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OPTIMIZATION OF CULTURE CONDITIONS FOR KOJIC ACID PRODUCTION IN SURFACE FERMENTATION BY *ASPERGILLUS ORYZAE* ISOLATED FROM WHEAT GRAINS

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Kojic acid is a natural organic acid synthesized during aerobic fermentation of carbohydrates as a secondary metabolite by some species of Aspergillus and has been used commercially in several industrial applications. The current investigation aims to optimize the culture conditions for kojic acid production from starch as a carbon source by the novel isolate Aspergillus oryzae 1034. Seventy-five isolates representing 11 species of Aspergillus were isolated from stored wheat grains and screened for kojic acid biosynthesis. Amongst, A. oryzae 1034 was selected as the most potent kojic acid producer from starch. This strain was subjected to different fermentation conditions to maximize the kojic acid production from starch versus glucose. The results concluded that glucose and starch substrates in concentrations 60 and 80 g/l, respectively were the optima for kojic acid production. The optimum phosphorus concentration was 0.5 and 2.0 g/l KH_2PO_4 in glucose and starch media, respectively. The maximum kojic acid yield was attained at 28ºC for 11 days of incubation in both glucose and starch media with pH 4.5 and 5.0, respectively. Supplementation of Pb^{+2} to glucose medium and Zn^{+2} to starch medium stimulated the biosynthesis of kojic acid to 79.3 and 68.8 g/l, respectively. Alternatively, kojic acid biosynthesis was decreased by supplementation of amino acids in the fermentation medium. These findings suggest the possibility of using A. oryzae 1034 as a promising.

INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is a natural organic acid was initially extracted from fermented steamed rice (known as "koji" in Japanese) in Japan by Saito in 1907[1]. Kojic acid is a secondary metabolite formed during aerobic fermentation of carbohydrates by several species of fungi as well as some bacterial strains of the two bacterial genera *Acetobacter* and *Brevibacterium*[2-4]. *Aspergillus* and *Penicillium* species are widely known among fungi as the best kojic acid producers[5]. Amongst the species of *Aspergillus*, *A. flavus*[6], *A. oryzae*[7], *A. tamarii*[8] and *A. parasiticus*[9] were regarded as the main kojic acid source in large amounts.

Nowadays, kojic acid has numerous industrial and biotechnological applications in food processing, chemicals, cosmetics and pharmaceuticals preparation[10]. For instance, kojic acid is now extensively used in cosmetic industries as a metal chelating agent due to its tyrosinase inhibition ability, thus it is used as a lotion for skin whitening and protection against the sunlight[11]. In the medicine, it is used as a pain killer, antibacterial against Gram-negative bacteria and anti-inflammatory drug[3&12]. In the food processing, it is used as a precursor of flavors and as a preserving material in agricultural products during storage, especially

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vegetables and fruits by inhibiting the polyphenol oxidase (PPO) action[13]. Kojic acid is documented in different studies as a precursor or an intermediate compound in chemicals production that can be used as therapeutic agents[3]. At the carbon 5 of pyrone ring, the hydroxyl group acts as a weak acid, make it is able to form salts with some metals like sodium, zinc, calcium, and cadmium[14].

Kojic acid has melting point ranges from 151-154°C and it crystallizes as colourless prismatic needles in water under cooling centrifugation[11]. It is characterized by its high solubility in water, ethanol and ethyl acetate while less soluble in ether and chloroform[15].

The apparently similar structure between the pyranose ring of glucose and the pyrone ring of kojic acid had paid the attention of researchers towards studying the pathways of its biosynthesis from glucose and other substrates. Some enzymes like glucose dehydrogenase, glucosen – 6-phosphate dehydrogenase, 6- phosphogluconate dehydrogenase, glucose oxidase and gluconate dehydrogenase have been detected in mycelial extracts of different isolates of *Aspergillus flavus* that gives kojic acid in large amounts. Also, kojic acid was detected with pentoses, glycerol, starch and dihydroxyacetone as substrates[5].

