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Isolation of citric acid-producing *Aspergillus niger* from soil and organic wastes

Fitri Ab Aziz Zakry[1,3*], Nurul Shaffee Syahidah[1], Masnindah Malahubban[2] and Pau Loke Show[4]

[1]Department of Crop Science, Faculty of Agricultural Science and Forestry, Universiti Putra Malaysia Bintulu Campus, Jalan Nyabau, 97008 Bintulu, Sarawak, Malaysia, [2]Department of Animal Science and Fishery, Faculty of Agricultural Science and Forestry, Universiti Putra Malaysia Bintulu Campus, Jalan Nyabau, 97008 Bintulu, Sarawak, Malaysia, [3]Institut Ekosains Borneo, Universiti Putra Malaysia Bintulu Campus, Jalan Nyabau, 97008 Bintulu, Sarawak, Malaysia, [4]Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus, Jalan Broga, Semenyih 43500, Selangor Darul Ehsan, Malaysia.

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***Corresponding Author:**
Fitri Ab Aziz Zakry,
Email: zakryfitri@upm.edu.my

ABSTRACT

Introduction of new *Aspergillus niger* strains that are more productive than those currently in use is one of the important steps in promoting more effective commercial citric acid production. The present study was conducted to isolate and characterise indigenous *A. niger* from selected soil and organic wastes such as soil with buried bamboo, soil with bamboo on the surface, soil under cattle grazing, mango orchard soil, rotting plum fruit and rotting bread. Morphological identification of *A. niger* was based on the length and width of the conidiophores, vesicles, phialides, and spores. Citric acid-producing *Aspergillus* isolates were screened based on the citric acid production index. The present study found that the highest fungal spore counts ($3.35\pm0.15 \times 10^7$ spores/g sample) were obtained from soil under cattle grazing, as were the highest *A. niger* counts ($7.25\pm0.05 \times 10^6$ spores/g sample). The lowest total fungal counts came from rotting plum fruit ($4.70\pm0.10 \times 10^5$ spores/g sample). A total of 14 isolates were collected, with five (NSA03, NSA06, NSA09, NSA12, and NSA14) showing morphological similarities with the reference isolate, *A. niger* Tiegh. All isolates were able to produce citric acid, but with varying efficiencies according to their citric acid production indices. The soil under cattle grazing area found to be the best site for sampling and isolation for citric acid-producing *A. niger* by using Czapek-Dox as a medium of isolation.

KEYWORDS: *Aspergillus niger*, citric acid, fungal morphology, organic waste, soil fungi.

INTRODUCTION

Citric acid is an ingredient found in processed foods, beverages and in medicinal and skin care products, reflecting its wide use in various major industries. While the demand for citric acid worldwide is increasing [1], supplies remain limited. Natural supplies of citric acid are supplemented by biotechnological fermentation processes, with about 80% produced through submerged fermentation by *A. niger* [2,3].

Aspergillus niger is a filamentous fungus that has maintained its position as the organism of choice for citric acid fermentation. *A. niger* can be easily found and isolated from various sources such as from soils [4], as well as rotting fruits and vegetables [5]. The fungus has the ability to thrive amidst a temperature range between 6ºC to 55ºC and it withstands low humidity well [6]. Such characteristics enable this species to be practically omnipresent. In addition, *Aspergillus* spores that are commonly found in the air can be dispersed over long distances [7].

Basic morphological identification of *Aspergillus* spp. is based on macroscopic and microscopic characteristics such as colony diameter, conidial colour, conidiophores, vesicles, metulae, phialides, and conidia [8]. Besides morphology, *Aspergillus* species have also been defined based on molecular and extrolite data in recent years [8]. *A. niger* can be described as having long, thread-like hyphae and does not show any crystal structure within the fungal pellet. The fungal mycelium aggregates and develops as beads [9].

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The speciality of *A. niger*, discovered by James Currie in 1916, is its ability to produce large amounts of citric acid, positioning it as the most widely used microorganism for citric acid production [10]. *A. niger* produces 0.131% m/v citric acid, this being the highest among known organisms, from a relatively low biomass [10]. Other characteristics that have rendered *A. niger* the microorganism of choice for this purpose lies in its ease of handling and ability to ferment various cheap substrates to obtain high yields [11].

