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Journal of Natural Sciences Research
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.3, No.4, 2013

Improvement of Kojic Acid Production by a Mutant strain of *Aspergillus flavus*

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Abstract

The ability of 58 different strains of Aspergillus, Mucor, and Penicillium to form kojic acid has been studied. Four Aspergillus species and *Penicillium cryzogenum* local isolates with high activity of kojic acid synthesis were screened for kojic acid production on five proposed kojic acid producing synthetic medium. *Aspergillus flavus* (AF) was found to be the highly active one for kojic acid production. The highest level of kojic acid productivity (0.234 g/ l.h) obtained by *A. flavus* using fermentation medium of 100g/l glucose, and 5.0 g/l yeast extract, and incubated at 30°C and 180rpm for 9 days. The study implicated the optimization of different carbon and nitrogen source of fermentation medium. Among the carbon sources tested, glucose gave the highest kojic acid yield (50.27 g/l) followed by sucrose (48.95 g/l). The use of 5g/l yeast extract resulted in the highest kojic acid production (50.21g/l) compared with the other nitrogen sources. Improvement of *Aspergillus flavus* by natural selection and random mutagenesis by using UV (for 5, 10, 20, 40 and 60 min) and various doses of gamma irradiation (20, 40, 60, 80, 100, 120, 140 and 160 Gy) has been done to obtain a potential mutant which produce kojic acid higher than its parent strain. Some of wastes hydrolyzates were used as carbon and nitrogen source, potato starch, and rice bran hydrolyzates were the best carbon and nitrogen source, respectively.

Key words: Kojic acid, *Aspergillus flavus,* mutation, UV, *Gamma radiation*

Introduction

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone; KA) is a major secondary metabolite produced by a limited range of microorganisms, including *A. oryzae*, *Aspergillus flavus,* and *Aspergillus tamarii*, as well as Penicillium species and certain bacteria (Ana Paula *et al*., 2011; Bentley, 2006). Kojic acid inhibits tyrosinase activity (Chang, 2009) and is used as a food additive (Blumenthal *et al*., 2004), a skin-whitening agent for the treatment of melasma (Mi Ha *et al*., 2007), antioxidant, antitumour agent (Gomes *et al*., 2001; Burdock *et al*., 2001; Tamura *et al*., 2006; Moto *et al*., 2006) and radio protective agent (Emami *et al*., 2007). Recently, in vitro antiproliferation and cytotoxic activities of KA derivatives have been reported (Fickova *et al*., 2008).

This acid can be produced from various carbohydrate and nitrogen sources under an aerobic condition by a variety of microorganisms especially *Aspergillus* spp. And use of agricultural wastes as cheap carbon and nitrogen sources for kojic acid production (El-kady *et al.,* 2009; Gad, 2003).

The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Wu *et al.,* 2004). Irradiation by gamma ray may cause some mutations to the genes of cells through the DNA repair mechanisms within cells (Ellaiah *et al*., 2002). After the 250 Gy of gamma irradiation, the C/G base repair substitutions were the main type of gamma ray induced mutations in E. coli (Wijker and Lafleur, 1998). The mutational spectrum depended on irradiated conditions and DNA repair mechanisms of host cells (Reitsma-Wijker *et al*., 2000). Microbial strains for the overproduction and improvement of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Karanam and Medicherla, 2008).

The present study was undertaken to investigate the effect of different media composition on the biotransformation of sugar to kojic acid, the effect of several parameters such as carbon, nitrogen, and agricultural wastes hydrolyzates as a cheap carbon and nitrogen sources on the performance of biotransformation. Improve the productivity of kojic acid fermentation using Aspergillus strain was carried out to find a hyper producer mutant of *A. flavus* through mutagenesis using UV and gamma irradiation.

Materials and Methods

Microorganisms and screening of kojic acid producers.

A total of 58 fungus strains, *Aspergillus orayzae*, *A. tamirii*, *Aspergillus flavus, Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus fumigatus, Mucor sp, Penicillium atrovenetum*, and *Penicillium cryzogenum*, isolated from soil, representing 6 species of Aspergiilus, one species of Mucor, and 2 species of Penicillium were screened for production of kojic acid in a defined solid medium: 0.25% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$–$7H_2O$, and 10% glucose; pH was adjusted to 4 before autoclaving. The medium supplemented with 1 mM ferric ion. When the kojic acid producer strain was grown on the medium, the color of the medium turned red at around 1 week.

Effect of culture medium composition.

Five different media were tested. The first medium contained (g/l): glucose, 100; yeast extract, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5 (media.1) and the other one contain 100 (g/l) sucrose instead of glucose and were designated as medium (2). Yeast extract /sucrose medium containing 2% yeast extract and 20% sucrose was medium YES (3). A modified Czapek-Dox liquid medium containing 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.0% KCl, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.1% yeast extract, and 10% glucose was designated as medium (4). Medium (5) contained 5% glucose, 0.6% peptone, 0.1 % KH_2PO_4, 0.1 % $MgSO_4.7H_2O$, and 0.001% $CaCl_2$.

Medium and fermentations

The optimized medium proposed by Madihah *et al.* (1992) for kojic acid production was used for inoculum preparation and kojic acid fermentation by *A. flavus* (as it gave high production of KA compared with the other tested isolates). The medium consisted of glucose (100 g/ l), yeast extract (5 g/ I), KH_2PO_4, (1g/ I), and $MgSO_4$, $7H_2O$ (0.5 g/ 1). All submerged batch fermentations were carried out using 250-ml Erlenmeyer flasks containing 100 ml medium. After inoculation with spores (A standard inoculum of 1 ml of spore suspension approximately 12 x $l0^7$ spores/ ml was used in all experiment), the flasks were incubated at 30°C on a rotary shaker agitated at 180 rpm.

