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STUDY OF THE OPTIMAL NUTRITIONAL CONDITIONS FOR THE PRODUCTION OF KOJIC ACID FROM THE LOCAL ISOLATION OF THE *ASPERGILLUS ORYZAE*

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Abstract

The cultivation conditions for respective ability to produce kojic acid from local *Aspergillus oryzae* isolates were studied. The obtained results showed that the best cultivation medium to produce kojic acid from local *Aspergillus oryzae* isolates included a modified molasses medium with a concentration of 5% reduced sugars, 0.5% sodium nitrate, 4.5 pH, fertilization (3.2 × 107 spore/ml), 0.1% potassiumdihydrogin phosphate and 0.05% of magnesium hydroxide sulfate.

Key words : Kojic acid, nutritional weather, pH, *Aspergillus oryzae*, molasses.

Introduction

Kojic acid was known as an organic acid produced by some microorganisms (Saleh *et al*., 2011) during the fermentation (EL. Aasar, 2006), which represents the fungal fungi group, particularly *Aspergillus* spp, *Penicillum* spp and bacterial strains *Acetobuctor* spp (Nohynek *et al*., 2004; Zborowski *et al*., 2004). Kojic acid was discovered by the researcher Saito from Japan in 1907, isolated from the *Aspergillus oryzae*, which is grown on steamed rice called Koji therefore, called kojic acid, it is produced from animal sources, plant sources or by chemical manufacturing. Through microbial fermentation, due to the potential increase in production by manipulating genetic genes of the microorganism through genetic mutations or by improving the environmental conditions of the organism (Demain and Dana, 20007). In order to obtain the highest production of kojic acid and the lowest cost, cheap raw materials were used to develop microorganisms, such as agricultural residues and plant waste. It is known that food processing processes have residues and byproducts after manufacturing. These wastes are often rich in sugars because of their organic nature and it could be used in microbial ferments to produce vital products, including the production of organic acids such as kojic acid (Demain and Dana, 2007). One of the byproducts of the food industry is molasses which play a role in some microbial ferments as in the production of organic acids such as citric acid and kojic acid. The total world production of molasses reached 45 metric tons. This shows the size of the important economic role played by molasses in the production of organic acids (Abubuker, 2012). Due to the importance of kojic acid in various fields including food, industrial and cosmetic, the present study aimed to produce kojic acid with high efficiency from local isolates *Aspergillus oryzae* through identifying optimal nutritional conditions and genetic improvement to produce the acid from isolation.

Cultivation and environmental conditions affecting the production of kojic acid

Carbony source

Many different sources of carbon were used in the production of kojic acid and the optimal source variation depending on the type of mold and it is strain. A group of sugars were used to produce kojic acid from mold *A. oryzae*, such as glucose, maltose, fructose, sugar mulberries, zeolose acid, Cloconol, Clactosolinositol, Sorbitol and Alanyolin were the highest acid producers at the consumption of molasses and maltose and the less productive with the use of Ainocetol and Kalkutos (Katagiri and Kitahara, 1929), while the best acid production was from A. oryzae 124A by using sucrose which reached 32.1 g/l (Rasmey and Basha, 2016). Additionally, the highest yield of kojic acid reached 0.43 g/1g glucose when the glucose concentration was 60 g/lby using *A. parasiticus* (El-Aasar, 2006).

Nitrogen source

To produce kojic acid many sources of nitrogen were used, both organic and inorganic sources, and the source of nitrogen does not enter directly in the process of acid production as in the case of carbon source, the source of nitrogen has a key role in the construction of biomass. Kitada *et al*. (1967) indicates that nitrogen sources differ in their effect on acid production and that organic nitrogen sources are better than inorganic sources in acid production ferments due to their contain of nucleic acids, amino acids, vitamins and buffer systems. These sources include peptone and the yeast extract. Inorganic sources, such as ammonia, lead to a reduction in the pH of the medium because of its positive ammonia ion, which reduces the production of biomass.

