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Research paper

Isolation and screening of kojic acid producing isolate of *Aspergillus oryzae* potentially applicable for production from sugarcane molasses

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Abstract

Kojic acid is an organic acid produced as secondary metabolite by different fungi specially *Aspergillus* species. Isolation of a novel fungal strain potential for kojic acid production from agro-industrial wastes was the main purpose of the present study. Kojic acid was estimated in the current investigation colorimetric by 2,6-dichlorophenolindophenol (DCIP). A total of 43 fungal isolates belonging to seven species of *Aspergillus* recovered from stored wheat grains and dust air were screened for their ability to produce kojic acid. Ten isolates of them belonging to *A. oryzae* and *A. flavus* var. *columnaris* produced high concentrations (16.818 ± 0.006 - 43.917 ± 0.389 g/l) of kojic acid from glucose. The secondary screening of these ten isolates for kojic acid production from glucose, sucrose, starch, maltose and cellulose as different carbon sources resulted that *A. oryzae* 124A was the highly producer on glucose and sucrose recording 44.189 ± 0.079 and 32.135 ± 0.298 g/l, respectively. *A. oryzae* 124A produced 15.022 ± 0.017 g/l of kojic acid from the pretreated semisynthetic sugarcane molasses. The maximum concentration (29.431 ± 0.001 g/l) of kojic acid production by *A. oryzae* 124A from sugarcane molasses was obtained when the fungus grown on 5 % sugarcane molasses adjusted at pH 3.5 and incubated at 28◦C for 19 days. The recorded results suggested that *A. oryzae* 124A could be used as a promising candidate for utilization in kojic acid fermentation from sugarcane molasses on industrial scale.

Keywords: *Fermentation; Kojic Acid; Molasses; Optimization; Pretreatment.*

1. Introduction

Aspergillus flavus are an important group of species in food mycology, medical mycology and biotechnology. Most species cause food spoilage, but on the other hand are also used in the fermentation industry to produce hydrolytic enzymes and organic acids. Non-aflatoxigenic species such as *A. oryzae* have been widely used in industry for food fermentation or enzymes production (Geiser *et al.* 1998). Different organic acids produced by *Aspergillus oryzae* such as citric, gluconic, itaconic and kojic acid. Kojic acid known as 5-hydroxy-2-hydroxymethyl-γ-pyrone is a weak organic acid produced as a major secondary metabolite by *Aspergillus oryzae* from carbohydrates in aerobic fermentation process (Rosfarizan *et al.* 2010, Hazzaa *et al.* 2013). This organic acid was originally isolated in Japan by Saito in 1907 from mycelia of *Aspergillus oryzae* grown on steamed rice (El-Aasar 2006).

Kojic acid has several economic uses in various fields. In food industries, Kojic acid is distributed naturally in traditional Japanese food such as miso, soy sauce and sake, thus, endowing these various food types with special tastes, colours and flavours (Wood 1998). kojic acid is now used as an additive to prevent browning of food materials, and as an antioxidant (Bentley 2006, Nohynek *et al.* 2004, Rosfarizan *et al.* 2010, Yasunobu *et al.* 2010) due to its inhibitory effect on polyphenol oxidase in different foods (Saruno *et al.* 1978, Chen *et al.* 1991). The inhibitory effect of kojic acid on polyphenol oxidase is associated to the inhibition of melanosis by interfering the uptake of oxygen required for enzymatic browning, and reduction of o-quinones to diphenols to prevent the formation of the final pigment (melanin) or the combination of the above actions (Rosfarizan *et al.* 2010).

Kojic acid and its derivatives possess antibacterial properties against gram-negative as well as gram-positive bacteria and antifungal agent (Kotani *et al.* 1976, Hassan *et al.* 2014). Besides its antibiotic functions, kojic acid also shows a certain insecticidal activity against *Heliothis zea* and *Spodoptera frugiperda* insects and it has been employed as a chelating agent for the production of insecticides (Buchta 1982, Dowd 1988). Also, kojic acid continues to attract attention because of its economic potential in medical field as an anti-inflammatory drug (Jignesh *et al.* 2014).

The most striking benefit of kojic acid is found in cosmetic and health care industries due to its ability to act as the ultra violet protector, whereby, it suppresses hyperpigmentation in human skins by restraining the formation of melanin through the inhibition of tyrosinase formation, the enzyme that is responsible for skin pigmentation (Noh *et al.* 2009). At present, kojic acid is primarily used as the basic ingredient for excellent skin lightener in cosmetic creams, where it is used to block the formation of pigment by the deep cells on the skins (Masse *et al.* 2001). In addition, kojic acid and its manganese and zinc complexes can potentially be used as radio protective agents, particularly against γ-ray (Emami *et al.* 2007). Recently, methods for the synthesis of various kojic acid derivatives, such as kojic acid ester, kojic acid laureate and kojic acid dipalmitate have been reported in many studies (Brtko *et al.* 2004, Lee *et al.* 2006, Khamaruddin *et al.* 2008, Ashari *et al.* 2009). Also, it has been reported that kojic acid can be easily conjugated with chitosan to produce kojic acid-chitosan conjugates, suggesting that kojic acid has a potential use in chemi-

cal industry (Guibal 2004, Synytsya *et al.* 2008). Moreover, in chemical industries it has been successfully used to make azo-dyes and biodegradable compounds (Hassan *et al.* 2014).
Among the used substrates for kojic acid production is the sugarcane molasses as by-product of sugar industries in Egypt. Molasses are cheap raw materials, readily available, and ready for conversion with limited pretreatments as compared with starchy or cellulosic materials. Sugarcane molasses is a dark viscous fluid and rich in sugar and some nutrients required by most microorganisms. Therefore, the current study was conducted to screen for the kojic acid producing ability among different *Aspergillus* species isolated from starchy grains and air and also to optimize kojic acid production by the selected highly producer isolate from sugarcane molasses.