Effect of different carbon and nitrogen sources

The experiment to investigate the feasibility of using different carbon sources for kojic acid fermentation was carried out using 100 g/ 1 of each carbon source and 5 g/1 yeast extract as the sole nitrogen source. To investigate the effect of different nitrogen sources, 100 g/1 glucose was used as a carbon source and the concentration of each nitrogen source was 5g/1, pH was adjusted to 4.0. Kojic acid production by *A. flavus* was also studied using agricultural wastes hydrolyzates (20% w/v as carbon source and 5% w/v as nitrogen source) as a cheap carbon and nitrogen sources.

Natural Selection

The wild strain *Aspergillus flavus* was subjected to natural selection. The organism grown on potato dextrose agar (PDA) slants was scraped off into sterile phosphate buffer (0.02 M and pH 7.0) containing Tween 80 (1:5000) to give uniform suspension. The suspension was transferred into a sterile conical flask and thoroughly shaken for 30 minutes on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to remove vegetative mycelium from the suspension. The spore suspension was then serially diluted with phosphate buffer and the dilutions were used for plating.

One ml of the respective dilutions of spore suspension was added to the melted PDA medium at 40°C and after thorough mixing was poured into sterile Petri dishes. The plates were incubated at 30°C for 7 days. Colonies were selected on the basis of the morphological variations including sporulation and the presence of aerial hyphae. The selected colonies were transferred onto PDA slants and were allowed to grow at 30°C for one week. A total of 12 isolates were selected and were designated as natural selectants (AF1 to AF12). These selectants along with the wild strain were tested for the production of kojic acid.

UV Mutagenesis

The best natural selectant *Aspergillus flavus* (AFNS) was grown on potato dextrose agar (PDA) medium for 7

days at 30°C. Spores were harvested by washing the sporulated mycelia twice with phosphate buffer pH 7, and each time was centrifuged at 3500 rpm for 20 minutes. The spores were then resuspended in sterile water to concentration of 10^6 spores/ml. Four ml quantities of the freshly collected spore suspensions were exposed to UV light. The exposure to UV light was carried out in a "Dispensing – Cabinet" fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A^0. Optimum dose required to get maximum mutants was arrived by exposing the organism for different periods of time (5, 10, 20, and 40 min) at the distance of 20 cm from the UV source. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in phosphate buffer and plated on PDA medium. The plates were incubated for 7 days at 30°C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. A total of 12 colonies (designated as AF UV1 to AF UV12) were selected from the plates showing less than 1% survival rate (20 and 40 min UV exposure time) and tested for kojic acid production. Among the selectants, the stable mutants were selected for further studies in parallel with wild strain.

Gamma Radiation Mutagenesis.

Irradiation process was carried out at National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt. In the present study *Aspergillus flavus* was subjected to the γ-irradiation by C^{-137} Gamma Cell 40. The average dose rate of this gamma radiation source was 1 Gy/2.5 min at the time of the experiments. Five ml of cell suspension was transferred in each vial, sealed with paraffin and exposed to gamma irradiator. Different test doses of gamma radiation were selected which were 10, 20, 40, 60, 80, 100, 120, 140 and 160 Gy. From the treated cell suspension (0.1 ml) after different time intervals was transferred to PDA plates and incubated at 30°C for 7 days. A total of 12 colonies (designated as AFGR1 to AFGR12) were selected from the plates showing less than 1% survival rate and tested for kojic acid production. Among the selectants, the stable mutants were selected for further studies in parallel with wild strain.

Screening of Mutants

Screening of mutants was done in 96-well plates after incubating for 4 days, followed by addition of 1 drop of 1% ferric chloride. The strains which showed deep red-purple were collected. Increase of kojic acid concentration by the improved strains was confirmed by shake flask fermentation. The selected strains were preserved as the sources of further improvement by batch fermentation on wastes hydrolyzates.

Kojic acid isolation and purification

Extraction procedure

At the end of the growth period the mycelial mat was discarded and the culture liquid phase was filtered and ethanol and water (80:20) were added .Consecutive extractions were performed to produce a product concentrate through the evaporation process. The final product was obtained through crystallization. Spectrophotometer determination of kojic acid was made using its reaction with ferric chloride (Ana Paula *et al*., 2011).

Analytical methods

Culture media were decanted, the mycelium was washed several time with distilled water and oven dried (80°C for 24 hs) to get mycelial dry weight. The supernatant was used for kojic acid estimation according to colorimetric method of Bentley (1957). KA forms a chelated compound with ferric ions and subsequently generates a red color, the peak of which is approximately at 495 nm.

Results and Discussion

A total of 58 fungus isolates representing 6 species of Aspergiilus, one species of Mucor, and 2 species of Penicillium were screened for production of kojic acid in a defined medium containing 1 mM ferric ion. When the kojic acid producer strain was grown on the medium, the color of the medium turned red at around 1 week. Five isolates; *Aspergillus orayzae*, *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus fumigatus,* and *Penicillium cryzogenum*, were clearly distinguished visually by the color of the medium Photo (1).

