast extract as a nitrogen source (0.184 g/50 mL medium) into a 250 mL Erlenmeyer flask and moistened to set the75% moisture level. The prepared medium was sterilized by autoclaving at 121^{O}C for 20 min. After cooling at room temperature, each flask containing bagasse medium was

inoculated with 1 ml of the conidial suspension (10^6 conidia/mL) of the selected fungal isolate and incubated at 30°C, inside the humidity-controlled incubator for 7 days. At the end of the incubation period, the fermented mass in each conical flask was harvested and extracted with 100 mL of distilled water at 40^oC and 300 rpm for 30 min. Then the extract was filtered through a muslin cloth and oven-dried until constant weight. The filtrate was centrifuged (Optima, BHG 500, Russia) at 2415 xg for 15 min and the supernatant was used for the estimation of citric acid (Vandenbergheet al. 2000a). For improving the citric acid production from sugarcane bagasse, The highest end-cellulases (CMCase) producing isolate was grown on bagasse for four days for efficient hydrolysis of sugarcane bagasse; subsequently, the highest citric acid-producing isolate was inoculated on fermentation culture to perform a mixed culture process and enhance the efficacy of citric acid production.

Results and Discussion

Isolation and screening of acid producer *A. niger* isolates

One hundred twenty-five fungal isolates related to *A. niger* group isolated from sugarcane bagasse on potato dextrose agar and Czapek'scellulose agar media were screened for acid production and the data was recorded in Table 1. All the tested *A. niger* isolates showed a variable affinity for acid production and dissolution of $CaCO_3$ in the solid medium that leads to the formation of a clear zone. Whereas, out of the 125*A. niger* fungal isolates screened for acid production ability, 20 fungal isolates exhibited a high ability for acid production and formation of the clear zone. The highest capability for acid production was estimated for fungal isolate *A. niger* Am 260 recording a clear zone of 8.82 cm, followed by *A. niger* Am 502 (clear zone, 8.8 cm), *A. brasiliensis* Am 27 (8.74 cm), *A. niger* Am 271 (8.7 cm), *A. niger* Am 1 (clear zone, 8.65 cm), *A. niger* Am 4 (8.6 cm), *A. niger* Am 270 (8.54 cm), Am 255 (8.5 cm), *A. niger* Am 40 (8.45 cm each), *A. niger* Am 273 (8.4 cm), *A. niger* Am 33 (8.3 cm), *A. brasiliensis* Am 12 (8.3 cm), *A. niger* Am 275 (8.24

cm), *A. niger* Am 262 (8.16 cm), *A. brasiliensis* Am 258 (8.1 cm), *A. niger* Am 31 (8.05 cm), *A. niger* Am 34 (8 cm), *A. niger* Am 17 (8 cm), *A. eucalypticola* Am 19 (8 cm), and *A. niger* Am 32 (7.9 cm) as shown in Table 1.

It was also found that some *Aspergillus* isolates grow at moderate rates on $CaCO_3$ solid medium and produce high amounts of acids, producing a high clear zone. But other fungal isolates produce a low amount of acids resulting in a small clear zone although producing moderate growth rates as shown in *A. eucalypticola* Am 36. Some examples of these results are shown in Figure 1. Interestingly, several fungal species are well established for their capability to produce high quantities of different valuable organic acids **(**Liaudet al. 2014). Most of these fungi are related to *Aspergillus* species for the production of various organic acids e.g. citric, malic, gluconic and itaconic acids as well as the genus *Rhizopus* was recorded as acid producers for lactic and fumaric acids. Citric acid production by *A. niger* can be produced in large-scale bioprocesses, displaying the high potentiality of fungal species as organic acid production platforms (Magnuson and Lasure 2004; Li and Punt 2013). Consequently, the present paper aimed to assay the potentiality of the highest acid producers for citric acid production.

Table 1. Screening for acid production by *A. niger* group isolates grown on $CaCO_3$ containing medium.