Though kojic acid has been produced industrially and applied in different applications, its production is still an interesting research area. The main subjects must be considered for enhancement of kojic acid biosynthesis are the strain development and the development of fermentation process. This could be attained either by manipulation of the microbial strains or optimization of the culture conditions[16&17]. Thus, the current research is aimed to investigate the ability of some *Aspergillus* species isolated from wheat grains to produce kojic acid in batch culture. Subsequently, the optimization of cultural conditions for kojic acid production from glucose and starch by the highly producer isolate was conducted.

MATERIALS AND METHODS

Isolation of *Aspergillus* species

Thirty samples of wheat grains stored under wet atmosphere were collected from different shops in Suez city, Egypt, for isolation of *Aspergillus* species. The isolation was performed by pour plate method onto Czapek's agar according to Christensen[18]. Modified Czapek's agar medium contained (g/l dist. H_2O): starch, 10.0; $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; and agar, 18.0. Chloroamphenicol (50 µg/ml) and rose bengal (10 µg/ml) were added to inhibit the growth of bacteria. The prepared medium was adjusted to pH 6.5 using HCl 0.1 N and NaOH 0.1 N. This medium was autoclaved at 121°C for 15 minutes. The inoculated plates were incubated at 28°C for 7 days. The growing *Aspergillus* colonies were picked up, transferred to agar slants and stored at 4°C. The results for each species in the sample were recorded as colony forming units (CFU).

Identification of *Aspergillus* species

The isolated *Aspergillus* species were characterized and identified to the genus and species level according to Moubasher[19], based on the studied microscopic and macroscopic morphological characters of their colonies and mycelia.

Preparation of spore suspension

The spore suspension was prepared by growing the fungus on potato dextrose agar (PDA) for 5 days and their spores were collected using 1% (v/v) of tween solution. The spore suspension concentration was adjusted to be 5×10^6 spores per one ml.

Fermentation process for kojic acid production

The used fermentation medium for production of kojic acid consists (g/L): soluble starch, 50; yeast extract, 5.0; KH_2PO_4, 1.5 and $MgSO_4$, 0.5. This medium was adjusted to pH 3.5 using HCl 0.1 N. Each 50 ml medium were sterilized at 121°C for 15 min and inoculated with one ml spores suspension (5×10^6 CFU/ml). The cultures were incubated at 28±2°C for 8 days as a static cultivation[3].

Assay of kojic acid

The formed kojic acid was determined in culture supernatant by a colorimetric method using ferric chloride ($FeCl_3$) reagent according to Bentley[20]. The reagent was prepared by dissolving one gram of $FeCl_3.6H_2O$ in 100 ml of 0.1 N HCl. The reaction between the functional group of hydroxyl and pyrone rings in the samples gives a deep red colour with the reagent. The absorbance of the mixture was measured at 500 nm using spectrophotometer. The kojic acid equivalent was determined from kojic acid standard curve.

Kojic acid fermentation from glucose and starch as carbon sources

Glucose and starch were individually supplemented to the basal fermentation medium in amount equivalent to 50 g/l of carbon. The cultures were incubated at 28±2°C for 8 days.

Optimized culture conditions for kojic acid production from glucose and starch by *A. oryzae* 1034

The influences of different culture conditions included substrate concentration, phosphorus concentration, initial pH value, incubation temperature, incubation period, amino acids concentration and heavy metals concentration on kojic acid biosynthesis in the fermentation medium were investigated. Effect of different glucose and starch concentrations (10-80 g/l) with 10 g/l interval in the fermentation medium was studied. Also, the effect of phosphorus concentration in the form of KH_2PO_4 (0.5-3.0 g/l) with 0.5 g/l interval was tested. To determine the optimum pH for kojic acid production, initial pH of the medium was adjusted to 2.5, 3.0, 4.0, 4.5, 5.0 and 6.0. The culture was incubated at different temperatures (5, 20, 28, 37, and 45°C) to determine the optimum incubation temperature. The incubation period was optimized by incubating the culture at different periods (5, 6, 7, 8, 9, 10, 11, 12, 13, 20). Effect of different amino acids (arginine, histidine, methionine, lysine and alanine) at 0.2 g/l was determined. In addition, different heavy metals (manganese chloride, nickel chloride, zinc chloride, lead nitrate, copper hydroxide) at 0.003 g/l were tested. At the end of each parameter, kojic acid was assayed.