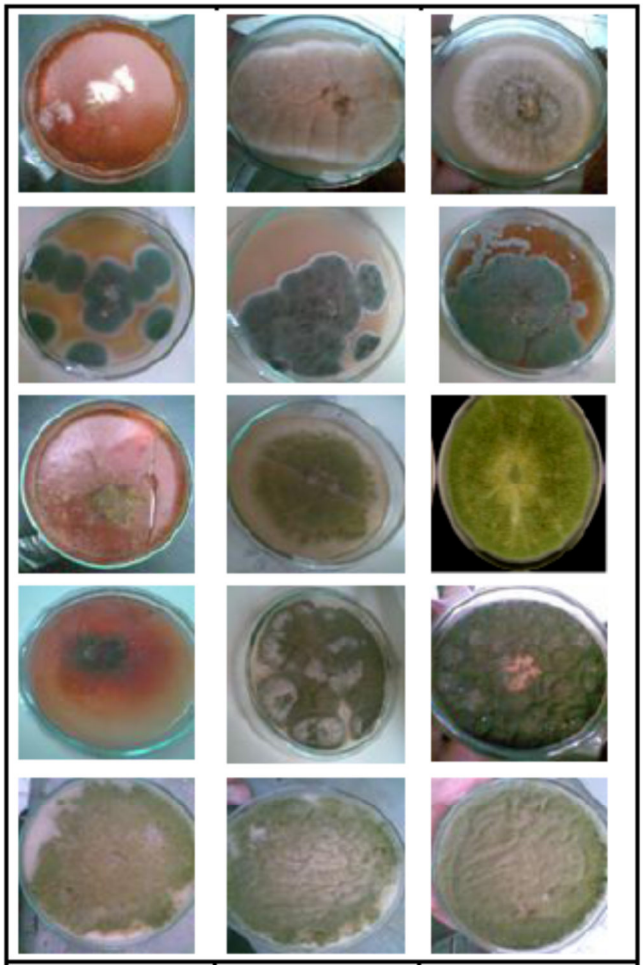

Photo (1). Culture morphology of *Aspergillus orayzae*, *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus fumigatus,* and *Penicillium cryzogenum* isolates on PDA plates containing 1 mM ferric ion. The color of the medium turned red at around 1 week.

Effect of culture medium composition.

Most of the media for kojic production contained glucose or sucrose as the sole carbon source; yeast extract or peptone as the sole nitrogen source in addition to magnesium sulphate and potassium phosphate. Futamura *et al*. (2001) suggested seventy six types of media to enhance the production rate of kojic acid by *A. oryzae* (Mk107-39) and selected two media from them. Many researchers reported that submerged fermentation conditions are more effective in kojic acid production than culture in the static flasks. In submerged culture, the yield of kojic acid ranges between 30-35 g/ 1 while in static fermentation it is about 17g/ 1 (Ariff *et al*., 1996).

The screened selected five fungal isolates were grown for 12 days on the five proposed media for kojic acid fermentation. It was found that media no 2(M-2) was the best in kojic acid production followed by M- 1, and M-3 and kojic acid reach its maximum yield after 9 days Table (1). Moreover; *A. flavus* followed by *A. parasiticus* were found to be the highly active for kojic acid production. These results show that 100g/l glucose followed by 100g/l sucrose induced maximum kojic acid production in addition to biomass yield in comparison with 200g/l sucrose. 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 decreased sugar utilization encountered with the highest concentration probably was due to osmosis effect. The decreased water activity and onset of plasmolysis combine to cause a decrease in the rates of fermentation (Rosfarizan and Ariff, 2007; Roukas, 1993).

Results conform to many early findings on the production of kojic acid by fermentation as secondary metabolite using *Aspergillus spp*. *A. effuses*, *A. glaucus, A, oryzae, A. flavus, A. gillus gymnosardae, A. awamori, A. clavatus, A. fumigatus, A. giganteus, A. albus, A. candidus, A. nidulans, A. parasiticus,* several species of Penicillium and Acetobacter (Wei *et al*., 1991; Coupland and Niehaus, 1987; Futamura *et al*., 2001; Gad, 2003). These differences in production of kojic acid and mycelial mat may be ascribed to either the culture conditions or

to species differences.

Table 1. Effect of culture medium composition on kojic acid production by selected fungal isolates

Fungal isolates	Media. no	X_m (g/l)	P_m (g/l)	$Y_{p/x}$(g/g)	$Y_{p/s}$(g/g)[a]	P (g/l.h)	Final pH
Aspergillus flavus	M-1	26.34	48.20	1.829	0.482	0.223	2.96
	M-2	33.55	50.57	1.507	0.505	0.234	2.90
	M-3	40.51	45.38	1.120	0.227	0.210	2.93
	M-4	12.45	16.37	1.284	0.164	0.0757	3.30
	M-5	17.58	16.16	0.919	0.323	0.075	3.63
Aspergillus parasiticus	M-1	27.60	40.31	1.461	0.403	0.187	2.01
	M-2	36.79	42.04	1.143	0.420	0.195	2.89
	M-3	55.63	39.12	0.703	0.196	0.181	2.96
	M-4	10.56	02.09	0.198	0.021	0.069	3.71
	M-5	14.71	14.48	0.984	0.289	0.067	3.13
Aspergillus orayza	M-1	23.30	10.08	0.433	0.101	0.047	2.79
	M-2	20.35	24.57	1.207	0.246	0.114	2.88
	M-3	25.03	32.88	1.314	0.164	0.152	3.03
	M-4	06.75	03.49	0.517	0.035	0.015	3.14
	M-5	13.11	08.44	0.644	0.169	0.039	3.37
Aspergillus fumigatus	M-1	20.40	06.85	1.316	0.134	0.124	2.86
	M-2	11.94	16.26	1.362	0.163	0.075	3.20
	M-3	15.77	12.24	0.776	0.122	0.057	2.95
	M-4	06.51	03.43	0.527	0.034	0.016	3.75
	M-5	10.532	07.72	0.733	0.154	0.036	3.39
Penicillium cryzogenum	M-1	21.95	11.40	0.519	0.114	0.053	2.95
	M-2	19.92	15.52	0.799	0.155	0.072	3.03
	M-3	24.80	15.05	0.607	0.075	0.070	3.11
	M-4	11.73	03.10	0.264	0.031	0.014	3.74
	M-5	12.62	09.74	0.772	0.195	0.045	3.40