Isolate No.	Fungal identification	Fungal growth	Presence of clear zone	Diameter of the clear zone (cm)	Index*
Am 1	*A. niger*	H	+	8.65	H
Am 2	*A. niger*	M	+	6	M
Am 3	*A. eucalypticola*	H	+	6.25	M
Am 4	*A. niger*	H	+	8.6	H
Am 5	*A. brasiliensis*	L	+	4.4	L
Am 6	*A. eucalypticola*	M	+	6.25	M
Am 7	*A. eucalypticola*	M	+	5	M
Am 8	*A. uvarum*	M	+	4.05	L
Am 9	*A. eucalypticola*	M	+	6.1	M
Am 10	*A. eucalypticola*	L	+	4.95	L
Am 11	*A. eucalypticola*	M	+	5.8	M
Am 12	*A. brasiliensis*	H	+	8.3	H
Am 13	*A. niger*	M	+	6.99	M
Am 14	*A. niger*	M	+	5.55	M
Am 15	*A. brasiliensis*	M	+	6.15	M
Am 16	*A. eucalypticola*	L	+	4.5	L
Am 17	*A. niger*	H	+	8	H
Am 18	*A. eucalypticola*	M	+	4.2	L
Am 19	*A. eucalypticola*	H	+	8	H
Am 20	*A. eucalypticola*	M	+	6	M
Am 21	*A. niger*	M	+	6.80	M
Am 22	*A. eucalypticola*	M	+	5.45	M
Am 23	*A. eucalypticola*	L	+	5.7	M
Am 24	*A. brasiliensis*	M	+	5	M
Am 25	*A. eucalypticola*	M	+	5.8	M
Am 26	*A. eucalypticola*	H	+	6.1	M
Am 27	*A. brasiliensis*	H	+	8.74	H
Am 28	*A. eucalypticola*	M	+	5.65	M
Am 29	*A. niger*	H	+	6.7	M
Am 30	*A. brasiliensis*	M	+	6	M
Am31	*A. niger*	M	+	8.05	H
Am32	*A. niger*	H	+	7.9	H
Am33	*A. niger*	H	+	8.3	H
Am 34	*A. niger*	H	+	8	H
Am 35	*A. niger*	M	+	6.1	M
Am 36	*A. eucalypticola*	M	+	4.85	L
Am 37	*A. eucalypticola*	H	+	6.2	M
Am 38	*A. eucalypticola*	L	+	3.7	L
Am 39	*A. brasiliensis*	M	+	6.6	M
Am 40	*A. niger*	H	+	8.45	H
Am 41	*A. niger*	M	+	6.1	M
Am 42	*A. neoniger*	M	+	6	M
Am 43	*A. eucalypticola*	M	+	6.3	M

Table 1. Continued

Isolate No.	Fungal identification	Fungal growth	Presence of clear zone	Diameter of the clear zone (cm)	Index*
Am 44	*A. brasiliensis*	M	+	6.4	M
Am 45	*A. eucalypticola*	M	+	6.25	M
Am 46	*A. eucalypticola*	M	+	6.35	M
Am 47	*A. eucalypticola*	M	+	5.8	M
Am 48	*A. eucalypticola*	L	+	5.45	M
Am 49	*A. eucalypticola*	M	+	6.45	M
Am 50	*A. eucalypticola*	M	+	6.4	M
Am 51	*A. eucalypticola*	M	+	6.3	M
Am 52	*A. brasiliensis*	M	+	5.95	M
Am 53	*A. eucalypticola*	M	+	6.15	M
Am 54	*A. brasiliensis*	M	+	6.05	M
Am 55	*A. eucalypticola*	L	+	5	M
Am 56	*A. eucalypticola*	M	+	4.95	L
Am 57	*A. eucalypticola*	H	+	6.2	M
Am 58	*A. brasiliensis*	L	+	6.25	M
Am 59	*A. eucalypticola*	M	+	5.75	M
Am 251	*A. eucalypticola*	M	+	5	M
Am 252	*A. eucalypticola*	M	+	5	M
Am 253	*A. neoniger*	H	+	5.55	M
Am 254	*A. eucalypticola*	M	+	6.35	M
Am 255	*A. niger*	H	+	8.5	H
Am 256	*A. eucalypticola*	M	+	5.65	M
Am 257	*A. brasiliensis*	M	+	7	H
Am 258	*A. brasiliensis*	H	+	8.1	H
Am 259	*A. neoniger*	M	+	4.5	L
Am 260	*A. niger*	H	+	8.82	H
Am 261	*A. eucalypticola*	M	+	5.85	M
Am 262	*A. niger*	H	+	8.16	H
Am 263	*A. niger*	M	+	6.4	M
Am 264	*A. eucalypticola*	L	+	4.1	L
Am 265	*A. niger*	M	+	5.8	M
Am 266	*A. brasiliensis*	M	+	5.35	M
Am 267	*A. eucalypticola*	M	+	5.05	M
Am 268	*A. niger*	H	+	7	H
Am 269	*A. eucalypticola*	M	+	4.5	L
Am 270	*A. niger*	H	+	8.54	H
Am 271	*A. niger*	H	+	8.7	H
Am 273	*A. niger*	H	+	8.4	H
Am 274	*A. niger*	L	+	4.1	L
Am 275	*A. niger*	H	+	8.24	H
Am 276	*A. niger*	H	+	7	H
Am 277	*A. eucalypticola*	M	+	5.2	M
Am 278	*A. niger*	M	+	5.55	M
Am 279	*A. brasiliensis*	M	+	4.55	L
Am 280	*A. eucalypticola*	M	+	5.6	M