2. Materials and methods

2.1. Samples collection and Fungi isolation

The investigated fungi in the present work were isolated from two habitats as following:

2.1.1. Dust air samples

Twenty dust air sampling from the outdoor of different sectors at Sues University was performed during the evening in winter season using the settle plate method according to Hoekstra *et al.* (2004). The plates were exposed for 15 minutes in each exposure, sealed and transferred immediately to the laboratory for incubation.

2.1.2. Wheat grain samples

Twenty samples of stored wheat grains were collected randomly from the markets at Suez, Egypt for isolation of associated mycoflora. The isolation was performed by dilution plate method according to Christensen (1963).
The collected fungi were isolated on Sabouraud dextrose agar (SDA) medium contained (g/l dist. H_2O): dextrose, 40.0; peptone, 10.0 and agar, 18.0 supplemented with chloroamphenicol (50 µg/ml) and rose bengal (10 µg/ml) (in order to minimize the appearance of bacteria). The culture medium was adjusted to pH 6.5 using HCl 0.1 N and NaOH 0.1 N and autoclaved at 121ºC for 15 minutes. The plates were incubated at 28^{o}C for 10 days. The growing fungal colonies were picked up, sub-cultured on fresh SDA plates, transferred to fresh agar slants and stored at 4^{o}C. The results were expressed as colony forming units (CFU).

2.2. Fungi Identification

The isolated fungi were identified at the genus and species level according to the detailed study of all the microscopic morphological characters and the macroscopic features of their colonies according to Moubasher (1993).

2.3. Fermentation medium for kojic acid production

The fermentation medium for kojic acid production consists (g/L): Glucose, 60; yeast extract, 5; KH_2PO_4, 1.5 and $MgSO_4$, 0.5 adjusted at pH 3.5 was used in screening experiments. The experimental cultures were grown in 250 mL Erlenmeyer flasks, each containing 50 mL of the synthetic medium. The flasks were sterilized at 121◦C for 15 min and inoculated after cooling with one mL of inoculum spores suspensions (5×10^6 spores per mL). The cultures were incubated at 28 ± 2◦C as static cultivation for 8 days.

2.4. Detection of kojic acid production

Kojic acid concentration was estimated quantitatively in culture filtrates by a colorimetric method based on the reaction between kojic acid and 2,6-dichlorophenolindophenol (DCIP) according to Tanigaki *et al.* (1980).

2.5. Kojic acid fermentation from different carbon sources

Different carbon sources (glucose, sucrose, starch, maltose and cellulose) were individually added to the basal fermentation medium in such amount equivalent to 60 g/l of carbon. The cultures were incubated at 28 ± 2◦C as static cultivation for 12 days.

2.6. Sugarcane molasses pretreatment

Sugarcane molasses was purchased from Sugars and Integrated Industries Egyptian Distillation Plants in Hawamdeia City, Giza, Egypt and stored at 4 ºC. The clarification of molasses was conducted according to Shashank (1994). The molasses were diluted using distilled water to prepare molasses with 6 % sugar concentration and the pH of the diluted molasses was adjusted to 3.5 using concentrated sulfuric acid. The molasses is heated to about 95^{o}C in water bath for 15 minutes and the heated diluted molasses was left to settle for at least 2 hours to precipitate the sludge. The treated molasses was filtered and the cleared supernatant was transferred to the fermentation flasks.

2.7. Optimized conditions for kojic acid production from sugarcane molasses by *A. oryzae* 124A

The effects of different initial pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5), different molasses sugar concentrations (3-10 %) with 1% interval, different incubation temperatures (5 - 45^{o}C) and different fermentation periods (7 – 21 days) on kojic acid production by the selected isolate were estimated.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed using CoStat V. 6.311 (CoHort software, Berkeley, CA94701). Kojic acid production mean values were compared at 5% significance level using Tukey's test.

3. Results and discussion

3.1. Isolation of fungi from dust air and wheat grains

The total count of fungi isolated from the air of Suez University area was 204 CFU/5h. A total of 20 fungal species plus to two species varieties belonging to 12 genera were isolated and identified. *Aspergillus* was the most dominant genus emerged in 18 out of 20 tested exposures and recorded 35.78 % of total fungal count. *Aspergillus* was represented by *A. carbonarius*, *A. flavus* var. *flavus*, *A. flavus* var. *columnarius*, *A. oryzae*, *A. sulphureus* and *A. niger*. The most common species of *Aspergillus* was *A. flavus* and recorded with moderate occurrence while the other isolated species were recorded as low or rare occurrence. Many researchers in different studies on isolation of airborne fungi reported that the most prevalent fungi were belonged to genus *Aspergillus* (Durugbo *et al.* 2013, Menezes 2004, Chakrabarti *et al.* 2012, Kalyoncu 2012, Sen and Asan 2009, Horner *et al.* 2004). *Alternaria* comes behind *Aspergillus* in the occurrence where isolated from 10 out of the 20 tested exposures representing 14.22 % of isolated total fungal count. It was represented by the two species *A. alternata* and *A. phragmospora* in low frequencies. *Cladosporium* and *Cochliobolus* ranked the third genera with regard to the number of cases of isolation. Both of the two genera were recorded with low occurrence. These results are in agreement with Shams-Ghahfarokhi *et al.* (2014). The predominance of *Aspergillus*, *Alternaria*, *Cladosporium* and *Cochliobolus* may be due to the ability of these genera produce numerous small and light spores that generally remain in the air for a long period of time, whereas other

fungal genera produce fewer, larger and heavier spores which tend to have faster settling (Vonberg & Gastmeier 2006). The other isolated genera were recorded with low frequencies and listed in table (1) and figure (1).