X_m, Maximum cell concentration obtained during fermentation; P_m, maximum kojic acid concentration; $Y_{p/x}$ yield of kojic acid (g KA/g DW); $Y_{p/s}$ yield of kojic acid (g KA/g sugar); p, Overall productivity; after 216 hs. The data are the means of 3 independent experiments. (a): Carbon sources were added at 100 g/ l. Initial pH 4± 0.2; shake flasks at 180 rpm and 30 ◦C.

Effect of carbon source

From the previous results Table (1) *Aspergillus flavus* was selected for KA production. The highest kojic acid production was found with glucose, with maximum kojic acid concentrations of 50.27 g/l. The overall productivity and yield achieved for this biotransformation using glucose were 0.233 g/l. h and 0.503 g kojic acid/g glucose, respectively (Table 2). The fungus also used sucrose and xylose as carbon sources; they produced maximum kojic acid on concentrations of 48.95 and 40.60g/1, respectively. The highest kojic acid production found with monosaccharides mixture of glucose and xylose (50 glucose: 50 xylose g/l) was 45.13g/l. Lower kojic acid over all productivity was achieved with maltose and fructose (0.136 and, 0.129 g/l.h), respectively. Kojic acid over all productivity was low (0.094 and 0.036 g/l.h) when lactose and arabinose was used as carbon sources. From alditols (sorbitol, manitol, and xylitol) manitol, and xylitol gave the lowest overall productivity (0.015and 0.013g/l.h, respectively) at the end of incubation as compared to other substrates.

Various carbon sources like glucose, sucrose, fructose, maltose and mixture of glucose and sucrose (have been utilized by most of the cultures and their mutants (Rosfarizan and Ariff, 2007; Madihah *et al*., 1992). Similar results were reported by Kitada *et al.* (1967) who found the best carbon sources for kojic acid production by *A. Oryzae* to be glucose and xylose. It has also been reported that kojic acid is formed directly from glucose involving a multi step enzyme reaction without any cleavage into small fragments (Almeida *et al*., 2007). Kojic acid production was very low when fructose, arabinose or lactose or were used as carbon sources. Fructose did not support as high kojic acid production as other monosaccharides; this may be due to the possibility that fructose in furanose form was not suitable for direct conversion to kojic acid (Palmqvist and Hahn-H¨agerdahl,

2000). This result can also be correlated to the requirement of C6 precursor in the pyranose form for direct conversion of kojic acid (Rosfarizan and Ariff, 2007).

Table 2. Effect of different carbon sources on kojic acid production by *Aspergillus flavus.*

Carbon Source	X_m (g/l)	P_m (g/l)	$Y_{p/x}$ (g/g)	$Y_{p/s}$ (g/g)	P (g/l.h)	Final pH
Glucose	33.55	50.27	1.498	0.503	0.233	2.93
Sucrose	26.60	48.95	1.840	0.489	0.226	2.10
Maltose	26.80	29.38	1.096	0.294	0.136	3.50
Fructose	16.87	27.89	1.654	0.279	0.129	2.93
Lactose	23.50	20.24	0.861	0.202	0.094	3.08
Arabinose	4.42	7.701	1.742	0.770	0.036	3.65
Sorbitol	8.47	14.91	1.760	0.149	0.069	3.42
Manitol	1.615	3.34	2.068	0.334	0.015	3.74
Xylitol	1.140	2.77	2.429	0.277	0.013	3.53
Xylose	14.44	40.60	2.812	0.406	0.188	3.04
Glucose+ Xylose (75:25)	21.025	44.26	2.105	0.442	0.205	2.89
Glucose+ Xylose (50:50)	18.812	45.13	2.399	0.451	0.209	2.83
Glucose+ Xylose (25:75)	11.534	40.30	3.495	0.403	0.187	2.94

X_m, Maximum cell concentration obtained during fermentation; P_m, maximum kojic acid concentration; $Y_{p/x}$ yield of kojic acid (g KA/g DW); $Y_{p/s}$ yield of kojic acid (g KA/g sugar); p, Overall productivity; after 216 hs. The data are the means of 3 independent experiments. (a): Carbon sources were added at 100 g/ l. all media contain 5g/l yeast extract as nitrogen source; Initial pH 4± 0.2; shake flasks at 180 rpm and 30 ◦C.

Effect of nitrogen source

The types of nitrogen source used greatly influenced both growth and kojic acid production. (Table 3). High maximum cell concentration (X_m) and kojic acid production were obtained when organic nitrogen sources (yeast extract, tryptone and peptone) were used. Growth of *Aspergillus flavus* was poor when inorganic nitrogen sources were used. Yeast extract and tryptone nitrogen sources favored and promoted kojic acid production compared with ammonium sulphate and ammonium nitrate. These results showed that the use of 5g/l yeast extract or tryptone resulted in the highest kojic acid production (50.21 and 48.95 g/l, respectively) compared with the other nitrogen sources (Table 3). In terms of yield, fermentation using peptone gave a slightly high value (38.21g/l) as compared to fermentations using yeast extract as the nitrogen source. On the other hand, the use of ammonium sulphate and ammonium chloride resulted in small amounts of production (2.630 and 15.43g/l, respectively).