Effect of pH

Bentley (2006) and Mohamad *et al*. (2010) pointed the need to pay attention to the effect of the pH of the cultivation medium, which is one of the most important factors affecting the growth of the molds producing the kojic acid in the medium of fermentation, producing acid in wide ranges of pH (ranging between 3-7). This range depends on the used mediums, the type of organism in addition to the method and type of fermentation used for production. Nurashikin *et al*. (2013) found that the highest yield of kojic acid was from *A. flavus* at pH 2.5. Moreover, the optimal production of kojic acid from *A. oryzae*when the pH was 4 (Hazzaa *et al*., 2013) as well as the optimal production of kojic acid from mold *A. parasiticus* was at pH of 5.5 (E-Aasar, 2006).

Materials and Methods

Four local fungal isolates of the genus *Aspergillu* spp and *Penicillium* spp were obtained as shown in table 1, which used fungal isolates to detect their ability to produce kojic acid.

Screening of fungal isolates

The isolates obtained on the PDB medium were developed in 300 ml. salts with 100 ml of nutritional medium and incubated at 30°C for 7 days. The liquid fungus was then filtered by a sterile filter unit (Whatman1 filter paper). The resulting leachate was used to detect kojic acid.

Table 1 : The used Microorganism in the research.

Mold name	The source
Aspergillus oryzae	Faculty of Agriculture, University of Baghdad
Aspergillus niger	Ministry of science and Technology, Department of Agriculture research
Aspergillus parasiticus	Ministry of science and Technology, Department of Agriculture research
Penicillium spp	Faculty of Agriculture/University of Baghdad

1. The initial screening of the fungal isolates was performed to determine the production of kojic acid by recording the resulting color (based on the consideration) of the reaction of 1 ml. of the fungal filtrate with 1 ml. of the ferric chloride which was prepared by dissolving 1 g of $FeCl_3.6H_2O$ in a certain volume of 0.1 of HCl and completed size to 100 ml (Bentley, 1957).

2. The second screening was based on the quantitative estimation according to the method (Bentley, 1957). By adding 1 ml of the fungal leachate to 1 ml. of ferric chloride solution and measuring the absorption of the spectrometer at a wavelength of 500 nm, pure kojic acid in quantitative determination of filtration (Mohamd *et al*., 2000).

Selection of the most efficient isolation

After the screening, the most efficient Isolation producing for kojic acid was selected that represented by the fungus *Aspergillus oryzae*, which was then physiologically filtered by UV rays and then used to study the optimal conditions for the production of kojic acid.

Preparation of the production medium

Use the medium of production described by ArIff *et al*. (1996) to produce kojic acid. This medium consists of 5% glucose, 1% potassium phosphate, 0.5% yeast extract and 0.1% potassium dihydrogin phosphate. After mixing the components, the pH was changed to 5 and the medium was distributed onto 300 ml flasks with 100 ml of the medium per flask. The medium was sterilized to 121°C for 15 minutes with the beater and cooled thereafter Vaccinated (2.57×10^7) and incubated at (30°C for 7) days.

Determination of kojic acid

The following four indicators were adopted for the estimation of kojic acid: quantitative estimation of kojic acid, estimation of residual sugars and the dry weight of mycelium and pH of the production medium to indicate increased acid production at each case studied and acid estimation by the above method.

Determination of reduced sugars

The reduced sugars were estimated at the medium of production in the manner described by Miller (1999) and based on the standard glucose curve as a reduced sugar.

Determination of dry weight

After each production process, the dry weight of the biomass was estimated by filtration of the fungus using the sterile filter unit (Whatman1 filter paper). The filter paper was weighed before drying after that weighed again with the mycelium and then placed in the oven at 95°C until the weight is stable then dry biomass was measured (Mohamad *et al*., 1998).

PH estimation

The pH of the production medium was read prior to incubation and after the end of the incubation process by a pH-meter device.

Preparation of molasses

The molasses were used after dilution. The molasses were diluted by using distilled water by 1: 4 (molasses to water) and 4 mL of sulfuric acid H2SO4 (N1) was added for each 100 mL diluted molasses. Then the boiling was done for 5 minutes, Left to the next day to observe the formation of a brown precipitate, which is eliminated by centrifugation at 5000 cycles/minute for 15 minutes, after that the precipitate is neglected and the filter content was estimated to be reduced sugars (Alaa and Ali, 2015).