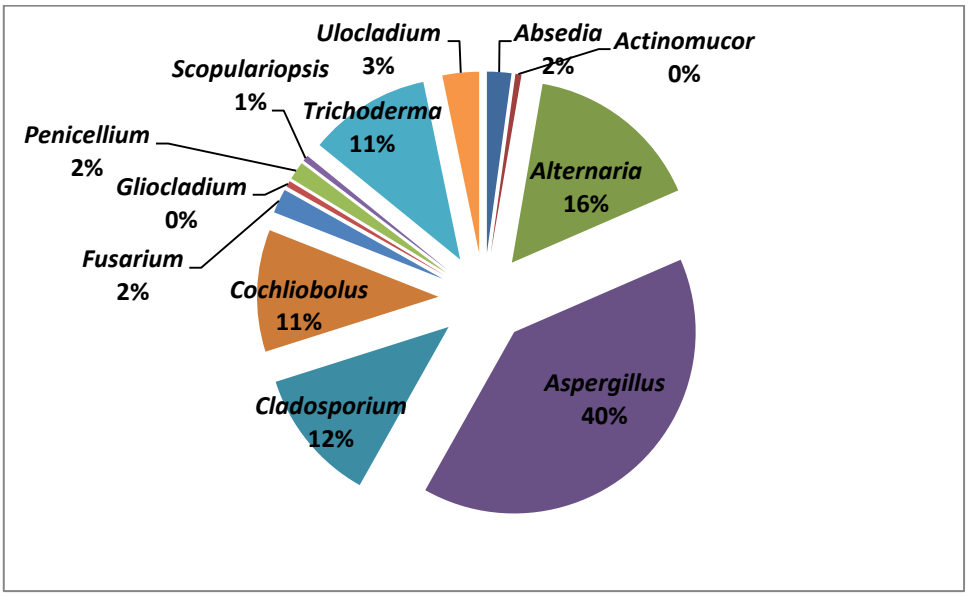

Fig. 1: Percentage of Colonies Forming Units of Fungi Isolated from Dust Air.

Table 1: Fungal Genera and Species Isolated from 20 Outdoor Air Samples Collected from Suez University

Genera & Species	Total CFU/5h	% CFU	NCI	OR
Absidia corymbifora	4	1.96	1	R
Actinomucor elegans	1	0.49	1	R
Alternaria	29	14.22	10	M
A. alternata	18	8.82	6	L
A. phragmospora	11	5.39	5	L
Aspergillus	73	35.78	18	H
A. carbonarius	11	5.39	8	L
A. flavus var. flavus	37	18.14	13	M
A. flavus var. columnaris	2	0.98	1	R
A. oryzae	7	3.43	5	L
A. sulphureus	1	0.49	1	R
A. niger	15	7.35	6	L
Cladosporium	22	10.78	8	L
C. cladosporiodies	2	0.98	1	R
C. herbarium	20	9.80	7	L
Cochliobolus	20	9.80	8	L
C. australiensis	1	0.49	1	R
C. luntus	1	0.49	1	R
C. spicifer	18	8.82	7	L
Fusarium lateritium	4	1.96	2	R
Gliocladium catenulatum	1	0.49	1	R
Penicellium oxalicum	3	1.47	2	R
Scopulariopsis brevicaulis	1	0.49	1	R
Trichoderma harzianum	20	9.80	1	R
Ulocladium atrum	6	2.94	3	R
Total	204	100.00		

CFU: Colony forming units, NCI: Number of cases of isolation, OR: Occurrence remark

In a survey for isolation of fungi from stored wheat grains during this study, a total of 12 species plus two species varieties belonging to five genera were isolated and identified. The isolated genera were *Absidia* (one species), *Aspergillus* (six species), *Candida* sp., *Cladosporium* (one species) and *Penicillium* (three species). Recent reports have been also made on the occurrence of fungi in stored seeds (Rasmey 2009, El-Shanshoury *et al.* 2014, Reddy *et al.* 2009, Rustemeyer *et al.* 2010, Soliman 2003). *Aspergillus* was the most common genus and recorded with high occurrence from 16 out of the 20 tested samples with 69.31 % of total fungal count. The most frequent species of *Aspergillus* were *A. niger* and *A. sulphureus* emerged in 11 and 10 samples out of 20 tested samples, respectively. The obtained results are consistent with Soliman (2003) who reported that *Aspergilllus* was the predominant and most frequent fungus in the collected wheat grains in his study. *Penicillium* was the second common genus where occurred in 10 out of the 20 tested samples and recorded 27.88 % of total fungal count. It was represented by the three species *P. brevicompactum*, *P. chrysogenum* and *P. duclauxii*. *P. duclauxii* was appeared with low frequency while the other two species were recorded as rare occurrence among the other isolated fungi. These obtained results are in agreement with that of Kacaniova & Tancinova (2001) who found that out of 24 genera isolated from feeding wheat, *Aspergillus* and *Penicillium* were the most frequently isolated. Also in a similar study, Al-Kahtani (2014) reported that the most common genera were *Alternaria* (isolated from 68.96% of the tested samples), *Aspergillus* (24.14%) and in a lesser extent *Fusarium* (6.9%). The remaining isolated genera from the tested wheat grain samples were recorded with rare occurrence and listed in table (2)and figure (2). Microorganism propagules get on grain in different ways, most often with dust from soil, from

the surface of plant remnants during harvesting, transportation, storage and processing (Klich 2002).