It has been reported previously that cell development and kojic acid production were higher in fermentations using organic nitrogen sources compared to fermentations using inorganic nitrogen sources. Several enzymes such as glucose-6.phosphate dehydrogenase, hexokinase and gluconate dehydrogenase are involved in the biosynthesis of kojic acid (Ariff *et al*., 1996). Free amino acids may be required for the enhancement of enzymes relevant to kojic acid synthesis. Yeast extract was the most favorable nitrogen source. However, this did not mean that yeast extract had better quality nitrogen, but probably contain higher levels of other essential components required for growth and fermentation, such as vitamins, amino acids and oligoelements (Futamura *et al*., 2001; Lin, 2001; Gad, 2003).The use of NH^{4+} ions as on inorganic nitrogen source may repress enzymes associated with kojic acid synthesis.

Table 3. Effect of different nitrogen sources on kojic acid production by *Aspergillus flavus.*

Nitrogen Source	X_m (g/l)	P_m (g/l)	$Y_{p/x}$(g/g)	$Y_{p/n}$ (g/g)	$Y_{p/s}$ (g/g)	P (g/l.h)	Final pH
Yeast extract	20.24	50.21	2.509	20.084	0.508	0.235	3.10
Tryptone	26.60	48.95	1.840	19.58	0.489	0.226	2.90
Peptone	23.51	38.21	1.626	15.284	0.382	0.177	3.15
Sodium nitrate	12.75	16.37	1.210	6.548	0.164	0.076	3.31
Amm. chloride	3.95	15.43	0.391	6.172	0.015	0.007	3.49
Amm. sulphate	6.85	2.630	0.384	1.052	0.026	0.012	3.60

X_m, Maximum cell concentration obtained during fermentation; P_m, maximum kojic acid concentration; $Y_{p/x}$ yield of kojic acid (g KA/g DW); $Y_{p/s}$ yield of kojic acid (g KA/g sugar); p, Overall productivity; after 216 hs. The data are the means of 3 independent experiments. (a): All media contain 100 g/ l glucose as carbon source. Nitrogen sources were added at 5 g/l. Initial pH 4± 0.2; shake flasks at 180 rpm and 30 ◦C.

Natural selectants and their kojic acid productivity

The KA production by the natural selectants is shown in Table 4. *Aspergillus flavus* natural selectant (AFNS9) showed highest KA production (51.50g/l), which was higher than the wild strain AF27 (50.21g/l). The natural selectant AFNS9 was chosen for further strain improvement.

Overproduction of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the process with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of UV and gamma radiation (physical mutagen) in strain improvement for enhanced KA productivity was investigated for the natural selectant AFNS9.

UV irradiation

Different samples of AFNS9 were processed to UV irradiation (5, 10, 20, 40 and 60 min). Irradiated samples were grown on potato/dextrose/agar for 4 days. The plates having less than 1% survival rate (20 and 40 min) were selected for the isolation of mutants. They were grown on potato/dextrose/agar for 4 days and subcultured in 96-well plates using the selection medium. The results showed few variants have a deep red. purple color, more intensive than parent strain (Photo 2). A total of 12 mutants were selected and tested for KA production (Table 4). The average kojic acid produced in shake flasks were 51.71 and 61.78 g/ l after 9 days as compared to 51.20 g/ l by the parent strain. The strain that produced 61.78 g kojic acid/ l (Table 4) was selected for further studies. The kojic acid yield of the best UV mutant (AFUV8) was 119.96% higher than the parent strain (AFNS9) and 123.04% times higher than the wild strain (AF27).

Gamma irradiation

Various doses of γ-radiation with range of doses (20 – 160 Gy) of γ-radiations at an interval of 20 Gy were applied to induce mutation in the cells of natural selection strain (AFNS9) in order to improve the KA production.). Irradiated samples were grown on potato/dextrose/agar for 4 days. The plates having less than 1% survival rate were selected for the isolation of mutants. They were grown on potato/dextrose/agar for 4 days and subcultured in 96-well plates using the selection medium (Photo 3). Of the 12 mutant isolated (40-60 Gy) AFG7 at 60 Gy gave maximum KA production (60.31g/l) which is 115% higher KA production than the parent strain at 60 Gy. Also, the kojic acid yield of the best gamma irradiation mutant AFG7 was 117 % higher than the parent strain (AFNS9) and 12.11% higher than the wild strain (AF) (Table 4). The results indicated that UV and gamma irradiation were effective mutagenic agents for strain improvement of *A. flavus* NS9 for enhanced kojic acid productivity.

Hopwood *et al*. (1985) suggested that 99.9% kill is best suited for strain improvement as the fewer survivors in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture.

Various strains exhibited different results in terms of enzymes production presumably because the enzyme activity was associated with the cell growth. It might be due to the fact that products of the reactions caused by ionizing radiations damage bases and, to lesser extent, damage sugars (Zhiqiang, 2005).

Table 4. Natural Selectants, UV mutants, and Gamma irradiation mutants, and their kojic acid productivity.