Study of nutritional conditions for the production of kojic acid

A number of factors have been studied in the production of kojic acid. These factors included:

1. Effect of the source and concentration of carbon : Six carbon sources were tested including glucose, sucrose, molasses, wheat bran, grape residues and molasses at concentration of 5% for all sources using the above productive medium. The molasses were adopted as an optimal source of acid production based on the obtained results as well as the optimum concentration of molten molasses was determined. The molasses were diluted with distilled water to make different concentrations (2.5, 5, 7.5, 10, 12.5, 15%) reduced sugars. And then, supported with nutrient that previously mentioned to determine the optimum concentration of molasses in acid production and the primary pH setting of the media to (5) and then sterilized at 121°C for 15 minutes. Thereafter, fertilized with an active isolates at the age of 7 days with fertilizer volume of 2.57×10^7 spore/ml and then the sterile mediums were incubated at a temperature of 30°C for 7 days. At the seventh day after the end of incubation period, then liquid mediums was nominated and the pH of the production medium, the dry weight of the biomass and the concentration of the sugars were measured and the concentration of kojic acid was estimated.

2. Effect of the optimal source and concentration of nitrogen : To determine the optimal nitrogen source in the production of kojic acid, five nitrogen sources were tested. These sources were the yeast extract, peptone, ammonium nitrate, sodium nitrate and ammonium sulfate, all the sources were used at the concentration of 0.5%. The optimum concentration of sodium nitrate, which was the best nitrogen source based on the previous experiment, was determined and the tested concentrations were (0.1, 0.25, 0.5, 0.75, 1%). After that, pH was adjusted and the mediums were sterilized, fertilized and incubated for 7 days and thereafter, the dry weight of the biomass, the concentration of the reduced sugars and concentration of Kojic acid were estimated.

3. pH effect : The pH of the medium was tested (2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5) using 0.1 N of both hydrochloric acid and sodium hydroxide to modify the pH and the mediums were incubated at a temperature 30°C for 7 days. After the end of incubation period for the seventh day the liquid mediums were nominated and the pH of the production medium was measured. The dry weight of the biomass, the concentration of the reduced sugars, and the concentration of the kojic acid was measured to study the optimal pH in the acid production.

Results and Discussion

A comparison of the degree of redness resulting from the color interaction of the fungal filtrate with the ferric chloride solution showed that the fungal isolates *A. oryzae* were more productive of the kojic acid than the other selected fungal isolates(Table 2).

Table 2 : Molds producing kojic acid.

Type of microorganism	Production	Kojic acid concentration g/l
Aspergillus oryzae	++	8.01
Penicillium spp	+	4.53
Aspergillus parasiticus	-+	2.78
A. niger	-	0

The results obtained from the quantitative estimation of kojic acid (Table 2) showed that the *A.oryzae* was exceeded compared to the other fungal isolates, which reached 8.01 g/l while the acid concentration was 0, 2.78,4.53g/l for *Penicillium Parasiticus*, *Aspergillus* and *A. niger* respectively. The isolation of *A. niger* did not record any production of kojic acid and this corresponds to the obtained results of Tariq et al.,(2017) who detected the lack of productivity of the kojic acid from *A. niger* isolation.

Aspergillus oryzae isolates was the most efficient in the production of acid (8.01 g/L), while the amount of acid produced from the other isolates was varied. The yield of this isolate increased (13.56 g/L) after exposure to UV ray. *A. oryzae* was isolated in our present study due to it is highly acidic, free from the production of fungal toxins and its widespread use in Japanese fermented foods (Bentley, 2006).