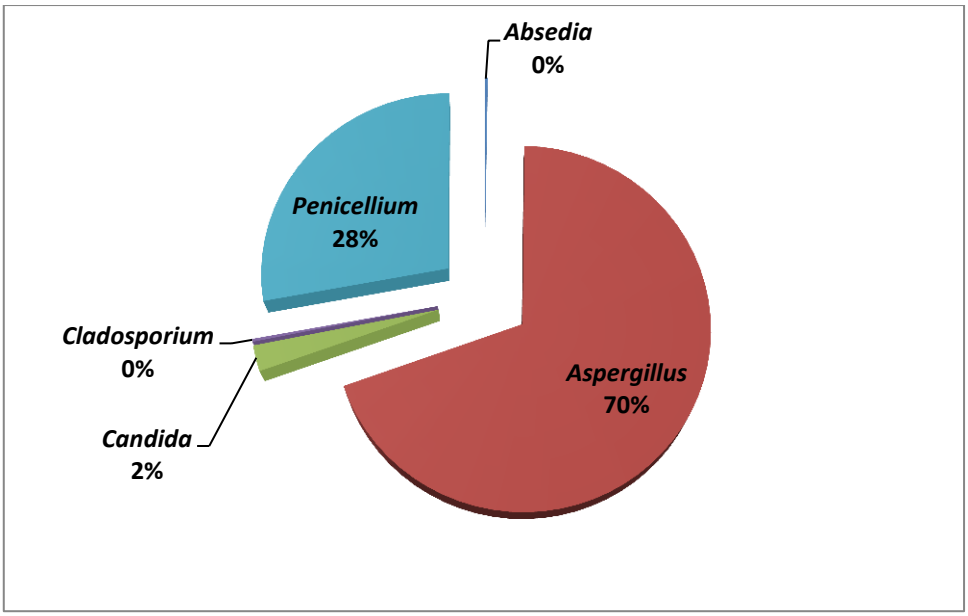

Fig. 2: Percentage of Colonies Forming Units of Fungi Isolated from Stored Wheat Grains.

Table 2: Fungal Genera and Species Isolated from 20 Wheat Grain Samples Collected from Suez University

Genera & Species	Total CFU/20 g	% CFU	NCI	OR
Absidia corymbifera	100	0.26	1	R
Aspergillus	27100	69.31	16	H
A. cervinus	100	0.26	1	R
A. flavus var. *flavus*	900	2.30	4	R
A. flavus var. *Columnaris*	800	2.05	1	R
A Oryzae	600	1.53	3	R
A. niger	8000	20.46	11	M
A. sulphureus	16600	42.46	10	M
A. terreus	100	0.26	1	R
Candida sp.	900	2.30	2	R
Cladosporium herbarium	100	0.26	1	R
Penicellium	10900	27.88	10	M
P. brevicompactum	300	0.77	1	R
P. chrysogenum	900	2.30	4	R
P. duclauxii	9700	24.81	5	L
Total	39100	100.00		

3.2. Screening of kojic acid production by the isolated *Aspergillus* species

A total of forty three isolates of the recovered fungi representing eight species with two species varieties belonging to *Aspergillus* were screened for their potentiality to produce kojic acid from glucose as the sole carbon source in liquid synthetic medium under aerobic condition. Glucose was used in screening of kojic acid producing *Aspergillus* species since it is the simplest sugar (El-Kady *et al*. 2014). The tested *Aspergillus* species were *A. flavus* var. *flavus* (six isolates), *A. flavus* var. *columnaris* (ten isolates), *A. parasiticus* (one isolate), *A. oryzae* (ten isolates), *A. niger* (ten isolates), *A. cervinus* (one isolate), *A. sulphureus* (one isolate), *A. carbonarius* (one isolate) and *A. terreus* (three isolates). All the tested isolates of each of *A. parasiticus*, *A. niger*, *A. cervinus*, *A. sulphureus*, *A. carbonarius* and *A. terreus* were negative for kojic acid production. Also, two isolates of each of *A. flavus* var. *flavus* and *A. oryzae* in addition to four isolates of *A. flavus* var. *columnaris* were unable to produce kojic acid. High concentrations (> 15 g/l) of kojic acid were produced by only two isolates of *A. flavus* var. *columnaris* (codes 120 and 218) and eight isolates of *A. oryzae* (codes 142, 152, 144, 124A, 120A, 231, 226 and 201). Moderate concentrations (5 – 15 g/l) of kojic acid were detected by only two isolates of *A. flavus* var. *flavus* (codes 146 and 110) while low concentrations (< 5 g/l) of kojic acid were obtained by two isolates of *A. flavus* var. *flavus* and four isolates of *A. flavus* var. *columnaris*. It is worth mentioning that the most highly producer isolates for kojic acid were belonging to *A. oryzae* (table 3). The above mentioned *Aspergillus* species were also recorded in different studies as kojic acid producers (Ogawa *et al.* 1995, Ariff *et al.* 1997, Burdock *et al.* 2001, Futamura *et al.* 2001, Sahasrabudhe and Sankpal 2001, Gad 2003, El-Aasar 2006, El-Kady *et al.* 2014). Among the kojic acid producing *Aspergillus* species, *A. flavus* (Bajpai 1982, Ariff *et al.* 1996) and *A. oryzae* (Kitada *et al.* 1967, Kwak & Rhee 1992, El-Aasar 2006) were reported to produce high concentrations of kojic acid. Kojic acid may be produced by different fungi especially *Aspergillus* species, but the commercial production is performed by *A. oryzae* and *A. flavus* using glucose as carbon substrate.