Table 1. Continued					
Isolate No.	Fungal identification	Fungal growth	Presence of clear zone	Diameter of the clear zone (cm)	Index *
Am 281	*A. eucalypticola*	M	+	6.1	M
Am 282	*A. eucalypticola*	M	+	6.15	M
Am 283	*A. eucalypticola*	L	+	4.15	L
Am 284	*A. eucalypticola*	M	+	5.5	M
Am 285	*A. eucalypticola*	M	+	6.05	M
Am 286	*A. eucalypticola*	M	+	5.6	M
Am 287	*A. tubingensis*	M	+	5.5	M
Am 288	*A. eucalypticola*	M	+	5.7	M
Am 289	*A. eucalypticola*	M	+	5.75	M
Am 290	*A. brasiliensis*	M	+	5.9	M
Am 291	*A. brasiliensis*	M	+	6.15	M
Am 292	*A. brasiliensis*	M	+	6.25	M
Am 293	*A. eucalypticola*	M	+	6.6	M
Am 294	*A. eucalypticola*	M	+	5.8	M
Am 501	*A. brasiliensis*	M	+	6	M
Am 502	*A. niger*	H	+	8.8	H
Am 503	*A. eucalypticola*	M	+	6.85	M
Am 504	*A. eucalypticola*	M	+	6.4	M
Am 505	*A. eucalypticola*	M	+	5.2	M
Am 506	*A. eucalypticola*	M	+	5.8	M
Am 507	*A. eucalypticola*	M	+	5.7	M
Am 508	*A. eucalypticola*	M	+	5.65	M
Am 509	*A. brasiliensis*	M	+	6.45	M
Am 510	*A. eucalypticola*	M	+	6.2	M
Am 511	*A. brasiliensis*	M	+	5.95	M
Am 512	*A. niger*	H	+	6.4	M
Am 513	*A. eucalypticola*	M	+	6.05	M
Am 514	*A. niger*	M	+	6.6	M
Am 515	*A. eucalypticola*	M	+	4.45	L
Am 751	*A. eucalypticola*	M	+	5.15	M
Am 752	*A. niger*	M	+	6.85	M
Am 753	*A. brasiliensis*	M	+	5.75	M
Am 754	*A. eucalypticola*	M	+	5.3	M
Am 755	*A. eucalypticola*	L	+	3.75	M
Am 756	*A. niger*	M	+	5.45	M
Am 757	*A. eucalypticola*	H	+	6.5	M
Am 758	*A. brasiliensis*	L	+	5.25	M

Index: H= equal to or more than 7 cm clear zone, M= from 5 to 7 cm, and L= less than 5 cm

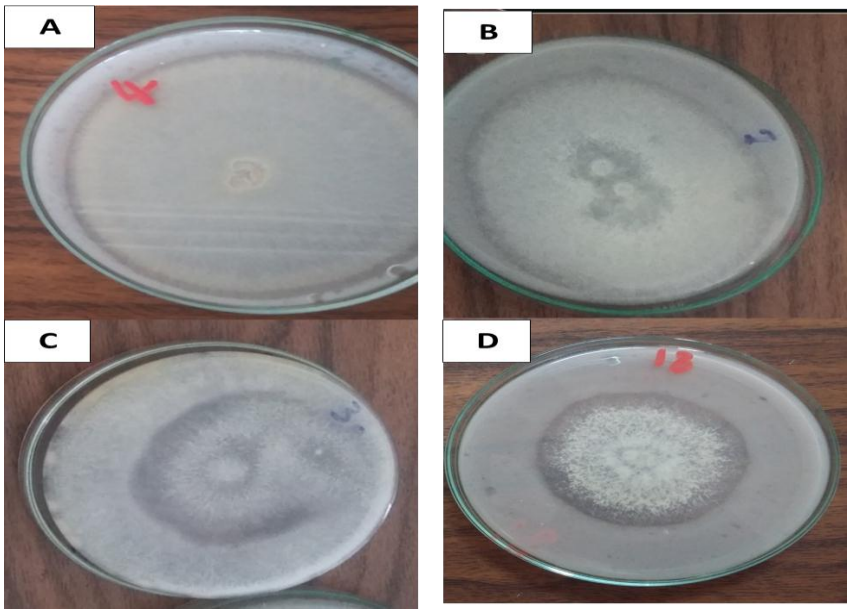

Figure 1. Acid production, dissolution of $CaCO_3$ and clear zone formation by some *Aspergillus* isolates A) *A. niger* Am4, B) *A. niger* Am1, C) *A. brasiliensis* Am30 and D) *A. eucalypticola* Am18.

