

SCREENING AND OPTIMIZATION OF SUBMERGED FERMENTATION OF *ASPERGILLUS* SPECIES FOR KOJIC ACID PRODUCTION

REHANA BADAR^{1,2}, SAMIYA YAQOOB¹, ASMA AHMED¹, ZILWA MUMTAZ², QURAT UL AAN SHAOOR^{1,3}

Corresponding ; asma.ahmed@imbb.uol.edu.pk;

ABSTRACT

Kojic acid is organic acid obtained from numerous species of *Aspergillus* through fermentation. This is among most demanding substances in cosmetic industries as an alternate to carcinogenic Hydroquinone and has grabbed a vital position in Pharmaceuticals, Food and Agriculture industries. Current experimental approach was designed for production and purification of *Kojic acid* crystals from *A. flavus* and *A. oryzae* and measured the effects of pH, temperature, static and non-static (shaker) condition on *Kojic acid* yield in submerged fermentation. Significant yield of *Kojic acid* crystal was obtained by *A. flavus* compared to *A. oryzae*. Optimized conditions were pH 4.5 for (*A. flavus*) and 3.5 for (*A. oryzae*) at 30 °C with 20 days of incubation. High yield of *Kojic acid* crystals were produced under static condition (16 g/L in *A. flavus* and 11 g/L in *A. oryzae*) in contrast to non-static (shaker) conditions (6 g/L in *A. flavus* and 5 g/L in *A. oryzae*). Quantitative estimation of *Kojic acid* was done through Bentley's colorimetric method followed by TLC, FTIR and HPLC. This analysis was found successful after achieving the high yield of *Kojic acid* under optimized conditions.

Keywords: *Kojic acid*, *A. flavus* and *A. oryzae*, pH, Quantitative, TLC, FTIR, HPL

¹Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Lahore, Punjab, Pakistan ²Superior University Lahore, Punjab, Pakistan

³University of the Central Punjab, Lahore, Punjab, Pakistan

INTRODUCTION

Kojic acid (5-hydroxy-2-hydroxymethylgamma-pyrone; KA) is considered as a major secondary metabolite which is produced by the carbohydrates through various microorganisms, such as *A. oryzae*, *A. flavus*, and *A. tamarii*, as well as *Penicillium* species and certain bacteria (Hazzaa *et al.*, 2013; Antonius *et al.*, 2019). *Kojic acid* is an organic acid which covers almost all the fields, act as antibacterial, antifungal, anti-melanosis, and a chelating agent. It can be applied in various areas of science such as chemistry, health, food, and cosmetics; however, its current, reported uses are limited at commercial scale, as its despite at the market level were being developed about 40 years ago. However it is also marked as an organic chelation Agent used as a decolorizing agent. It is well known for its uses in Cosmetics Industry. *Kojic acid* is reported to have an antioxidant potential due to which is marked as to be used in the cosmetics industry, which has been further described as an alternative to hydroquinone for the lightening of the skin (Kady *et al.*, 2014). This acid was basically discovered from the mycelia of *A. oryzae* which was grown on steamed rice (the term koji means steamed rice in Japanese) in the year 1907 (Burnett *et al.*, 2010). Koji stands for rice in Japanese language and likewise the name of *Kojic acid* was given to this acid (Brtko *et al.*, 2004).

Kojic acid can be produced in large amounts by using various carbon and nitrogen base sources, based on agriculture waste under aerobic fermentation process. Till now among others glucose has been marked as the one of the best source as a raw material in order to attain higher yields of *Kojic acid*. Therefore use of some mutated strains of *A. flavus* had shown remarkable peaks of *Kojic acid* used potato, sago and corn starch etc. (Chaudhary *et al.*, 2014). *Kojic acid* has many economic applications in different eras, such as in the field of medical, it has been reported as having anti-bacterial and anti-fungal potentials. While in case of chemical industries it has been also used auspiciously for the synthesis of azo-dyes and biodegradable compounds. Furthermore in the field of food industries, it has been used as an anti-speck and anti-melanosis (blacking of product) agent for agricultural products (Hassan *et al.*, 2014).