Natural selectants	kojic acid (g/l)	Gamma irradiation mutants*	kojic acid (g/l)	UV mutants**	kojic acid (g/l)
AFNS1	50.64	AFG1	59.91	AFUV1	58.46
AFNS2	50.31	AFG2	54.98	AFUV2	56.91
AFNS3	50.90	AFG3	58.53	AFUV3	58.60
AFNS4	51.09	AFG4	52.87	AFUV4	59.53
AFNS5	50.69	AFG5	59.84	AFUV5	57.95
AFNS6	50.12	AFG6	54.29	AFUV6	51.71
AFNS7	50.25	AFG7	60.31	AFUV7	56.89
AFNS8	50.47	AFG8	59.47	AFUV8	61.78
AFNS9	51.50	AFG9	56.22	AFUV9	54.44
AFNS10	51.24	AFG10	54.69	AFUV10	56.54
AFNS11	51.13	AFG11	55.31	AFUV11	51.97
AFNS12	50.82	AFG12	58.46	AFUV12	53.89
Wild strain (AF)	50.21	Parent strain (AFNS9)	51.50	Parent strain (AFNS9)	51.50

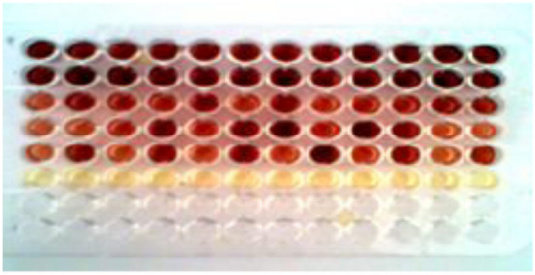

Photo (2). Screening results of VU induced mutants after addition 1% $FeCl_3$. From upper; 5, 10, 20, 40, 60 minute contact and control.

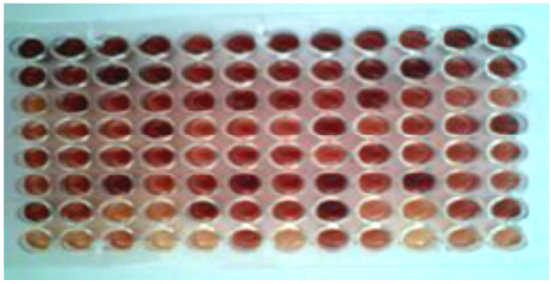

Photo (3). Screening results of gamma irradiation induced mutants after addition 1% $FeCl_3$. From upper the irradiation doses were; 20, 40, 60, 80, 100, 120, 140, and 160 Gy.

Effect of different wastes hydrolyzates on kojic acid production

The potential mutant strain AFUV8 was used for fermentation with 100 ml of 20 % w/v wastes hydrolyzates medium (as carbon source) and 5% w/v wastes hydrolyzates (as nitrogen source) in a 250 ml Erlenmeyer flask and grown at 180 rpm and 30°C for 9 days. The result showed that flask culture of potato starch and molasses produced a high concentrations of kojic acid which were 40.67 and 32.31g/l, respectively and higher than that of sugar cane bagasse and barely (23.58 and 16.45g/l). It was also found that potato starch resulted in small uniformed pellets, which is usually preferred in fungal fermentation studies. From the results it is cleared that rice bran and wheat bran were the best nitrogen sources as they give KA by 44.0 and 39.88g/l, respectively (Table 5).

The result showed that flask culture of adapted mutant (repeated growth of the mutant in the same media for 5 times) produced a high concentration of kojic acid which was 54.0 g/l, about 128.76% higher than that of the parent strain (AFUV8) by combining potato starch and rice bran as carbon and nitrogen source, respectively (photo 4).

The utilization of waste hydrolyzates as a source of nutritional components (carbohydrate, protein and other basic nutrients) needed for microbial activities in the production studies of valuable industrial compounds, not only provides the cheap substrates to industrial fermentation studies but also helps the solution of environmental problems by reducing the amount of wastes, which may cause serious ecological hazards (Mosier *et al*., 2005).

Use of lignocellulosic feed stocks for production of bio-products requires pretreatment, in addition to sugars (glucose, xylose, and arabinose); pretreated biomass may also contain aromatic aldehydes and phenolic compounds released from lignin, and organic acids such as acetic acid from hemicelluloses. Dilute acid pretreatment in particular also may cause formation of furfural and 5-hydroxymethylfurfural (HMF) from the dehydration of released sugars. These side-products are a concern because they act as microbial inhibitors and negatively affect fermentation of the sugars (Futamura *et al*., 2001; Almeida *et al*., 2007).

Some molasses sucrose during sugar processing is hydrolyzed into reducing sugar glucose and fructose in beet molasses 22% (w/w) reducing sugar. Gad (2003) found that beet molasses at 20% (w/v) concentration was suitable for kojic acid production by *A. parasiticus.*

El-kady *et al*. (2009). Found that rice fragments was the best suitable medium followed by molasses medium for kojic acid production by 2 isolates tested of *A. flavus* var. columnaris. Rice fragments and molasses as by-products were the more favorable substrates for kojic acid production by the tested five isolates. They also found that, *Aspergillus flavus* was used and grown on molasses medium, maximum level (53.5 g/I) of kojic acid was obtained after eight days of incubation.