Production of citric acid by selected isolates of *A. niger* group grown on Cz-Dox liquid medium

The highest acid producers of *Aspergillus* isolates (20 isolates) were screened for citric acid production by growing on a Cz-Dox liquid medium, and the results were recorded in Table 2. The highest citric acid producer isolate was *A. brasiliensis* Am 27 recording 8.49 ±2 g/L, followed by Am 260, AM 270, Am32, Am1, Am4, AM 262 and Am19 recording, 3.53±0.8, 1.99±0.5, 1.88±0.4, 1.73±0.4, 1.35±0.2, 1.29±0.2 and 1.29±0.2 g/L citric acid, respectively. Whereas the other fungal isolates exhibited a lower ability of citric acid production on Cz-Dox medium ranging from 0.97±0.2- 0.37±0.02 g/L.As well, the highest yield of citric acid from initial sugar was recorded for fungal isolate AM27 (29.22% of initial glucose). On the other hand, citric acid yields from initial glucose by all tested isolates other than *A. brasiliensis* Am 27 were very low (Table 2). Furthermore, specific productivity

of the highest citric acid producers was estimated for fungal isolates Am27, Am 260, Am 1, Am31, and Am 270 recording 0.81±0.2, 0.37±0.02, 0.23±0.01, 0.22±0.1 and 0.21±0.01 g/g DW fungal biomass, respectively. Data in Table 2 revealed a decrease in the pH value of fungal fermentation culture compared with the initial pH of the cultivation medium. As well changes in pH values of the fungal culture with different fungal isolates were observed. On the other side, the fungal biomasses formed by the fungal isolates under study were ranged from 7.46 to 11.08 g/L.

Nearly similar results were recorded by Almousaet al. (2018). They tested ninety-six *A.niger* isolates for their ability to produce citric acid on agar plates. Also, they found that all *A. niger* isolates were varied in their capability to produce citric acid on Cz-Dox broth medium and based on the citric acid productivity, the highest productivity was recorded by *A. niger* MH368137. Also, they noticed that there is no correlation between the harvested fungal biomass of all tested fungal isolates and the amounts of citric acid produced. Where they observed the measurements of dry fungal biomass were ranged from 4.8 to 8.2 g/L and the pH values decreased to the acid range (3.19-3.63). Interestingly, many studies were stated that *A. niger* is the most efficient fungal species for citric acid production owing to ease of handling processes, the ability to utilize and ferment different varieties of cheap raw materials because of its well-developed enzymatic system producing a high yield of citric acid (Schuster et al. 2002; Boominathanet al. 2012; Femi-Ola and Atere 2013).

The utilization of sugars such as glucose and sucrose for commercial citric acid production is the most commonly used substrates for fungal fermentation processes, but they are not cost-effective and can be substituted by various cheap and available substrates, including agro-industrial wastes and other by-products (Prasad et al. 2014). So, there is an urgent need for choosing suitable and easily available raw materials (Soccolet al. 2006**).** Thus, the aim of the current study was to check the potentiality of *A. niger* isolates to use sugarcane bagasse raw materials as a cheap substrate for citric acid production.

Table 2. Fungal biomass and citric acid production by *Aspergillus* isolates.