Effect of carbon source in the production of kojic acid : Kojic acid was extracted from the isolation of *A. oryzae* using different types of carbonic sources and different concentrations in the liquid medium of the production, and through the results shown in fig. 1, it was found that the molasses gave the highest yield of acid compared to the other sources (57.272 g/l) and the percentage of reduced sugars 1.914(g/l) followed by grape waste with a production rate of 47.309 g/land reduced sugars 1.952 g/L. The rest of the sources were below this percentage. The sucrose was in terms of productivity by 35.709 g/l for kojic acid and (2.852 g/L) for the reduced sugars. The suitability of the molasses to produce the acid was due to its components that support the growth of mold, containing 8% inorganic content, 20% water, 10% non-sugary ingredients, related acids, nitrogenous substances, soluble and sugary substances about 62%. Fructose was 16%, sucrose 14% and glucose 32% (El-Aasar, 2006). In addition to the presence of certain vitamins such as riboflavin and thiamine (Khalifa, 2003), the presence of these compounds in molasses can be catalysts for the production of kojic acid (El-Kady *et al*., 2014).

Fig. 2 shows a variation in the dry weight of the mycelium when using different sources of carbon to produce acid. Mohammad and Ariff (2006) found that there is no significant change in the dry weight of mycelium for the acid-producing molds at different carbon sources used.

Fig. 2 showed the use of molasses at a concentration of 5%, the biomass was 4.23 g/L with a decrease in pH of 3.70, followed by grape residue of 3.75 g/l for biomass and pH of 3.75, while the lowest biomass obtained was 2.25 g/L when sucrose was used at pH of 4.40. Therefore, the molasses was selected to be the optimal source of carbon and different concentrations were tested included

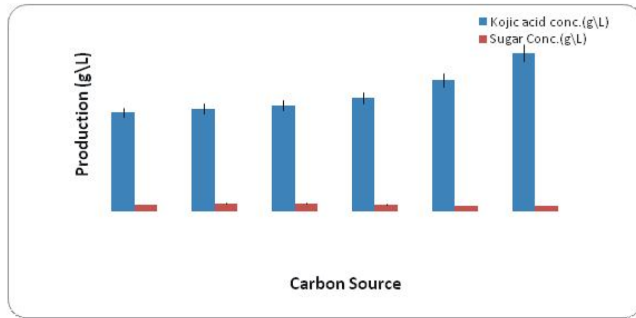

Fig. 1 : Effect of carbonic sources in the production of kojic acid from the isolation of *A. oryzae*.

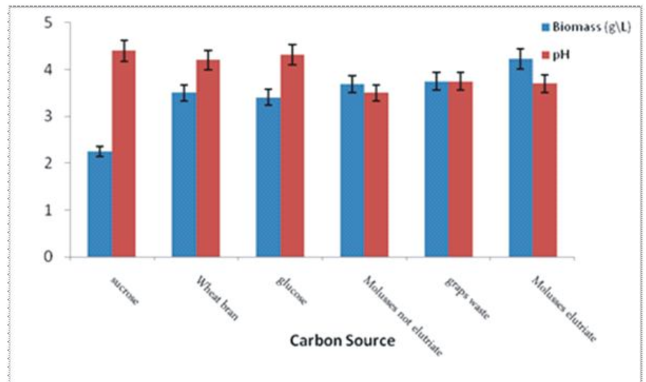

Fig. 2 : Effect of carbonic sources on dry mycelium weight and pH of isolation *A. oryzae*.

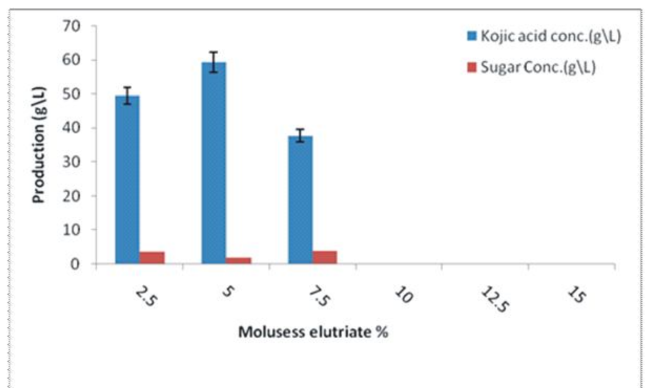

Fig. 3 : The effect of molasses on the production of kojic acid from isolates *A. oryzae.*

2, 5, 7.5, 10, 12.5 and 15% of reduced sugars in order to determine the optimal concentration. The results showed that the highest value of kojic acid was59.272 g/lat concentration of 5% from *A. oryzae* isolation and reduced sugars (1.899 g/l), while acid production decreased at the concentration of 7.5% recorded 37.363 g/l with reduced sugars value of 3.977 g/l (fig. 3).