3.3. Kojic acid production from different carbon sources by the highly producer isolates

The influence of various carbon sources such as glucose, sucrose, starch, maltose and cellulose on kojic acid production by the selected highly producer isolates of *Aspergillus* was studied. The results in table (4) revealed that glucose was the most suitable carbon source for kojic acid production by the all ten tested isolates of *Aspergillus* followed by sucrose and starch. *A. oryzae* 142 was able to produce high concentration of kojic acid from starch as sole carbon source and recorded 31.576 ± 0.404 g/l. kojic acid

was not detected when the tested isolates grown on maltose or cellulose as sole carbon source in the fermentation medium. Carbon source plays the most important role in kojic acid production since it supplies the energy for the cell, substrate for kojic acid production and functions as biosynthesis of cellular constituents like carbohydrates proteins, lipids, nucleic acids and many more. Utilization of different carbon sources such as glucose, starch, sucrose, maltose and cellulose by different *Aspergillus* species for kojic acid production were studied by different authors (Kitada *et al*. 1967, Rosfarizan & Ariff 2000). Sahasrabudhe & Sankpal (2001) reported that Kojic acid producing do not require specific carbon sources since all of the cultures are able to utilize various carbon sources like glucose, fructose, sucrose, maltose, and mixture of glucose and sucrose. It is well known that glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid (Kitada *et al.* 1967, Basappa *et al.*1970). Kitada *et al.* (1967) and Megalla *et al.* (1986) suggested that, during the fermentation, kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments. Kitada *et al.* (1967) reported that the highest yield of kojic acid produced by *A. oryzae* was obtained in the fermentation using glucose as carbon source, followed by sucrose. In a similar study, El-Aasar (2006) reported that glucose also have yield highest kojic acid production by *A. parasiticus* and followed by sucrose and beet molasses. *A. oryzae* 124A was the most highly producer isolate in the current investigation for kojic acid production from glucose and sucrose, so it was selected for production of kojic acid from sugarcane molasses in the further experiments.

Table 3: Screening of Kojic Acid Production by Some *Aspergillus* Isolates Recovered From Wheat Grains and Dust Air

Fungal species	Isolation source	Isolate code	Kojic acid (g/L) ± SE	Remark
A. flavus var. *flavus*	Air	146	13.693 ± 0.065	M
		157	0.000	N
		110	12.399 ± 0.130	M
		116	3.584 ± 0.045	L
	Wheat grains	233	0.000	N
		242	3.519 ± 0.013	L
A. flavus var. *columnaris*	Air	133	0.000	N
		149	1.937 ± 0.013	L
		136	0.000	N
		102	0.000	N
		108	0.000	N
		147	0.674 ± 0.006	L
	Wheat grains	120	32.437 ± 0.006	H
		239	0.958 ± 0.194	L
		230	1.150 ± 0.039	L
		218	32.673 ± 0.019	H
A. parasiticus	Air	129	0.000	N
		142	16.818 ± 0.006	H
	Wheat grains	124	0.000	N
		103	0.000	N
A. oryzae	Air	152	43.917 ± 0.389	H
		144	39.678 ± 0.324	H
		124A	43.904 ± 0.045	H
		120A	30.518 ± 0.001	H
	Wheat grains	231	38.918 ± 0.026	H
		226	35.964 ± 0.130	H
		201	17.906 ± 0.065	H
A. niger	Air	104, 112, 126, 101, 134, 121, 148, 151, 130 & 176	0.000	N
A. cervinus	Wheat grains	212	0.000	N
A. sulphureus	Wheat grains	204	0.000	N
A. carbonarius	Wheat grains	205	0.000	N
A. terreus	Wheat grains	208, 203 & 211	0.000	N

H: High kojic acid production (> 15 g/l)
M: Moderate kojic acid production (5 – 15 g/L)
L: Low kojic acid production (< 5 g/l)
N: negative

Table 5: Kojic Acid (G/L) Production from Different Carbon Sources by the Selected Highly Producer Ten *Aspergillus* Isolates

Isolates Code	Kojic acid (g/l) Glucose	Sucrose	Starch	Lactose	Cellulose
A. oryzae 152	40.736 ± 0.023	19.925 ± 0.447	12.522 ± 0.365	- ve	- ve
A. oryzae 144	42.222 ± 0.127	25.527 ± 0.251	7.058± 0.675	- ve	- ve
A. oryzae 231	25.719 ± 1.273	9.716 ± 0.229	0.169 ± 0.029	- ve	- ve
A. oryzae 226	26.567 ± 0.623	8.754 ± 0.927	0.0591 ± 0.000	- ve	- ve
A. oryzae 124A	44.189 ± 0.079	32.135 ± 0.298	11.735 ± 0.070	- ve	- ve
A. oryzae 120A	43.3581 ± 0.005	29.688 ± 0.093	6.491 ± 0.721	- ve	- ve
A. oryzae 201	43.393 ± 0.150	14.724 ± 0.332	0.126 ± 0.038	- ve	- ve
A. oryzae 142	39.958 ± 0.175	21.787 ± 0.277	31.576 ± 0.404	- ve	- ve
A. flavus var. *columnaris* 218	34.504 ± 0.294	10.398± 0.753	8.256 ± 0.490	- ve	- ve
A. flavus var. *columnaris* 120	38.245 ± 0.649	25.344 ± 0.846	12.915 ± 0.0491	- ve	- ve