Table 5. Effect of different wastes hydrolyzates on kojic acid production by *Aspergillus flavus.*

Wastes hydrolyzate used as carbon source	X_m **(g/l)**	P_m **(g/l)**	$Y_{p/x}$**(g/g)**	P **(g/l.h)**	**Final pH**
Potato Starch	23.99	40.67	1.695	0.188	3.83
Molasses	33.90	32.31	0.953	0.149	3.69
Sugar Cane Bagasse	15.13	23.58	1.558	0.109	3.50
Barely	7.80	16.45	2.109	0.076	2.55
Rice Straw	3.19	05.96	1.869	0.027	3.64
Wastes hydrolyzate used as nitrogen source	X_m **(g/l)**	P_m **(g/l)**	$Y_{p/x}$**(g/g)**	P **(g/l.h)**	**Final pH**
Rice Bran	22.79	44.00	1.930	0.204	3.14
Wheat Bran	25.49	39.88	1.564	0.184	3.30
Corn Steep Liquor	27.28	23.30	0.854	0.108	3.45
Molt Bran	16.5	14.94	0.905	0.069	2.49
Wastes hydrolyzate used as carbon and nitrogen source	X_m **(g/l)**	P_m **(g/l)**	$Y_{p/x}$**(g/g)**	P **(g/l.h)**	**Final pH**
Parent strain (AFUV8)	21.736	41.94	1.929	0.194	3.65
Adapted mutant	22.79	54.0	2.369	0.450	3.61

X_m, Maximum cell concentration obtained during fermentation; P_m, maximum kojic acid concentration; p, Overall productivity; $Y_{p/s}$ yield of kojic acid after 216 hours. Initial pH 4± 0.2

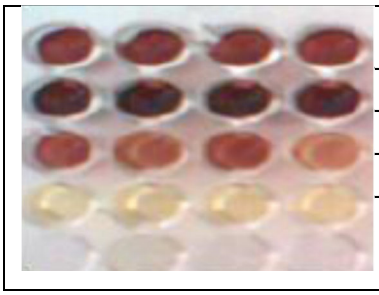

Photo (4). Screening results of kojic acid production by *Aspergillus flavus.*

Isolation and purification of kojic acid

Kojic acid, isolated from the culture medium by ethanol extraction (ethanol: water (80:20)) (Photo 5). During this study, much yellowish- brown crystals were present in the flasks containing the culture filtrate after one night of storage in refrigerator .The crystals were collected dried at 80˚C for 24hs. Crystals per each flask were obtained. They were combined and purified by repeated crystallization from a matrix of water and acetone,

yielding colorless long needles (Ana Paula *et al*., 2011).

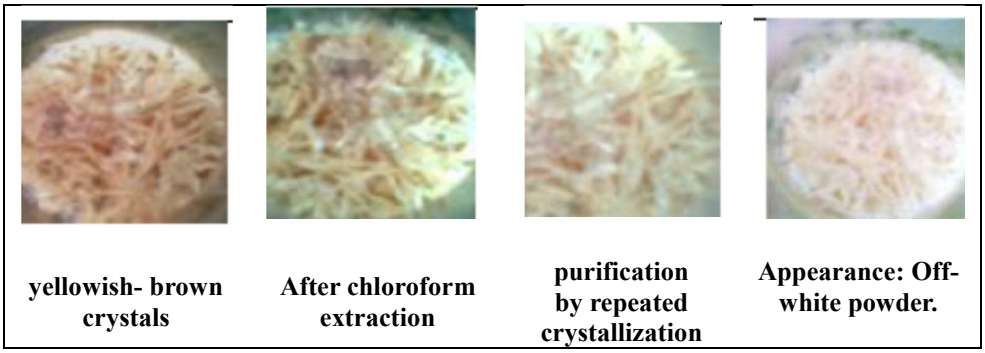

Photo (5). Isolation and purification of kojic acid

Conclusion

It is suggested that the high yielding fungal mutant strain of the *Aspergillus flauvs* (AFUV8), can be exploited commercially for large-scale industrial production of kojic acid.

These results recommended us to use combination of potato starch and rice bran instead of glucose as carbon source and yeast extract as nitrogen source to decrease costs of fermentation.

References

Almeida, J.R.M., Modig, T., Petersson, A., Hahn-Ha¨gerdal, B., Lide´n, G., and Gorwa- Grauslund, M.F. (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J Chem Technol Biotechnol*., 82:340–9.

Ana Paula D. Rodrigues, Antoˆ nio Sergio C. Carvalho, Alberdan S. Santos, Claudio N. Alves, Jose´ Luiz M. do Nascimento, and Edilene O. Silva1.(2011). Kojic acid, a secondary metabolite from Aspergillus sp, acts as an inducer of macrophage activation. *Cell Biol. Int*., 35: 335–343.

Ariff, A.B., Salleh, M.S., Ghani, B., Hassan, M.A., Rusul, G., and Karim, M.I.A. (1996). Aeration and yeast extract requirements for kojic acid production by *Aspergillus flavus* Link, *Enzyme Microb. Technol.*, 19: 545–550.

Bentley, R. (2006). From miso, sake and shoyu to cosmetics: a century of science for kojic acid. *Nat Prod Rep*., 23:1046–62.

Bentley, R. (1957). Preparation and analysis of kojic acid. In: Colowick, S.P. and N.O. Kaplan, (eds.), *Methods in Enzymology*, Academic Press, New York, 3: 238–41.

Blumenthal, C.Z. (2004). Production of toxic metabolites *in Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regul Toxicol Pharmacol*., 39:214–28.

Burdock, G.A., Soni, M.G., and Carabin, I.G. (2001). Evaluation of health aspects of kojic acid in food. *Regul Toxicol Pharmacol*., 33:80–101.

Chang T. (2009).An updated review of tyrosinase inhibitors. *Int J Mol Sci*., 10:2440–75.

Coupland, K., and. Niehaus, W.G. Jr. (1987). Effect of nitrogen supply, Zn2+ and salt concentration on kojic acid and versicolorin biosynthesis by *Aspergillus parasiticus*. *Exp. Mycol*., 11: 206–13.