To obtain optimum yields of citric acid, many factors have been considered such as the strain of *A. niger*, initial fermentation pH, source of carbon, initial concentration of sugar, temperature and the presence of other metal elements in the fermentation process [5] (Lee *et al.*, 2015). These variables have been tested by their adjustment under culture, with factors such as agitation and aeration, pH, and the concentrations of important trace metals and inoculum level being varied [12]. Lee *et al.* [5] found sucrose to be an important carbon source in submerged fermentation with citric acid measured at pH 2. Similar findings were also reported by Chirova *et al*. [13]. It is also important to note that the cell growth and consequent metabolic activity of a pelleted suspension occur mainly at the culture medium surface where there is access to adequate oxygen and nutrients [12].

With little information available on indigenous citric acid-producing *A. niger* strains in Malaysia, procuring the best *A. niger* isolates for citric acid production can be expected to be challenging, especially at the isolation and screening steps. The aims of the present study were to isolate and morphologically characterise indigenous isolates of *A. niger* from soils and agricultural wastes for citric acid production.

MATERIALS AND METHODS

Collection of samples for the isolation of *Aspergillus niger* was from various sites in the Universiti Putra Malaysia Bintulu Sarawak Campus. These included rhizospheric soil under the tree canopy of a mango orchard (3°12’33.60”N, 113° 4’53.80”E), soil under cattle grazing (3°12’35.14”N, 113°04’58.65”E), and rhizospheric soil covered with leaf litter under bamboo plants (3°12’20.80”N, 113°05’23.70”E). Rejected blemished fruits from fresh market in Bintulu town of Sarawak, Malaysia and bread which had been exposed to air at ambient temperature were other sources of *A. niger.*

The direct sampling method was employed on soil samples such as the rhizospheric soil from a mango orchard and soil under cattle grazing. The baiting method was used for soil samples under bamboo plants. The baiting technique was conducted by placing banana-jackfruit mixtures (1:1, v/v) pre-treated with molasses on the soil surface or buried in about five cm of soil. After five days, the rotting fruit mixture on the soil surface was removed and the soil under it was collected by a steel soil sampling tube with a sharp end. The surface over which the sample was collected was classified as a ‘treated soil surface,’ while the sub-surface soil was classified as ‘soil treated with buried waste’. Plum fruit and bread in plastic containers were left incubated at room temperature for several days and observed for fungal spore emergence. The sources of sampling and isolation were eventually classified as (1) soil treated with buried waste, (2) treated soil surface, (3) rotting bread, (4) soil under cattle grazing, (5) mango orchard soil, and (6) rotting plum fruit.

Rotting fruit and bread were cut into small pieces. A total of 10 g of each sample (fruit, bread or soil) was placed in a 250 mL Erlenmeyer flask, made up to 100 mL with double distilled water and then subjected to ten-fold serial dilutions. The media used to determine total fungal count were potato dextrose agar (PDA) and Czapek-Dox agar (CzDA), and the isolates were plated with spread plate technique. Of the two media, the latter has been reported to be especially suited to *A. niger* [2]. After 96 hours incubation, the plates were visually observed for *Aspergillus*-like mycelia that were then aseptically collected and transferred on to fresh agar plates of PDA and CzDA. Subsequently, the inoculated plates were incubated for up to 96 hours before further examination for culture consistency and contamination. Subsequent subculture was undertaken until the fungal cultures were deemed purified.

Morphological characterization of the fungal cultures was based on macroscopic and microscopic observations. A compound microscope fitted with a digital colour camera (Leica Microsystem, USA) was used in microscopic observations. Examination of fungal macro and microconidia characteristics was made by pressing a piece of Scotch tape onto the surface of the culture and staining with lactophenol cotton blue. Morphological differences that were recorded included the length of conidiophores, vesicles, metulae, phialides, and conidia. In this study, *A. niger* var. Tiegh [14] was used as the reference strain for morphological measurements with newly isolates. This strain is a widespread fungus can be found in soil environment specifically in soil-plant ecosystem [15].