Statistical analysis

The different experiments were achieved using one-way analysis of variance. The comparison of means was performed using Duncan's multiple range test for the difference between means at $p < 0.05$.

RESULTS AND DISCUSSION

Recently, the microbial source for production of significant commercial products was prevailed as an alternative way for the chemical methods, this is due to that microbial metabolites can be produced on large scale fermentation with low cost. Among these products, kojic acid could be produced in large amounts in simple fermentation process by different species of *Aspergillus*, particularly *A. oryzae*. The isolation and selection of new fungal strains from suitable substrates will help to increase the productivity. Also, the cost can be minimized by using suitable starting substrate. Therefore, this study has been performed to isolate a new strain of *Aspergillus* has the potentiality to produce kojic acid in large amount on starch in comparison with glucose as carbon sources in addition to optimize the culture conditions for production.

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

Eleven *Aspergillus* species were isolated and identified from 30 wheat grain samples collected from Suez city (Table 1). These species were *A. aculateus, A. carneus, A. flavus, A. oryzae, A. fumigatus, A. niger, A. ochraceous, A. parasiticus, A. sydowii, A. terreus* and *A. ustus*. *A. flavus*, *A. fumigatus* and *A. niger* were the highest occurrence three species followed by *A. terreus* with moderate occurrence while the rest species were recorded as rare occurrence. In a similar study[3], they reported that six species of the genus *Aspergillus* were isolated from wheat grains and the most predominant species was *A. flavus*. These data are similar with Soliman[21], who mentioned that the genus *Aspergillus* was the predominant in the collected wheat grains. Also, different recent studies were conducted on the associated fungi of stored grains and reported that *Aspergillus* was among the highest occurrence fungal genera on these grains[3&21-25]. The associated fungi on cereal grains might be come from different ways such as dust from air, soil, harvesting, transportation and storage[26].

Table 1: Isolation and screening of kojic acid production by *Aspergillus* species from 30 samples of stored wheat grains collected from Suez city.

Fungal species	CFU*/ g wheat grains	CFU%	NCI** (out of 30 samples)	OR***	Kojic Acid (KA) detection		
					Tested isolates	+ve isolates	-ve isolates
Aspergillus aculateus	18	0.91	1	R	6	2	4
A. carneus	2	0.10	1	R	2	0	2
A. flavus	673.3	34.06	19	H	16	10	6
A. oryze	8	0.40	2	R	8	8	0
A. fumigatus	326	16.49	15	H	12	3	9
A. niger	838	42.39	28	H	15	8	7
A. ochraceous	2	0.10	1	R	3	1	2
A. parasiticus	6	0.30	2	R	2	2	0
A. sydowii	5.3	0.27	2	R	2	0	2
A. terreus	92	4.65	11	M	7	5	2
A. ustus	6	0.30	1	R	2	1	1
Total	1976.666				75	40	35

CFU*: Colony forming units, NCI**: Number of cases of isolation, OR***: Occurrence remark, R: rare, H: high and M: moderate.

Seventy-five isolates of the obtained *Aspergillus* species were tested for their capacity to produce kojic acid in starch medium under aerobic conditions. The tested species with isolates of *Aspergillus* were *A. aculateus* (six), *A. carneus* (two), *A. flavus* (sixteen), *A. oryzae* (eight), *A. fumigatus* (twelve), *A. niger* (fifteen), *A. ochraceous* (three), *A. parasiticus* (two), *A. sydowii* (two), *A. terreus* (seven) and *A. ustus* (two). The isolates of *A. carneus* and *A. sydowi* were kojic acid non producers. Also, two isolates of both *A. ochraceous* and *A. terreus* besides one, four, six, nine and seven isolates of *A. aculateus*, *A. flavus*, *A. fumigatus* and *A. niger* were non producers of kojic acid. On the other hand, the all tested isolates of both *A. oryzae* and *A. parasiticus* were kojic acid producers. These species were also studied for kojic acid production in different previous investigations[3,9&27-34]. *A. oryzae* was reported as the most potential producer species of the genus *Aspergillus* for kojic acid production from various substrates[3,9&35]. Thus, the eight isolates of *A. oryzae* were selected for quantitative detection of kojic acid production on starch as the sole carbon source in the fermentation medium as shown in table 2. These isolates proved to produce high concentrations of kojic acid, of which *A. oryzae* 1034 produced the highest concentration and recorded 41.678 ± 0.324 g/l. *Aspergillus oryzae* has been regarded in different studies as being of low pathogenicity, and no aflatoxins or any other carcinogenic metabolites producer; therefore, it is an excellent starter for the safe production of kojic acid. Rosfarizan and Ariff[33] recorded that *Aspergillus* species have the ability to produce amylase enzyme that hydrolyze starch into simple sugars for subsequent its conversion into kojic acid. The amount of produced kojic acid depend on the type of fermentable sugars available in the culture medium[3].