El-kady, I.A., Mostafa, E.M., and Ragab, S.R.H. (2009). Kojic acid production by some fungi using agro-industrial by-products. *Assiut University Journal of Botany*, Assiut Univ. (June, 2009).

Ellaiah, P., Prabhakar, T., Ramakrishna, B., Taleb, A.T., and Adinarayana, K.M. (2002). Strain improvement of *Aspergillus niger* for the production of lipase. *Indian J. Microbiol*., 42: 151-153.

Emami, S., Hosseinimehr, SJ.,Taghdisi, S.M., and Akhlaghpoor, S. (2007).Kojic acid and its manganese and zinc complexes as potential radio protective agents. *Bioorg Med Chem*., 17:45–8.

Fickova, M., Pravdova, E., Rondhal, L., Uher, M., and Brtko, J. (2008). In vitro antiproliferative and cytotoxic activities of novel kojic acid derivatives: 5-benzyloxy-2- selenocyanatomethyl-and 5-methoxy-2-selenocyanatomethyl-4-pyranone. *J Appl Toxicol*., 28:554–9.

Futamura, T., Ishihara, H., Tamura, T., Yasutake, T., Huang, G., Kojima, M., and Okabe, M. (2001). Kojic acid production in an airlift bioreactor using partially hydrolyzed raw corn starch. *J. Biosci. Bioeng*., 92: 360–365.

Gad, A.S. (2003). Modification of molasses for kojic acid production by *Aspergillus parasiticus*. *N. Egyptian J.*

Microbiol., 5: 14–26.
Gomes, A.J., Lunardi, C.N., Gonzalez, S., and Tedesco, A.C. (2001). The antioxidant action of *Polypodium leucotomos* extract and kojic acid: reactions with reactive oxygen species. *Braz J Med Biol Res*, .34: 1487–94.
Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., and Schrempt, H. (1985). Genetic Manipulation of Streptomyces – A Laboratory manual. The John Innes Foundation., Norwick.
Karanam, S.K. and N.R. Medicherla. (2008). Enhanced lipase production by mutation induced Aspergillus japonicus. *African Journal of Biotechnology*, 7(12): 2064-2067.
Kitada, M., Veyama, H., Suzuki, E.., and Fukmbara, T. (1967). Studies on kojic acid fermentation I. Cultural condition in submerged culture. *J. Ferm. Technol*., 12: 1101–7.
Lin, Chi-Chu. (2001). The effect of equipping a non-waven fabrics in the fermenter on the production of kojic acid by *Aspergillus flavus*. M.Sc Thesis, Chemical Engineering, China.
Madihah, M. S., Baharuddin, A. G., Abdul Karim, M. I., Hassan, M. A., Mitsugi, K., Tano, T., Sugio, I., Tada, M., and Kanzaki, H. (1992). Cultivation characteristics during kojic acid fermentation by local fungus, mutant strain 44-l. Second UNESCO Nat. Workshop Promot. Microbial. Malaysia, UPM Serdang, Malaysia.
Mi Ha, Y.M., Chung, S.W., Song, S., Lee, H., Suh, H., and Chung, H.Y. (2007). 4-(6-Hydroxy-2-naphthyl)-1, 3-bezendiol: a potent new tyrosinase inhibitor. *Biol Pharm Bull*, 30:1711–5.
Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., et al. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol, .96:673–86.
Moto, M., Mori, T., Okamura, M., Kashida, Y., and Mitsumori, K. (2006). Absence of liver tumor-initiating activity of kojic acid in mice. *Arch Toxicol*,. 80:299–04.
Palmqvist, E., and Hahn-H¨agerdahl, B. (2000). Fermentation of lignocellulosic hydrolysates. II. Inhibitors and mechanisms of inhibition. *Bioresour Technol*., 74: 25–33.
Reitsma-Wijker, C.A., Slotman, B.J., and Lafleur, M.V.M. (2000). Mutagenic effect by phenylalanine during g-irradiation of plasmid DNA in aqueous solution under toxic conditions. *Mutat*. *Res*., 454: 71-76.
Rosfarizan, M., and Ariff, A.B. (2007). Biotransformation of various carbon sources to kojic acid by cell-bound enzyme system of A. flavus Link 44-1. *Biochemical Engineering*, 35: 203–209.
Roukas, T. (1993). Ethanol production from carob pods by *Saccharomyces cerevisiae*. *Food Biotechnol*., 33: 805–10.
Tamura, T., Mitsumori, K., Totsuka, Y., Wakabayashi, K., Kido, R., Kasai, H. et al. (2006). Absence of in vivo genotoxic potential and tumor initiation activity of kojic acid in the rat thyroid. *Toxicology*., 222:213–24.
Wei, C., Huang, T. S., Chen, J. S., Marshall, M. R., and Chung, K. T (I991). Production of kojic acid by *Aspergillus candidus* in three culture media. *J. Food Prot*., 54, 546-548.
Wijker, C.A., and Lafleur, M.V.M. (1998). Influence of the UV-activated SOS response on the γ-radiation-induced mutation spectrum in the lacI gene. *Mutat. Res*., 408(3): 195-201.
Wu, W-T., Wan, H-M., Chen, C-C., Chang,, T.S., and Giridhar, R.N. (2004).Combining Induced Mutation and Protoplasting for Strain Improvement of *Aspergillus oryzae* for Kojic Acid Production. *Biotechnol. Lett*., 26: 1163-1166.
Zhiqiang, A. (2005). Handbook of Industrial mycology. Marcel Dekker., 22: 539-561.