CzDA medium was supplemented with methyl red as an indicator for acid production, changing in colour from yellowish orange to pink (acid). This simple and rapid technique, modified from Bose *et al*. [16], was used to screen for the fungal isolates that were the most promising in producing citric acid. An index to measure the capacity of fungal isolates to produce citric acid was formulated using as variables the colony diameter after two days and colour-change (pink) diameter surrounding the colony:

$$\text{Citric acid production index} = \frac{\text{colony diameter} + \text{diameter of colour change zone}}{\text{colony diameter}}$$

Analyses of variance (ANOVA) were carried out on measurements on the morphology and citric acid screening parameters. The Duncan’s Multiple Range Test (DMRT) was applied to determine differences between means at the 1% significance level. Statistical procedures were conducted using the Statistical Analysis System (SAS), version 9.3.

RESULTS AND DISCUSSION

Aspergilli are generally found in co-habitation with humans, animals and plants. Several species of Aspergilli are good and beneficial, especially *A. niger* which is extensively used for commercial and industrial purposes, besides several strains

that are important in human health. In the present study, 14 fungal isolates with black mycelia, and morphologically defined as *A. niger,* were collected from different sources such as farm soils and rotting fruits. These were coded NSA01 to NSA14 (Table 1). The highest total fungal counts were obtained from soil under cattle grazing (335.00±15.00 x 10^5 spores/g from isolate NSA08). The *A. niger* fungal counts were in the range of 18 to 725 x 10^4 spores/g substrate with isolate NSA08 showing the highest (725 x 10^4 spores/g soil) and isolate NSA06 the lowest at 18 x 10^4 spores/g rotting bread grown on CzDA. The 14 isolates obtained in the present study were relatively low in number as compared with those from several reports where several hundred Aspergilli isolates were obtained. For example, Qi *et al.* [17] successfully isolated 253 black Aspergilli from vineyards in several attempts from 2012 to 2014. The sparse collection in this study could be due to low sampling frequency attempted. The present study also found that samples from the natural environment yielded higher spore counts than ex-situ sources such as stored rotting bread and fruit. This phenomenon might be due to natural exposure and interaction of organic substrates (such as soil under cattle grazing in this case) to natural microflora which is denser and more diverse as compared with other substrates that are usually limited in space and resources for microbial growth [18]. Moreover, the population of *A. niger* that was higher from soil under cattle grazing than from several sources might be associated with the presence of cattle manure which contains nutrients, organic matter and microflora that attract microorganisms including Aspergilli [19]. According to Rakkar *et al.* [20], grazing improves soil properties, including the biomass of most microbial groups present in the soil.

Table 2 shows the morphological differences among the *A. niger* isolates. Five morphological parameters were measured, namely conidiophore length, conidiophore width, vesicle diameter, phialide radius, and spore diameter. Morphological data of *Aspergillus niger* var. Tiegh [14] were used as the reference strain in this study for comparison. Some reports have suggested that the morphology of citric acid-producing microorganisms is related to the control and augmentation of citric acid formation [21,22].

Of the 14 isolates, five isolates were morphologically similar to the reference strain. These were NSA03, NSA06, NSA09, NSA12, and NSA14, where three out of five morphological parameters were within the measurement range of the reference strain shown in Table 2. Nevertheless, these isolates were not found to be significantly different in several parameters when compared with the remaining isolates that were dissimilar from the reference isolate *A. niger* var. Tiegh. Accordingly, seven isolates, namely NSA04, NSA06, NSA07, NSA09, NSA10, NSA12, and NSA14, were similar in four out of five parameters studied. In this regard, NSA03 did not fit into this group despite being linked to the reference isolate. NSA03 was similar in only three parameters when compared with those in the group of seven. The group of seven *A. niger* isolates could be divided into two sub-groups based on morphology – specifically the vesicle diameter and the conidiophore length. The vesicle diameters of one sub-group comprising NSA04 (26.81±2.78 µm), NSA07 (20.46±1.69 µm), NSA09 (18.65±1.47 µm), NSA12 (18.65±1.22 µm), and NSA14 (17.22±2.46 µm) were significantly lower than that of *A. niger* var Tiegh that ranged between 55.00 and 75.00 µm (Table 2). The conidiophore lengths of another sub-group comprising NSA06 (1.87±0.07 mm) and NSA10 (1.73±0.14 mm) were much greater than that of *A. niger* var Tiegh, which reached only 0.74 mm. It should be noted that such observed morphological differences among the *A. niger* isolates were not necessarily controlled genetically but could be due to the influence of habitat and environment. Hence, *A. niger* might change morphologically according to the local environment, this being a survival mechanism that would allow the fungus to adapt to the prevailing surroundings to survive in diverse environments [23]. Alternatively, the morphological differences among *A. niger* in this study might also indicate that strain diversity had indeed been induced by mutational changes in response to the environment [24].