Table 2: Kojic acid production from starch by the isolated *Aspergillus oryzae*.

Isolate No.	Kojic acid (g/l)
A. oryzae 1008	32.7 ± 0.003^{c}
A. oryzae 1021	37.3 ± 0.239^{b}
A. oryzae 1034	41.7 ± 0.324^{a}
A. oryzae 1052	33.7 ± 0.033^{c}
A. oryzae 1054	28.1 ± 0.001^{d}
A. oryzae 1071	38.9 ± 0.026^{b}
A. oryzae 1074	36.8 ± 0.350^{bc}
A. oryzae 1096	26.3 ± 0.027^{d}

Values are means of three replicates; values assigned the same letters are not significantly different ($p < 0.005$) in Tukey's test.

For enhancing kojic acid production from starch versus glucose by the potential producer isolate *A. oryzae* 1034, the effects of some nutritional and environmental conditions were investigated.

Optimization of culture conditions for kojic acid production

Effect of phosphorus concentration on kojic acid production

The effect of phosphorus concentration in the medium on kojic acid biosynthesis from starch and glucose by *A. oryzae* 1034 was tested and the obtained data were presented in figure 1. It was noticed that kojic acid yield from starch was increased by increasing the phosphorus in the medium until reaching to 2.0 g/l then declined after this concentration. In contrast, kojic acid production from glucose was decreased by gradual increasing of phosphorus concentration. The maximum kojic acid concentration in starch medium was 55.36 g/l at 2.0 g/l KH_2PO_4 while the maximum production with glucose medium was 58.9 g/l at 0.5 g/l KH_2PO_4. Shakibaie *et al.*[36] indicated that the measured *p*-value of phosphorus in their study was less than 0.05 and concluded that this factor was considerably affected the kojic acid production. The phosphorus element is an important cellular cation for numerous enzymatic reactions. The high concentration of phosphorus might be towards the microbial cell to form phosphate interacted compounds rather than kojic acid especially with glucose as carbon source[37].

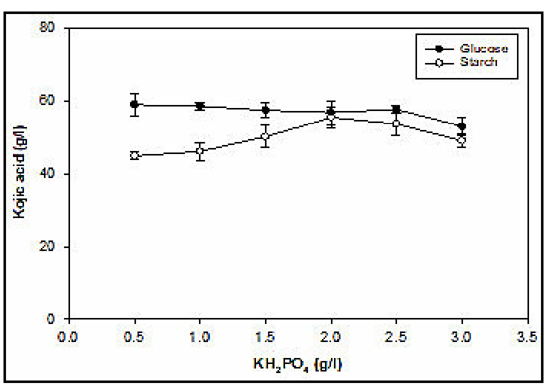

Fig. 1: Kojic acid production by *A. oryzae* 1034 at different KH_2PO_4 concentrations in the fermentation medium 28±2°C for 8 days.

Incubation temperature effect on kojic acid production

Kojic acid yield by *A. oryzae* 1034 from starch and glucose was affected by varying the incubation temperature. This isolate was able to grow well and produce considerable kojic acid amount the range from 20°C to 30°C (Fig. 2). The optimum temperature for kojic acid yield during the culture incubation was 28°C for both glucose and starch media giving 68.08 and 65.54 g/l kojic acid, respectively. The optimum temperature for formation of kojic acid by different fungal species was in the range 25-30°C in different studies[30,32,34&38].