These results agreed with the results of Rasmey and Basha (2016), who demonstrated that the production of kojic acid increased with increasing the concentration of molasses whereas the maximum production value of kojic acid was 19.930 g/L at 5% concentration of molasses by

A. parasiticus. Gad (2003) investigated the effect of increasing the concentration of molasses on the production of acid by *A. parasiticus*, which resulted in a decrease in the acid production, due to the Osmosis effect, which led to a decrease in water activity and plasma dislocation, resulting in low fermentation rates and then reduced concentration of acid produced (Roukas, 1993). Additionally, the excessive concentration of carbon source affects the production of kojic acid and resulted in an increase in the remaining sugars due to the inability of fungi to digest high levels of sugars (Rosfarizan and Ariff, 2000). In contrast, Hassan *et al*. (2014) showed that the best carbon source used in the kojic acid production from isolates *A.oryzae* was glucose. Glucose 5% comes fourth in terms of it is effect on the acid production from *A.oryzae* recorded mean of 38.363 g/L, which was similar to the result achieved when 5% of wheat bran was added and recorded value of 37.09 g/L. The pH of the molasses was reduced to 3.70 at a concentration of 5% and a biomass of 4.23 g/l. The highest pH value was 0.75 and the lowest biomass was 0.93 g/l.

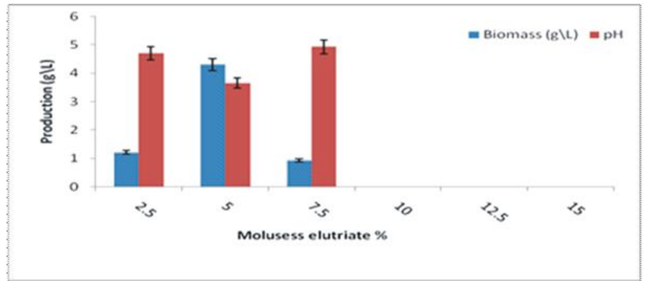

Fig. 4 : The effect of Molasses on the dry weight of mycelium and pH for isolates *A.oryzae.*

The effect of the nitrogen source

Fig. 5 showed that the highest production of kojic acid was 65.054 g/L and achieved when sodium nitrate was added while the yeast extract, peptone, and ammonium sulfate recorded means of 45.08, 42.02, and 33.51g/L of kojic acid, respectively. The sources of organic nitrogen in general better than the sources of inorganic nitrogen to produce kojic acid. It may contain complex organic nitrogen sources such as peptone extract and yeast vitamins, which act as precursors to produce kojic acid. In addition, some organic nitrogen sources have a good storage system (Kitada *et al*., 1967). For the remaining reduced sugars, the recorded values of kojic acid were 0.682, 0.780, and 1.08 g/l when sodium nitrate (5%), yeast extract, and ammonium nitrate were added.

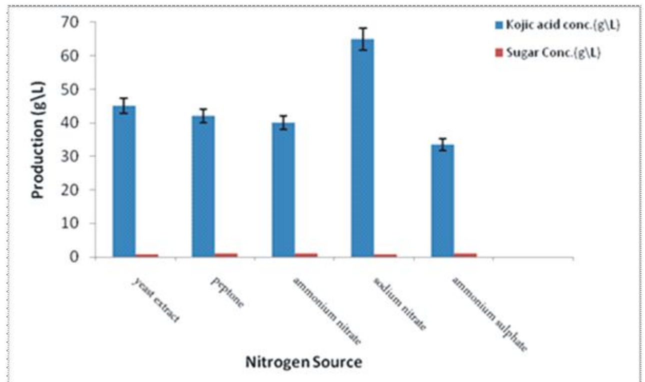

Fig. 5 : The effect nitrogen sources in the production of kojic acid from isolates *A. oryzae*.