Isolate No.	Amount of citric acid (g/L)	The yield of citric acid (%)*	Citric acid specific productivity (g/g DW)	Fungal biomass (g/L)	pH	Residual sugar (mg/mL)
Am 27	8.49 ±2	29.22	0.81 ±0.2	10.52 ±2	1.94 ±0.6	0.94±0.2
Am 260	3.53 ±0.8	11.80	0.37 ±0.02	9.78 ±2	2 ±0.8	0.08±0.002
Am 270	1.99 ±0.5	6.71	0.21 ±0.01	9.32 ±2	2.6 ±1	0.34 ±0.03
Am 31	1.88 ±0.4	6.30	0.22 ±0.01	8.5 ±1	3.13 ±1	0.16 ±0.01
Am 1	1.73 ±0.4	5.85	0.23 ±0.01	7.46 ±1	3.4 ±1	0.41±0.1
Am 4	1.35 ±0.2	4.54	0.143 ±0.008	9.46 ±2	2.09 ±0.8	0.29 ±0.005
Am 262	1.31 ±0.2	4.39	0.138 ±0.008	9.48 ±2	3.04 ±1	0.19 ±0.005
Am 19	1.29 ±0.2	4.32	0.172 ±0.01	7.46 ±1	5.7 ±1	0.17 ±0.004
Am 32	0.97 ±0.2	3.25	0.107 ±0.01	9.04 ±2	8 ±2	0.19±.005
Am 34	0.89 ±0.1	3.01	0.081 ±0.001	11.08 ±3	3.51±1	0.4 ±0.1
Am 273	0.83 ±0.1	2.79	0.082 ±0.002	10.08 ±3	2.20 ±0.8	0.23 ±.005
Am 275	0.81 ±0.1	2.72	0.085 ±0.002	9.5 ±2	2.41 ±1	0.2 ±0.005
Am 12	0.77 ±0.1	2.58	0.084 ±0.002	9.08 ±2	2.40 ±1	0.19 ±0.004
Am 271	0.61 ±0.08	2.06	0.066 ±0.001	9.1 ±2	1.98 ±1	0.46 ±0.008
Am 33	0.48 ±0.05	1.61	0.049 ±0.001	9.6 ±2	2.27 ±0.8	0.23 ±0.005
Am 502	0.46 ±0.05	1.56	0.046 ±0.001	10 ±3	1.83 ±0.6	0.49 ±0.1
Am 40	0.39 ±0.05	1.31	0.048 ±0.001	8.26 ±2	2.13 ±1	0.24 ±0.005
Am 17	0.37±0.02	1.24	0.035 ±0.0008	10.58 ±2	3.81 ±1	0.16 ±0.002
Am 255	0.36 ±0.02	1.22	0.039 ±0.0008	9 ±1	2.16 ±1	0.39 ±0.1
Am 258	0.33 ±0.02	1.11	0.037 ±0.001	8.76 ±1	3.05 ±2	0.34 ±0.008

***The yield of citric acid is calculated as percent from initial glucose.**

Assay for endo-cellulases (CMCase)enzyme activity on carboxy methyl cellulose containing liquid medium

A total of selected twenty fungal isolates recovered from sugarcane bagasse were screened for cellulolytic activity by growing on carboxy methyl cellulose-containing liquid medium to detect their potentiality for endo-cellulases production. Data in Table 3 showed that the 20fungal isolates exhibited various

degrees of cellulolytic activities under submerged culture. The highest cellulolytic production was recorded by fungal isolate Am 270 (0.73±0.2 IU/mL) followed by AM 1 (0.44±0.1 IU/mL), Am19 (0.39±0.1IU/mL), Am40 (0.38±0.1IU/mL), Am32 (0.32±0.08 IU/mL), Am 271 (0.32±0.08 IU/mL), Am 258 (0.29±0.05 IU/mL) and Am 34 (0.27±0.05 IU/mL). The highest endo-cellulases (CMCase) specific activity 19.59±1 IU/mg protein was recorded by Am 270 and followed by fungal isolate Am 40 (10.97±0.5 IU/mg protein), Am32 (10.57±0.7IU/mg protein), Am 271 (10.03±0.5 IU/mg protein), Am19 (8.84±0.7 IU/mg protein), Am34 (7.97±0.4IU/mg protein), Am4 (7.24±0.4 IU/mg protein) and Am1 (6.38±0.4 IU/mg protein).

In agreement with our results, Mrudula and Murugammal (2011) stated that approximately all fungal species of the genus *Aspergillus* produce cellulase. Consequently, this fungal genus has a great potential for enzyme industry. The fungal species of *Aspergillus* and *Trichoderma* spp. are well documented for the efficient production of cellulases (Van-Peijet al. 1998). Traditionally, the important industrial enzymes have been obtained through submerged fermentation processes (SMF) because of the ease of handling and better control of environmental and culture conditions such as temperature, pH, and required nutrients. On the other hand, solid-state fermentation (SSF) processes can enhance the enzymatic production yield and decrease the cost processes of enzyme production (Ghildyalet al. 1985; Huiet al. 2010). Reports from India revealed that the highest production of cellulase by *A. niger* was achieved after 72 h in SSF and 96 h in SMFrecording 8.89 U per g of dry mycelial bran in solid-state fermentation and 3.29 U per mL culture broth for submerged fermentation. As well, the productivity of extracellular cellulase by *A. niger* in solid-state fermentation was 14.6 fold higher than in submerged fermentation (Mrudulaand Murugammal 2011).