3.4. Kojic acid production from sugarcane molasses by *Aspergillus oryzae* 124A

An attempt to use sugarcane molasses as natural medium for kojic acid production by *A. oryzae* 124A was investigated in the current study. The possibility of using waste carbon sources as suitable substrates for kojic acid production using *Aspergillus* species was studied by El-Kady *et al.* (2014). Egyptian sugarcane molasses contain about 52% as total sugar (glucose, sucrose, and fructose), 0.46% as total nitrogen in addition to detectable amounts of some vitamins such as riboflavin and thiamin (Khalifa 2003). Presence of these compounds in each of molasses and may favor kojic acid production (El-Kady *et al.* 2014). Three different molasses media

for kojic acid production by the selected isolate *A. oryzae* 124A were tested. The three media were; M1 (untreated 6 % molasses sugar without any nutrients supplementation); M2 (pretreated 6 % molasses sugar without any nutrients supplementation) and M3 (pretreated 6 % molasses sugar supplemented with 5.0 g L^{-1} yeast extract, 1.0 g L^{-1} KH_2PO_4 and 0.5 g L^{-1} $MgSO_4.7H_2O$). The results in figure (3) revealed that the pretreated sugarcane molasses supplemented with yeast extract as nitrogen source in addition to salts was suitable than the untreated molasses for kojic acid production by *A. oryzae* 124A. Generally, kojic acid production levels by the tested fungal isolate grown on sugarcane molasses under investigation was relatively low comparing to those levels produced by the same fungal isolate on synthetic medium of glucose or sucrose.

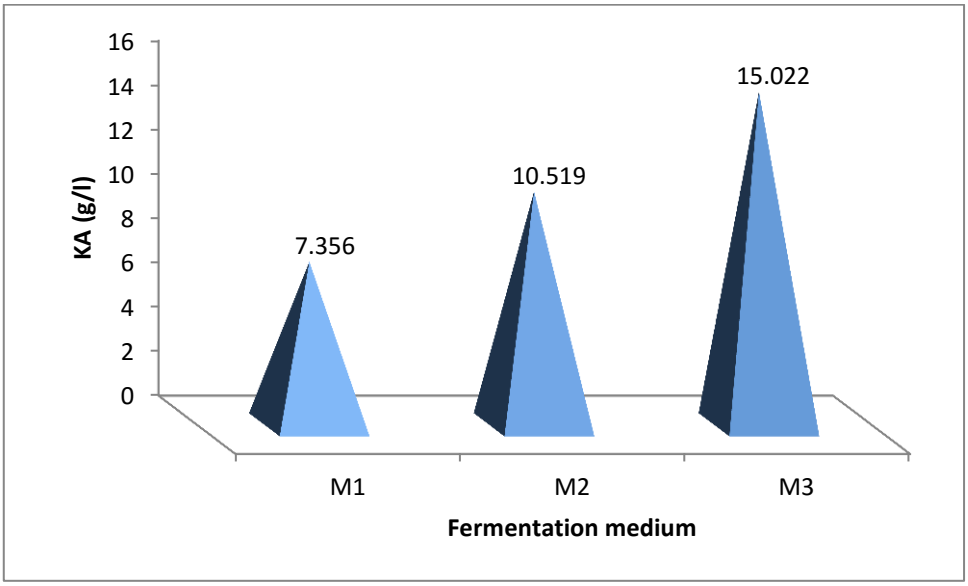

Fig. 3: Effect of Different Sugarcane Molasses Media on Kojic Acid Production by *A. Oryzae* 124A.

Sugarcane molasses as byproduct was relatively suitable substrate for kojic acid production by *A. oryzae* 124A. Therefore, the pretreated semisynthetic medium (M3) of sugarcane molasses was selected to determine the effect of cultural conditions on the efficiency of kojic acid production by *A. oryzae* 124A in order to maximize the produced amount of kojic acid by the tested isolate.

3.5. Optimization of kojic acid production from sugarcane molasses by *A. oryzae* 124A

3.5.1. Effect of pH on kojic acid production

The effect of initial pH value in molasses fermentation medium on the production of kojic acid from sugarcane molasses by *A. oryzae* 124A was studied and the obtained results were presented in figure (4). The optimum pH for kojic acid production by the tested isolate was 3.5. The production of kojic acid began to decrease above pH 3.5. Most studies conducted on the effects of culture pH towards the growth and production of kojic acid was based on the initial culture pH (Lin *et al.* 1976, Clevstrom & Ljunggren 1985). Several fungi like *A. oryzae* and *A. flavus* have the ability to produce kojic acid at pH range of 3 to 7 (Rosfarizan *et al.* 2000). Lin (2001) reported that pH optima for kojic acid production were 4.5and 6.5 for *A. flavus* and *A. oryzae* respectively. Lekha & Lomane (1997) reported that enzymes, being proteins, contain ionizable groups; consequently, the pH of the culture medium effects their structure and function. The optimal pH is very important to be determined since it can greatly affected the optimum production of enzymes required for kojic acid production. Filamentous fungi, generally, are characteristically tolerant to acidic pH and most of them have an optimum pH between 5 and 6 for cellular growth and several metabolic activities (Rosfarizan *et al.* 2000). High pH may lead to the growth of microorganism or inhibition of enzymes activities responsible for the biosynthesis of kojic acid.