Fig. 6 detected that sodium nitrate is the best source for the growth of mold *A. oryzae* given a biomass of 3.95 g/l while the yeast extract was given 2.97 g/L, and this is due to an increase in the concentration of sodium nitrate which led to increase the biomass so the concentration of the nitrogen source in the medium should be determined for the purpose of determining the growth in the biomass of the selected isolation and in turn will stimulate the consumption of carbon source on the production of acid and thus control the phenomenon of biomass increasing.

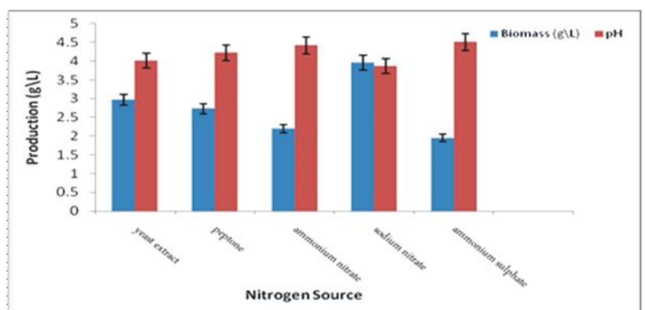

Fig. 6 : The effect of nitrogen sources on dry weight of Mycelium and pH for the isolates *A. oryzae*.

Mohmad *et al*. (2010) determined the need to control the growth of acid-producing molds by determining the concentration of the nitrogen source in the production medium and thus achieving a metabolic balance in favor of the process of kojic acid production. After production of kojic acid by using sodium nitrate (0.5%), medium pH was decreased to 3.87,whereasit was 5 before production. However, the pH was increased with the use of ammonium sulfate reaching 4.51 after production as well as the pH of the sodium nitrate reached 4.42 after production. Therefore, sodium nitrate was selected as the best nitrogen source of acid production and several concentrations were tested, which were limited between 0.1, 0.25, 0.5 and 0.75% to determine the optimum concentration. The results shown in Fig. 7 detected that the highest production of kojic acid was at the concentration of 0.5% with mean value of 67.25 g/l and 0.682 g/l for the reduced sugars. The current study showed that sodium nitrate ($NaNO_3$) gave the highest

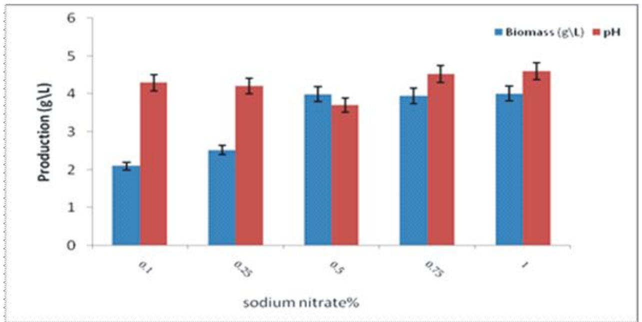

Fig. 7 : The effect of sodium nitrate on the production of kojic acid from isolates *A. oryzae*.

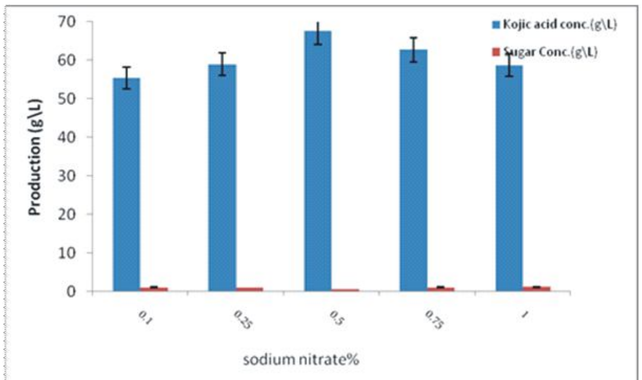

Fig. 8 : Effect of sodium nitrate concentrations in the dry mycelium of *A.oryzae* and pH.