Table 3. Endo-cellulases (CMCase) production and specific activity of some fungal isolates.

Isolate No.	Total protein (mg/mL)	Enzyme activity (IU/mL)	Specific activity (IU/mg protein)
Am 270	0.04 ±0.01	0.73±0.2	19.59±1
Am 1	0.07 ±0.01	0.44±0.1	6.38±0.4
Am 19	0.05 ±0.02	0.39±0.1	8.84±0.7
Am 40	0.04 ±0.02	0.38±0.1	10.97±0.5
Am 32	0.03 ±0.01	0.32±0.08	10.57±0.7
Am 271	0.032 ±0.006	0.32±0.08	10.03±0.5
Am 258	0.04 ±0.01	0.29±0.05	7.54±0.5
Am 34	0.034 ±0.008	0.27±0.05	7.97±0.4
Am 4	0.03 ±0.01	0.21±0.04	7.24±0.4
Am 33	0.03 ±0.01	0.15±0.06	5.92±0.3
Am 255	0.04 ±0.01	0.15±0.05	4.23±0.2
Am 262	0.021 ±0.005	0.14±0.04	6.59±0.4
Am 502	0.035 ±0.008	0.13±0.03	3.65±0.1
Am 27	0.027 ±0.005	0.13±0.03	4.72±0.2
Am 275	0.04 ±0.008	0.05±0.01	1.31±0.2
Am 17	0.06 ±0.01	0.03±0.01	0.61±0.1
Am 260	0.04 ±0.008	0.03±0.01	0.896±0.2
Am 12	0.05 ±0.01	0.03±0.01	0.52±0.08
Am 273	0.03 ±0.008	0.024±0.01	0.87±0.1
Am 31	0.029 ±0.003	0.006±0.001	0.21±0.05

Citric acid production on carboxy methyl cellulose containing liquid medium

The nine highest endo-cellulases (CMCase) producers were selected to assay their potentiality for citric acid production on CMC containing medium in submerged culture fermentation. Data in Figure 2 showed that the *Aspergillus* fungal isolates exhibited various capabilities for citric acid production from CMC liquid medium in submerged fermentation. The highest citric acid yield (% of CMC) was recorded by fungal isolate Am 258 (14.74%) followed by Am40 (10.80%), Am19 (8.6%), Am32 (8.40%) and Am34 (6.40%).

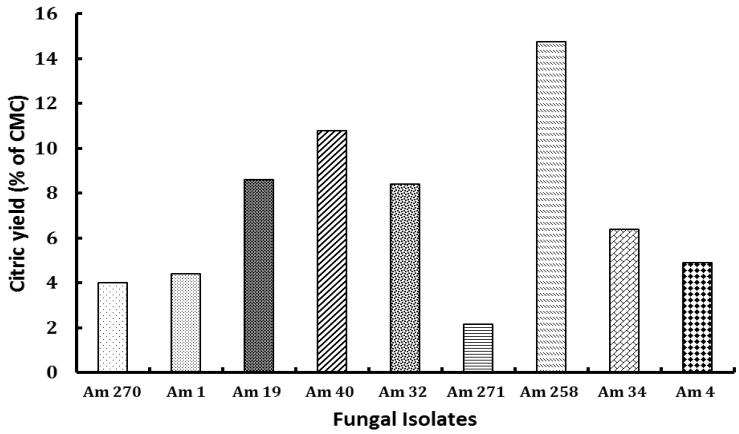

Figure 2. Citric acid yield on carboxy methyl cellulose containing liquid medium.

Citric acid production from sugarcane bagasse

The highest citric acid-producing isolate Am 258 recorded in the previous experiment was screened for its capability for citric acid production from sugarcane bagasse in solid-state fermentation. The highest amount of citric acid by this isolate was obtained 10 days after incubation, recording 34 g/L citric acid and by increasing the incubation period, the obtained amount of citric acid decreased, as shown in Figure 3. As well, a mixed culture of the highest endo-cellulases (CMCase) producing isolate Am 270 and the highest citric acid-producing isolate Am 258 was performed to enhance the amount of citric acid produced. The obtained data revealed a slight enhancement of citric acid production by mixed culture recording 35.1 g/L citric acid after 8 days of the total incubation period (4 days after inoculation of fungal isolate Am 258 on fermentation culture of Am 270), then citric acid decreased by increasing incubation time (Figure 4). The depletion of the obtained citric acid with the extension of the fermentation period may be due to many factors, including the declined available nitrogen and sugar contents in the fermentation medium and the age of fungi. Yadegary et al. (2013) and Francisco *et al*. (2020) found that increasing incubation periods reduce