3.5.2. Effect of incubation temperature on kojic acid production

Kojic acid production from sugarcane molasses by the selected isolate was effected by the incubation temperature and it was noticed from the data that *A. oryzae* 124A was able to grow considerable well and produce kojic acid within a temperature range from 20°C to 35°C (figure 5). The optimum incubation temperature for kojic acid production was recorded at 28°C. The optimum temperature for kojic acid production by fungi indifferent studies was found to be 25 - 30°C (Futamura *et al.* 2001, Lin 2001, Gad 2003, El-Kady *et al.* 2014).

3.5.3. Effect of sugar concentration on kojic acid production

Data presented in figure (6) showed that the concentration of kojic acid increased along with the increase in molasses sugar concentration and reached the maximum kojic acid (19.930 g/l) at 5 % sugar. Further increasing in sugar molasses concentration resulted in decreasing the produced kojic acid concentration. Gad (2003) studied the effect of beet molasses concentration on kojic acid production by *A. parasiticus*. Some molasses sucrose during sugar processing is hydrolyzed into reducing sugar glucose and fructose in sugarcane molasses which converted to kojic acid. Above a critical substrate concentration, a decreased water activity and onset of plasmolysis combine to cause a decrease in the rates of fermentation (Roukas 1993). Increasing initial sugar concentration resulted in a significant increase in residual sugar, which may be due to inability of the microorganisms to metabolize high levels of sugar. The osmotic pressure apparently had an unfavorable effect since the production of the acid dropped off sharply. These results agreed exactly with the early results found by May *et al.* (1931). Kojic acid synthesis only started after growth reached stationary phase and stopped when glucose in the medium was depleted (Kitada *et al.* 1967, Megalla *et al.*1985, Rosfarizan *et al.* 1998, Futamura *et al.* 2001). Rosfarizan& Ariff (2000) and El-Aasar (2006) reported that the excess concentration of carbon source affects the production of kojic acid and resulted in significant

increase in residual sugar due to the inability of fungi to metabolize high levels of sugar.

3.5.4. Effect of incubation time on kojic acid production

Influence of incubation time on kojic acid formation from sugarcane molasses by *A. oryzae* 124A was studied and the recorded results in figure (7). Data showed that the formation of kojic acid gradually increased by increasing the incubation time of the culture until reached the maximum value (29.431 g/l) at 19 days then decreased. These results are in harmony with that previously obtained by Ariff *et al.* (1997), El-Kady *et al.* (2014) and El-Assar (2006). The reduction of kojic acid with extension of incubation time may be attributed to degradation of kojic acid to oxalic and acetic acid by the mycelium under glucose depleted conditions (Bajpai 1982, Mohamed & Ariff 2007).

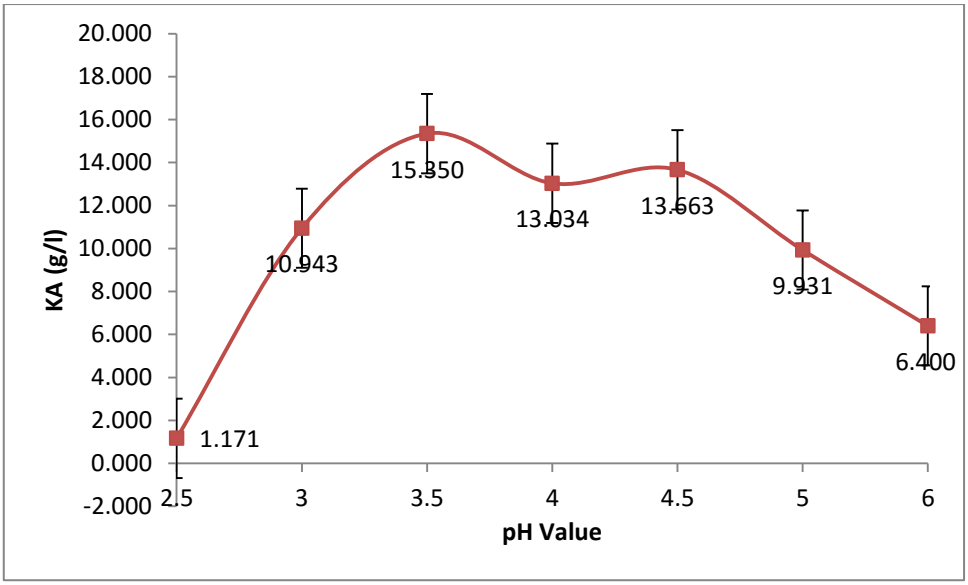

Fig. 4: Effect of Different Initial pH Values on Kojic Acid Production from Sugarcane Molasses by *A. Oryzae* 124A.