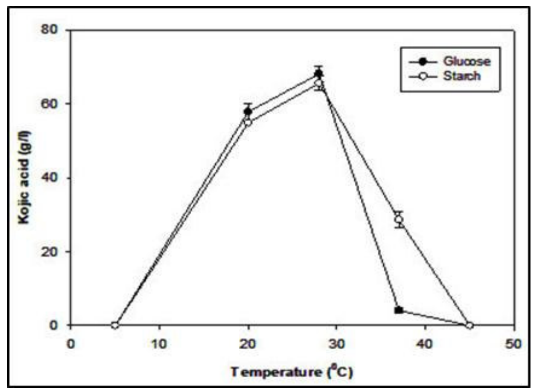

Fig. 2: Kojic acid production by *A. oryzae* 1034 at different incubation temperatures (°C) for 8 days.

Effect of pH on kojic acid production

Kojic acid production by *A. oryzae* 1034 was studied at different initial pH value and the obtained results were presented in figure 3. The maximum production (70.89 g/l) was obtained in glucose medium at 4.5 while the maximum production (64.71 g/l) in starch medium was obtained at 5.0. Kojic acid yield was declined above pH 4.5 and 5.0 for glucose and starch media, respectively. Most of the previous studies that were carried out to determine the optimum pH for the culture growth and kojic acid biosynthesis were based on the initial medium pH[39&40]. The highest yield of kojic acid was achieved at acidic medium with pH ranged from 4 to 6 in similar to the previous studies[12&41]. Rasmey and Basha[3] informed that enzymes as proteins contain ionizable groups thus their structure and function can be affected by the initial pH of the medium. Determination of the optimal pH for culture growth and kojic acid biosynthesis is very important since it affects on production of enzymes responsible on kojic acid pathway[37]. Alkaline pH may lead to the inactivation of enzymes responsible for kojic acid biosynthesis.

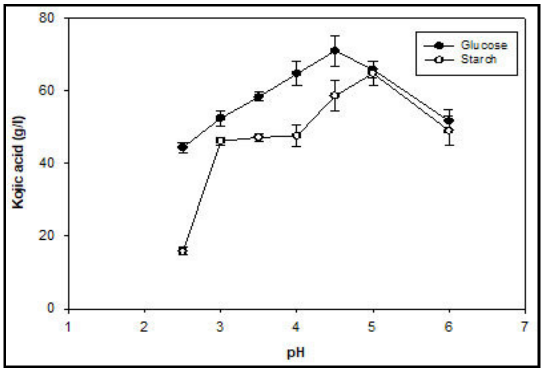

Fig. 3: Kojic acid production by *A. oryzae* 1034 at different initial pH values of the fermentation medium at 28°C for 8 days.

Incubation period effect on kojic acid production

Influence of incubation period on kojic acid yield from starch and glucose by *A. oryzae* 1034 was considered in the current investigation (Fig. 4). The presented data shows that the formed kojic acid increases by the gradual increasing of incubation period till reached the maximum production (69.9 and 68.78 g/l for glucose and starch, respectively) at 11 days. The obtained results are in consistent with the previous results obtained by different investigations[3,9,28,34&37]. The decrease in kojic acid production after the optimum incubation time might be due to the kojic acid degradation or conversion into other organic compounds[3&13].

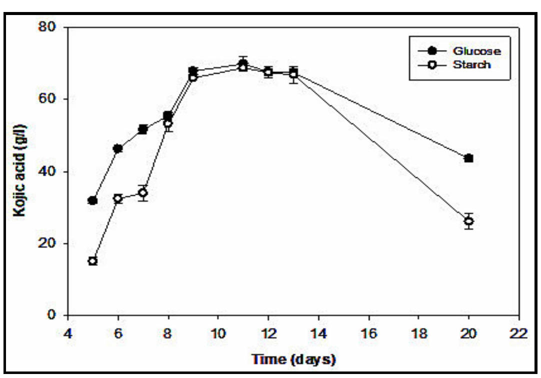

Fig. 4: Kojic acid production by *A. oryzae* 1034 at different incubation periods (days) at 28°C.