nitrogen and sugar contents in the fermentation substrate, and consequently a decrease in citric acid production. This indicates that the suitable fermentation periods for citric acid production which a higher citric yield is obtained at early stages and consequently reduces the cost required and timesaving for maintaining the longer time fermentation processes (Yadegaryet al. 2013; Mahadiet al. 2021). Tuquerres et al. (2017) reported that *A. niger* isolated from the Ecuadorian Amazon region showed a high capability to produce citric acid using sugarcane bagasse as raw material and the highest production was 9.9 g citric acid/kilogram bagasse. They also recorded that the highest sugar utilization and citric acid production from sugarcane bagasse were estimated by *A. niger* 318 recording 196.21 µg/g and followed by fungal isolate *A. niger* 14/20 (103.06 µg/g) in the presence of Prescott salt. On the other hand, the highest citric acid production was recorded by *A. niger* 14/20 (50.01 µg/g) in absence of Prescott salt (Al-Mahinet al. 2008).

Recently, extensive interest has been focused on exploiting less expensive agricultural wastes as an alternative renewable feedstock for citric acid production by *A. niger* using solid-state fermentation techniques (Bastos and Ribeiro 2020; Roukas and Kotzekidou 2020). A variety of agro-industrial residues and by-products have been investigated using SSF techniques for their potential to be used as substrates for citric acid production. As well, many cost-effective raw materials have been achieved for the production of citric acid, such as sugarcane bagasse (Kumar et al. 2003), cassava bagasse (Prado et al. 2005), coffee husk (Vandenbergheet al. 2000b) wheat bran (Kumar et al. 2003), and okara residue (Khareet al. 1995). All these agriculture residues are very well adapted to solid-state fermentation strategy attributable to their structural composition of cellulosic and starchy nature (Amato et al. 2020). The maximum amount of citric acid produced from sugarcane bagasse by *A. niger* isolates in this study is quite good when compared with citric acid amounts reported in the previous literature using different organic substrates (Table 4).

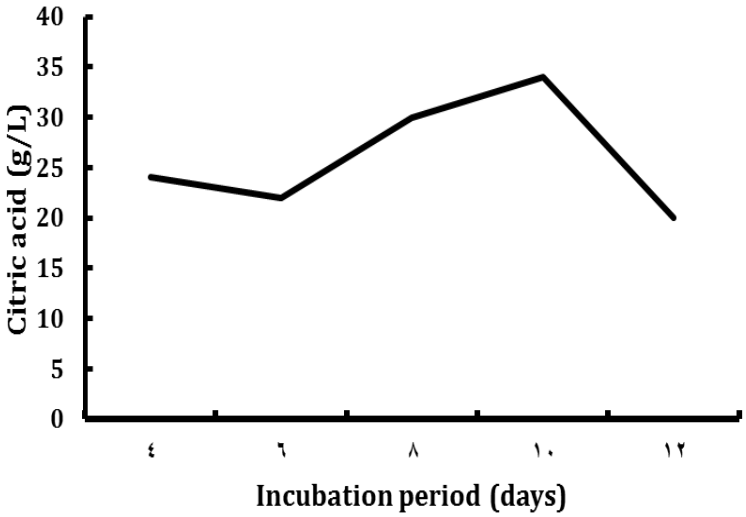

Figure 3. Citric acid production by fungal isolate Am 258 grown on sugarcane bagasse using solid-state fermentation strategy.

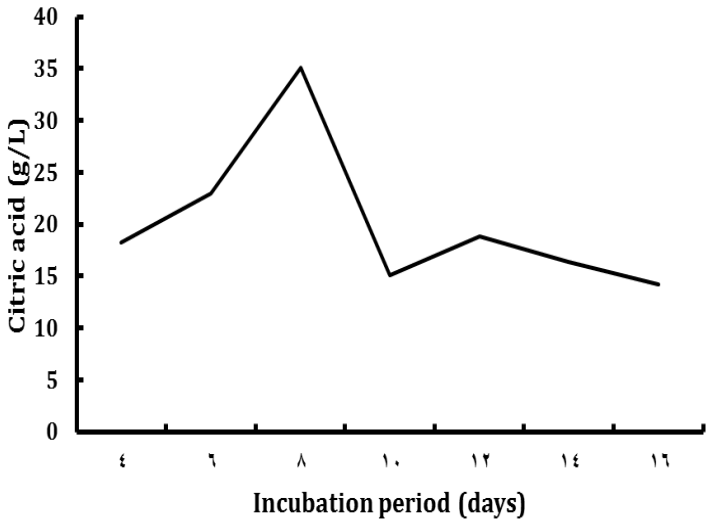

Figure 4. Citric acid production by mixed culture of fungal isolates Am270 and Am 258 grown on sugarcane bagasse using solid-state fermentation strategy.