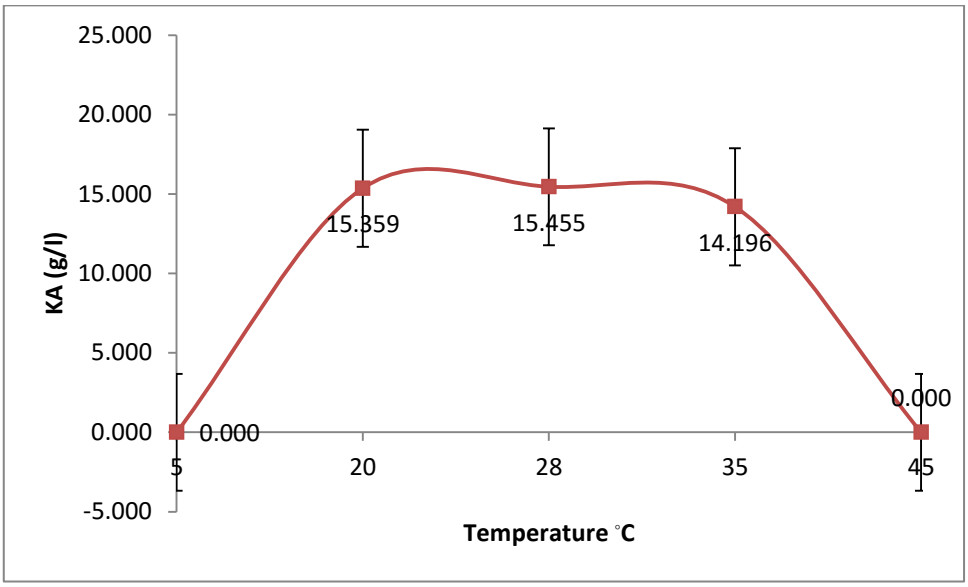

Fig. 5: Effect of Different Incubation Temperatures on Kojic Acid Production from Sugarcane Molasses by *A. Oryzae* 124A.

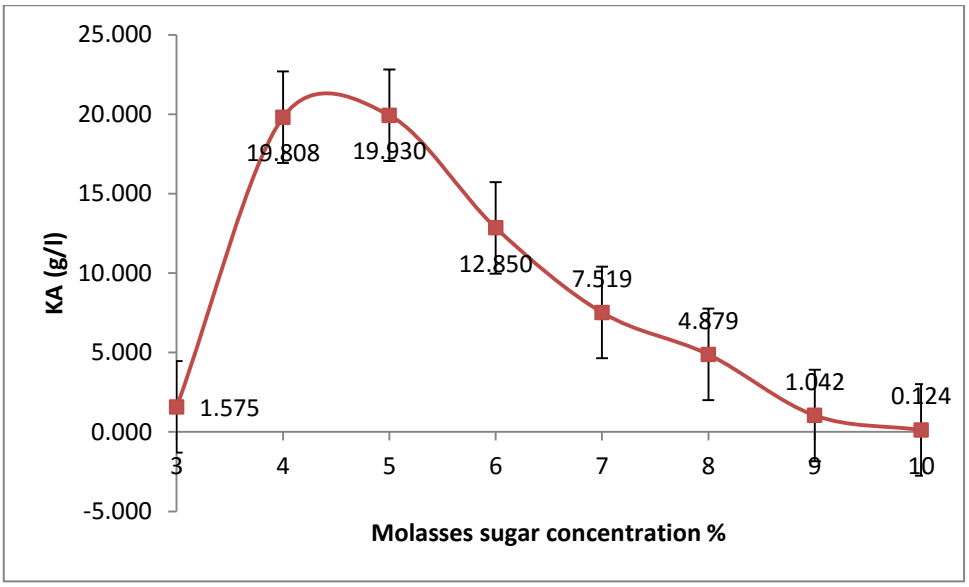

Fig. 6: Effect of Different Molasses Sugar Concentrations on Kojic Acid Production from Sugarcane Molasses by *A. Oryzae* 124A.

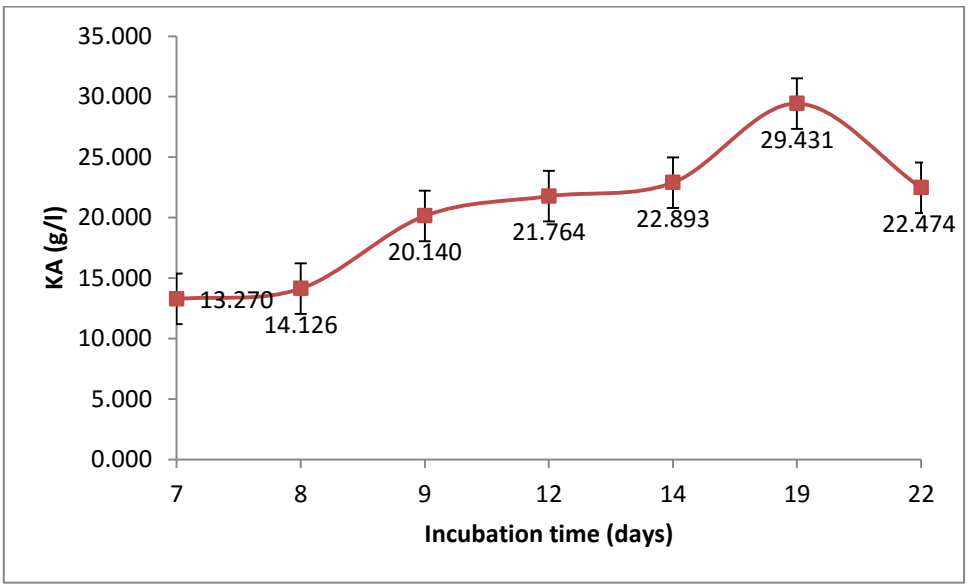

Fig. 7: Effect of Different Incubation Times on Kojic Acid Production from Sugarcane Molasses by *A. Oryzae* 124A.

This study explained that optimal fermentation conditions for kojic acid production by *A. oryzae* 124A were 5 % sugarcane molasses supplemented by 5.0 g/L yeast extract, 1.5 g/L KH_2PO_4 and 0.5 g/l $MgSO_4$ substrate and nutrients. The optimal pH, temperature, and incubation period as environmental conditions were pH 3.5, 28 ◦C, and 19 days, respectively. These results are completely similar to those recorded by several investigators (Arrif *et al.* 1997, Rosfarizan & Ariff 2000, El-Kady *et al.* 2014).

4. Conclusion

Aspergillus oryzae 124A is very active and promising fungal strain for production of kojic acid from agro-industrial wastes such as sugarcane molasses and this will help in recycling the wastes by production of commercial products.

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