After a two-day incubation, the capacity to produce citric acid by *A. niger* isolates was assessed from the citric acid production index, based on the diameter of the colour change zone caused by citric acid on the agar plate and the diameter of the fungal colony (Table 3). Since efficient acid-producing candidates

Table 1: Total fungi and *Aspergillus niger* collected from different locations and grown on PDA and Czapek media

Source of isolation	Media	Total Fungi ×10^5 spores/g sample	Isolate code	*Aspergillus niger.* ×10^4 spores/g sample
Soil treated with buried waste	Czapek	33.50±1.50^{cde}	NSA01	610.00±10.00^{c}
	Czapek		NSA02	26.00±2.00^{fgh}
	PDA	65.00±1.00^{b}	NSA03	21.50±0.50^{gh}
Treated soil surface	Czapek	50.50±1.50^{bc}		
	PDA	7.85±0.95^{f}	NSA04	32.00±2.00^{fgh}
Rotting bread	Czapek	35.00±1.00^{cd}	NSA05	20.00±3.00^{gh}
	Czapek		NSA06	18.00±1.00^{h}
	PDA	31.50±3.50^{cde}	NSA07	38.50±0.50^{efg}
Soil under cattle grazing	Czapek	335.00±15.00^{a}	NSA08	725.00±5.00^{a}
	PDA	18.00±2.00^{def}	NSA09	54.50±2.50^{de}
Mango orchard soil	Czapek	32.50±3.50^{cde}	NSA10	26.50±0.50^{fgh}
	PDA	44.50±0.50^{bc}		
Rotting plum fruit	Czapek	4.70±0.10^{f}	NSA11	700.00±10.00^{b}
	PDA		NSA12	21.50±1.50^{gh}
	PDA	11.60±1.40^{ef}	NSA13	43.50±1.50^{ef}
	PDA		NSA14	65.00±1.00^{d}

Values in columns not followed by the same letter are significantly different according DMRT ($p<0.01$)

Table 2: Morphological comparison of indigenous *Aspergillus niger* isolated from different sources and conditions with the reference strain, *A. niger* var. Tiegh [14]

Isolate code	Conidiophore length (mm)	Conidiophore width (μm)	Vesicle diameter (μm)	Phialide radius (μm)	Spore diameter (μm)
Reference strain	Up to 0.74	10.00-14.00	55.00-75.00	5.00-13.80	3.70-4.50
NSA01	1.52±0.03[bc]	17.03±0.47[ab]	73.57±2.23[a]	32.78±3.74[a]	7.78±0.73[a]
NSA02	1.32±0.09[c]	17.36±1.20[ab]	40.21±1.92[de]	22.51±2.43[bcd]	5.02±0.28[b]
NSA03	1.36±0.04[c]	13.72±0.93[bc]	41.98±3.42[de]	12.67±1.76[d]	4.40±0.24[bc]
NSA04	0.58±0.02[d]	15.38±0.87[bc]	26.81±2.78[fg]	17.19±0.80[cd]	4.21±0.09[bc]
NSA05	1.87±0.12[a]	17.80±0.62[ab]	68.51±3.53[ab]	30.33±1.98[ab]	4.25±0.39[bc]
NSA06	1.87±0.07[a]	15.62±0.69[bc]	57.95±3.14[bc]	13.72±2.74[d]	4.15±0.27[bc]
NSA07	0.57±0.04[d]	14.85±0.98[bc]	20.46 ±1.69[gh]	13.24±1.11[d]	4.02±0.25[bc]
NSA08	1.72±0.10[ab]	16.96±1.61[ab]	32.53±2.40[ef]	20.40±1.70[bcd]	3.94±0.45[bc]
NSA09	0.61±0.05[d]	13.77±0.20[bc]	18.65±1.47[gh]	13.06±1.17[d]	3.09±0.26[c]
NSA10	1.73±0.14[ab]	18.04±1.50[ab]	48.21±4.28[cd]	15.14±2.27[d]	3.36±0.18[c]
NSA11	1.79±0.11[ab]	20.89±0.93[a]	64.81±5.09 [ab]	27.02±3.20[abc]	3.72±0.21[bc]
NSA12	0.70±0.06[d]	11.73±0.20[c]	18.65±1.22[gh]	19.84±5.02[cd]	4.22±0.31[bc]
NSA13	1.68±0.04[ab]	14.75±0.57[bc]	15.25±0.60[h]	11.76±0.47[d]	3.73±0.21[bc]
NSA14	0.66±0.02[d]	13.74±0.89[bc]	17.22±2.46[gh]	12.34±1.05[d]	3.36±0.18[c]