Effect of substrate concentration on kojic acid production

The results obtained from studying the effect of substrate (starch and glucose) concentration on kojic acid yield are shown in figure 5. Kojic acid amount was gradually increased by increasing the concentration of the two substrates under investigation. The maximum production was obtained at 60 g/l glucose. While, the maximum production in case of starch was obtained at 80 g/l starch. It was revealed that the high concentrations of carbon in the fermentation medium may result in increasing of residual sugar due to the inability of the used microbe to convert the all amounts of sugar into the desired product, based on the osmosis phenomenon[9,17&42]. Above a critical concentration of the tested sugar, water activity decreasing in combine with cells plasmolysis occur causing a decrease in the fermentation rate[16&43].

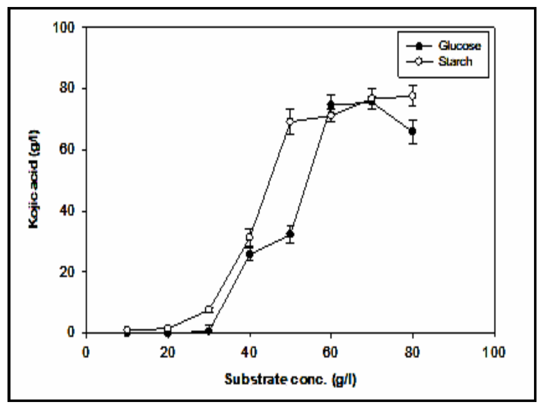

Fig. 5: Kojic acid production by *A. oryzae* 1034 at different substrate (glucose and starch) concentrations in the fermentation medium at 28°C for 11 days.

Effect of amino acid in the medium on kojic acid production

In the present study, the effect of five amino acids (arginine, histidine, methionine, lysine and alanine) on kojic acid production from glucose and starch was studied (Fig. 6). It is obvious that the kojic acid biosynthesis was decreased by addition of amino acids in the fermentation medium. The decrease of kojic acid might be that amino acids catalyzed other enzymes involved in formation of metabolites rather than kojic acid. Also, amino acids may activate the anabolism of other metabolites needed for fungal growth and adaptation not for production of secondary metabolites[44].

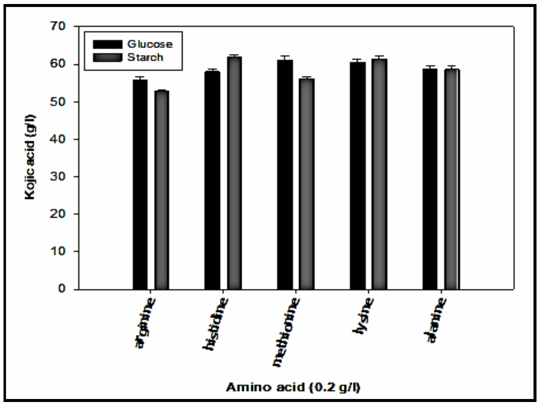

Fig. 6: Kojic acid production by *A. oryzae* 1034 in the fermentation medium supplemented with different amino acids individually at 28°C for 11 days.

Effect of heavy metals in the medium on kojic acid production

The effect of heavy metals (copper hydroxide, lead nitrate, zinc chloride, nickel chloride and manganese chloride) on kojic acid production from glucose and starch was also investigated (Fig. 7). Kojic acid production from glucose was stimulated by addition of heavy metals as trace elements in the fermentation medium. Kojic acid production was increased to 79.3 g/l by addition of lead nitrate in glucose medium while the maximum production of kojic acid (68.83 g/l) was obtained by addition of zinc chloride in starch medium. Pb^{2+} and Zn^{2+} might be strongly activated the enzyme activity responsible for kojic acid biosynthesis[45]. Also, production of an extracellular alpha-amylase enzyme may be activated by the presence of trace elements as cofactors in the fermentation medium.