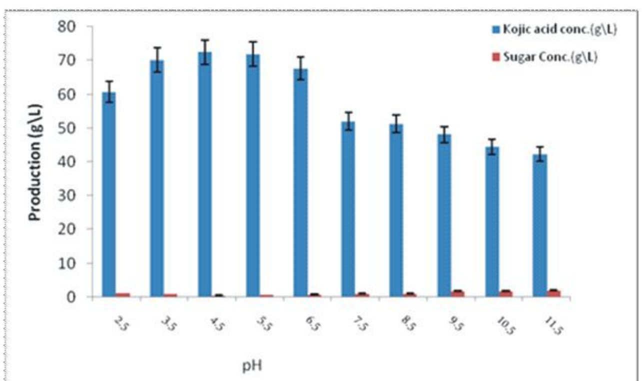

Fig. 9 : The effect of optimal pH on the production of kojic acid from isolates *A. oryzae*.

yield of kojic acid. Conversely, the production of kojic acid decreased when sodium nitrate was added as a nitrogen source (El-Aasa, 2006).

Fig. 8 revealed that post-production pH of sodium nitrate (0.5%) was decreased to 3.70 and gave a biomass of 3.99 g/l, while at concentration of 1.0% sodium nitrate pH recorded the highest value of 4.60 and 4.01 g/l for biomass.

The results obtained from this study indicated that the inorganic nitrogen sources (sodium nitrate) exceeded the organic sources. Therefore, sodium nitrate was selected as the best nitrogen source used in subsequent experiments.

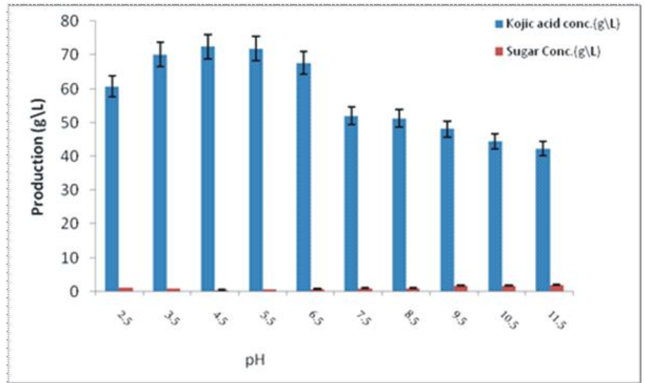

Fig. 10 : The effect of the optimal pH on the dry weight of mycelium for isolates *A. oryzae* and pH.

Effect of pH

The primary pH was studied in the production of kojic acid. Fig. 9 showed that the optimal pH in the production of kojic acid from the isolation of *A.oryzae* reached (4.5) and the amount of acid produced was 72.436 g/l with remained reduced sugars of 0.551 g/l. The optimal pH obtained from the present study was consistent with results of Lin *et al*. (1976), who found that the optimal pH was 4.5 in the production of kojic acid from mold *A.parasiticus*. Similarly, Hazzaa *et al*. (2013) detected that the ideal pH number for the production of kojic acid from *A. oryzae* was 4. On the other hand, the results obtained from this study were not consistent with other studies, where the highest conversion from glucose to kojic acid was obtained at very low pH (1.9- 3.0) (Clevstrom and Liunggren, 1985). While the optimal production was pH 5.0 - 7.0 (Rosfarizan *et al*., 2002; El-Aasar, 2006). Furthermore, at pH value of 4.5-6.0 the mold appears to be in growth stage which consist of using the enzymes to convert glucose to kojic acid (Rosfarizan *et al*., 2002) while at the pH of 2.0 to 3.0 the mold was constant where the kojic acid was produced (Rosfarizan *et al*., 2000). The difference in the results of the current study with the results of previous studies can be explained by the difference in the source of nitrogen and the carbon source. Rosfarizan *et al*. (2010) showed that the optimal pH for acid production depends on the type of nitrogen source and carbon source used in the cultivation medium.

As shown in fig. 10, pH had an effect on biomass with the highest biomass at pH 4.5 was 6.23 g/l, followed by pH value of 11.5 with biomass at 5.40 g/l , While the lowest biomass was obtained at pH 2.5 with biomass of 2.56 g/l. The process of acid production and quantification was influenced by the pH change of the medium used to produce the acid. Using a high pH may help the growth of the microorganism, inhibits the action of enzymes responsible for acid synthesis (El-Aasar, 2006). According to the results obtained from this study PH 4.5 was installed to produce the kojic acid of *A. oryzae* isolation and was used in all subsequent experiments.

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