Values in columns not followed by the same letter are significantly different according to DMRT ($p<0.05$)

Table 3: Isolates of *Aspergillus* spp. screened for citric acid production

Isolate code	Diameter of citric acid-induced colour zone (cm±SE)	Diameter of fungal colony (cm±SE)	Citric acid production index±SE
NSA01	4.04±0.19[cde]	0.65±0.00[h]	7.35±0.35[a]
NSA02	4.42±0.19[bcde]	0.65±0.00[h]	7.81±0.29[a]
NSA03	2.18±0.33[gh]	1.98±0.27[fg]	2.10±0.10[ef]
NSA04	3.53±0.29[ef]	5.05±0.17[a]	1.70±0.05[f]
NSA05	5.50±0.12[a]	2.73±0.24[ef]	3.07±0.21[cd]
NSA06	4.66±0.15[abcd]	1.15±0.03[h]	5.07±0.22[b]
NSA07	2.76±0.49[fg]	5.45±0.25[a]	1.51±0.10[f]
NSA08	5.29±0.24[ab]	2.25±0.13[f]	3.38±0.20[c]
NSA09	3.98±0.21[de]	4.93±0.23[ab]	1.82±0.07[f]
NSA10	4.91±0.22[abcd]	1.31±0.06[gh]	4.75±0.17[b]
NSA11	5.04±0.19[abc]	3.31±0.24[de]	2.55±0.15[de]
NSA12	1.74±0.32[h]	3.83±0.35[cd]	1.48±0.12[f]
NSA13	1.65±0.13[h]	3.48±0.26[cde]	1.49±0.06[f]
NSA14	4.24±0.12[cde]	4.20±0.21[bc]	2.01±0.04[ef]

Values in columns not followed by the same letter are significantly different according to DMRT ($p<0.01$)

are important in citric acid fermentation [24], this simple measurement was used to screen the collection of *A. niger* isolates for their capacity to produce citric acid.

In future studies, these collections would be subjected to further detailed examination on several variables related to citric acid productivity such as the effect of aeration, carbon source, nitrogen and phosphate, pH, and trace elements. In the present study, all the isolates were found to be able to produce citric acid at varying rates, with the colour zone diameter ranging from 1.65 cm to 5.50 cm, and citric acid production indices in the range of 1.48 to 7.81. *A. niger* isolate NSA05 recorded the largest colour zone diameter at 5.50±0.12 cm while the smallest was seen in isolate NSA13. The colour zone diameter exhibited by NSA05 in the present study was much wider than as reported by Sutradhar *et al.* [25] who recorded much lower diameter at 15 mm or 1.5 cm. However, the study conducted by Sutradhar *et al.* [25] which collected only a single strain of *A. niger* from laboratory collection, therefore, the observation on citric acid production capacity potentially limited.

All the *A. niger* isolates varied in colony size. Greater colony size may be important that cause for *A. niger* to have greater capacity to produce citric acid [26]. Different in colony size might be due to that *A. niger* isolates were collected from different isolation sources which contained different media or samples composition [27]. In terms of the citric acid production index, *A. niger* isolates NSA01 and NSA02 were the best performers, with indices of 7.35 ± 0.35 and 7.81±0.29, respectively. On the other hand, the lowest citric acid producer was isolate NSA12, at 1.48±0.12. It should be reiterated here that the ability of *A. niger* to produce citric acid is widespread, and it remains the fungus' greatest asset for citric acid production on an industrial scale in view of its amenity to handling and ability to ferment various raw materials to achieve high yield [28].

CONCLUSION

The total number of fungi recovered from cattle-farm-soil and cultured on Czapek medium was the highest among the various sources compared in this study. The isolates were identified as *A. niger*, although morphological differences were observed among them. Five isolates, namely NSA03, NSA06, NSA09, NSA12, and NSA14 were morphologically most similar with reference strain, *A. niger* var Tiegh.

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