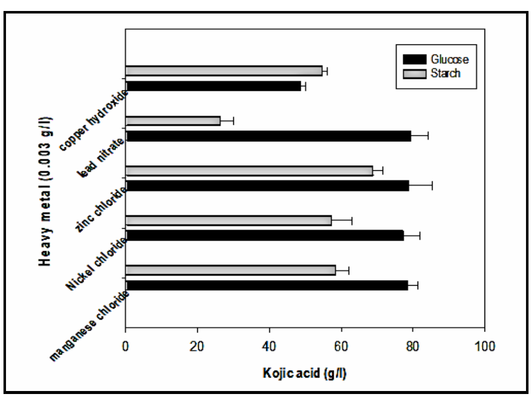

Fig. 7: Kojic acid production by *A. oryzae* 1034 in the fermentation medium supplemented with different heavy metals individually at 28°C for 11 days.

Conclusion

Kojic acid has much industrial enforcement and its demand is increasing extremely with the growing industries related to its applications. *Aspergillus oryzae* 1034 is a promising fungal strain for kojic acid production from cheap carbohydrates. Various fermentation mechanisms were applied for kojic acid production from glucose and starch media. Our findings indicated that kojic acid biosynthesis is highly affected by substrate concentration, phosphorus concentrations, amino acids and heavy metals in the fermentation medium. The highest kojic acid concentration (79.3 and 68.8 g/l) was obtained by incubation the culture at 28 ₒC for 11 days in glucose and starch media with initial pH 4.5 & 5.0, respectively. This isolate should be subjected to other metabolic and genetic engineering processing to enhance its kojic acid production from different agro-industrial wastes.

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نشرة العلوم الصيدليـــة
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تحسين ظروف الاستزراع لإنتاج حمض الكوجيك في التخمير السطحي بواسطة أسبيرجلس اوريزى المعزولة من حبوب القمح

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حمض الكوجيك هو حمض عضوي طبيعي يتم تخليقه أثناء التخمير الهوائي للكربوهيدرات كمركب ايضى ثانوي بواسطة بعض أنواع فطرة الاسبيرجلس ، وقد تم استخدامه تجاريًا في العديد من التطبيقات الصناعية. يهدف البحث الحالي إلى تحسين ظروف الاستزراع لإنتاج حمض الكوجيك من النشا كمصدر للكربون بواسطة العزلة الجديدة لفطرة أسبيرجلس اوريزى رقم ١٠٣٤. تم عزل عدد ٧٥ عزلة فطرية تمثل ١١ نوعًا تابعة لفطرة الاسبيرجلس من حبوب القمح المخزنة واختبار قدرتها على التخليق الحيوي لحمض الكوجيك. من بين هذه الأنواع ، تم اختيار أسبيرجلس اوريزى رقم ١٠٣٤ كافضل عزلة منتجة لحمض الكوجيك من النشا. تعرضت هذه السلالة لظروف تخمير مختلفة من اجل اكثار إنتاج حمض الكوجيك من النشا مقابل الجلوكوز. خلصت النتائج إلى أن اوساط الجلوكوز والنشا بتركيزات ٦٠ و ٨٠ جم/لتر على التوالي هي الأمثل لإنتاج حمض الكوجيك. وكان تركيز الفوسفور الأمثل ٠,٥ و ٢,٠ جم/لتر من فوسفات البوتاسيوم ثنائى الهيدروجين مع اوساط الجلوكوز والنشا ، على التوالي. تم تحقيق أقصى إنتاجية لحمض الكوجيك عند ٢٨ درجة مئوية لمدة ١١ يومًا من التحضين في كل من وسط الجلوكوز والنشا مع درجة حموضة ٤,٥ و ٥,٠ على التوالي. تم تحفيز التخليق الحيوي لحمض كوجيك إلى ٧٩,٣ و ٦٨,٨ جم/لتر ، على التوالي عند اضافة ايونات الرصاص Pb^{+2} إلى وسط الجلوكوز وايونات الزنك Zn^{+2} إلى وسط النشا. على النفيض ، تم تقليل التخليق الحيوي لحمض الكوجيك عند إضافة الأحماض الأمينية في وسط التخمير. تشير هذه النتائج إلى إمكانية استخدام عزلة أسبيرجلس اوريزى رقم ١٠٣٤ كعزلة فطرية واعدة لانتاج حمض الكوجيك من المواد النشوية.