Table 4. Comparison of citric acid production from different organic substrates by *A.niger*.

Organism	Substrate	Citric acid (g/L)	References
A. niger (MTCC 281)	Waste apple pomace	46	Kumar et al. 2010
A. niger (FUO2)	Cassava peel malted sorghum	1.93	Adeoyeet al. 2015
A. niger (ATCC 9142)	Cocoyam starch	108.00	Amenaghawonet al. 2015
A. niger (KA88)	Corn cobs solid substrate	138.24	Addoet al. 2016
A. niger	Mango peels	7.52	Abbas et al. 2016
A. niger	Sweet orange peels	11.01	Abbas et al. 2016
A. niger	Rice straw	50.20	Ali et al. 2012
A. niger	Oat bran	62.00	Rao and Reddy 2013
A. niger (UABN 210)	Banana peels	82.12	Kareem and Rahman 2011
A. niger	Sugarcane bagasse	9.9	Tuquerreset al. 2017
A. niger 14/20	Sugarcane bagasse	5.001	Tuquerreset al. 2017
A. niger Am 258	Sugarcane bagasse	34	This study
Mixed culture (Am 270 and Am 258 isolates)	Sugarcane bagasse	35.1	This study

Conclusion

The obtained results indicated that citric acid production from sugarcane bagasse can be enhanced in fermentation processes involving active cellulolytic isolates of *A. niger* (e.g. *A. niger* Am 270, highest endo-cellulases (CMCase) producer and *A. niger* Am 258, highest citric acid producer). A higher yield of citric acid (35.1 g/L versus 34 g/L) in a shorter incubation period (8 versus 10 days) was recorded.

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الملخص العربي

إنتاج حامض الستريك بواسطة فطريات الأسبرجيللس السوداء المحللة للسليلوز والمعزولة من باجاس قصب السكر

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في هذه الدراسة تم تقييم مقدرة فطر ياتالاسبرجيللس السوداء التابعة لقسم Negri والمعزولة من باجاس قصب السكر ،على إنتاج الأحماض والإنزيمات المحللة للسليلوز وحمض الستريك. كما تم دراسة إمكانية تحسين إنتاج حامض الستريك من باجاس قصب السكر بإستخدام مزارع مختلطة من المعزولات الأعلى إنتاجا لإنزيم السليوليز وحمض الستريك. أظهرت النتائج أن جميع المعزولات الفطرية المختبرة لها المقدرة على إنتاج كميات متفاوتة من الحمض على وسط غذائى صلب من الشاببيكس ديكستروز يحتوي على كربونات الكالسيوم. كما أظهرت فطرة A. brasiliensis Am27 أعلى إنتاجا لحمض الستريك من الوسط الغذائى السائل من الشاببيكس ديكستروز (8.49±2 جم / لتر) وهذه الكمية من حامض الستريك تعادل ٢٩.٢٢٪ من السكر االبادئ. علاوة على ذلك ، تم دراسة مقدرة المعزولات الفطرية على إنتاج إنزيم السليوليز وكذلك إنتاج حمض الستريك على وسط غذائى من الكربوكسي ميثيل السليلوز. أظهرت النتائج أن المعزولة الفطرية Aspergillus niger Am 270 هى الأعلى إنتاج لإنزيم السليوليز (٠.٢ ±0.73 IU/mL) بينما أظهرت المعزولة الفطرية A. niger Am 258 أعلى قدرة إنتاجية لحمض الستريك (١٤.٧٤٪). ولذلك تم إختيار هذه المعزولات لتحسين إنتاج حامض الستريك من باجاس قصب السكر. وتم الحصول على أعلى كمية من حامض الستريك من باجاس قصب السكر بواسطة المعزولة الفطرية A. niger Am 258 بعد ١٠ أيام من عملية التخمر مسجلا ٣٤ جم / لتر حامض الستريك وبزيادة فترة التخمرإنخفضت الكمية المتحصل عليها من حامض الستريك. في حين أظهرت المزارع المختلطة لـ A. niger (Am 270 , Am 258) تحسنًا طفيفًا في إنتاج حامض الستريك مسجلاً ٣٥.١ جم / لتر حامض الستريك بعد ٨ أيام من فترة التخمر الكلية. لذلك قد توفر هذه الدراسة إستراتيجية مناسبة لتحسين إنتاج حامض الستريك من قصب قصب السكر.