On commercial scale, *Kojic acid* synthesis was initiated by Charles Pfizer and Company of USA in 1955 which was the first company for this production. This finding was useful for further enhancement at commercial value, applications and production (Brtko *et al.*, 2004; Bentley, 2006). Recently the production of KA is marked by two Chinese and three companies in Japan, Switzerland, and the USA. Rapid growth of industries and discovery of the potential applications of KA along with its derivatives, generated a demand for the manufacture of this product KA greatly (Saeedi *et al.*, 2019).

According to global marketing report the use and production of cosmetic is increasing day by day. KA is used as primary constituent in skin products as it obstruct the synthesis of pigment, responsible for skin blackening (Masse et al. 2001). It is a good for the prevention of melanin production which ultimately safe man from freckles. However it was banned in Asia, later on due to carcinogenicity in by Drug Authority (Emami *et al*, 2007).

Kojic acid is present in colorless prismatic needle form that can sublime in vacuum with no alteration in morphology. It has melting point ranging from 151°C to 154°C, while the boiling point of this substance is 401.67°C at 760 mmHg. Furthermore the extensive use of this acid in the production of cosmetic are due to the ability of acting as a UV protector, which extensively work as a suppressor of hyperpigmentation in human and could have great potential to restrainer the formation of melanin, which is basically associated with its *tyrosinase* inhibition. This could also be developed as a chemo sensitizer in order to increase the potential of antifungal drugs or fungicides at market levels (Saeediet al. 2019).

According to Cryoscopy Method, molecular weight of *Kojic acid* is 142.1 and its maximum peak of UV Absorption Spectra is at 260-284 nanometer. KA is marked assoluble in Water, Acetone, Ethyl Acetate, and Ethyl Ether & Ethanol and also slightly soluble in Ether, Alcohol-Ether mixture, Chloroform & Pyridine etc. The solubility of this acid in Pyrimidine is very low, however, it is completely insoluble in Benzene (Neil, 2006).

Kojic acid has properties of weak acids and it is categorized as a multifunctional, reactive Gamma-Pyrone. Its reactivity predominantly could be observed at all positions on a ring and a number of valuable industrial products can be made from it such as metal chelates, Ether, Pyrimidine, Pyridones, Azodies, Mannich Base etc. This is also reported as a strong antibacterial component, and had shown very strong control against gram positive bacteria (Zohri *et al.*, 2018). Since beginning, several numbers of its chemical reactions have been studied for research purpose and its deliberate use for the benefit of humankind. It has ability to form salt with rare metal such as sodium, zinc, cooper, calcium, nickel and cadmium as its Hydroxyl group acts as weak acid at carbon 5 position. Carbon 5 side chain acts as primary alcohol whose reactivity is increased by neighboring oxygen atom in the nucleus (Chaudhary *et al.*, 2014).

It has strong reactions with amino acids to form conjugates, which exhibit a higher Tyrosine inhibition activity and stability as compared to KA alone, its derivatives are also reactive. (Mohammad *et al.*, 2010; Noh *et al.*, 2009). These derivatives are efficiently utilized in human medicines, veterinary medicines and synthesis of new and high biologically active compounds with preferable properties (Brtko, 2004). Secondary metabolites are the organic compounds which are not directly involved in the procedure of normal growth, progression and reproduction of an organism. Primary metabolites are responsible for the production of Secondary metabolite. Secondary metabolites play vital role in an organism's growth, reproduction as well as defense mechanism of living organism.

Plants use secondary metabolites in their defense system against herbs and humans utilize secondary metabolite as medicine, recreational drugs, texture preservative, food thickening and flavoring agent. (Pichersky and Gang, 2000; Gök *et al.*, 2013). Natural selection has been very important in the discovery of vital compounds which can give rise to new products. Organisms that inhabit vital biotopes produce natural products that perform specific functions and they can be used for vital secondary metabolite. (Schulz *et al.*, 2002). However certain plants of

Solonaceae had been reported to be used in drugs to cure diarrhoea and vomiting, phytoestrogens is secondary metabolite found in seed of Neem plant and act against insects (Chizzali and Beerhues, 2012).

Aspergillus is one of the oldest known fungus known for the production of metabolites, acids and enzymes. Industrial utilization of microbial enzymes started 100 years ago in western world with the synthesis of Alpha Amylase by *A. oryzae*. These enzymes are used for numerous applications like cheese production, starch production, clarification of juice, food preservation and instant tea making. Though the strains of *oryzae* had been also reported for the production of lipase (Ahmed et al. 2019). *Aspergillus* is widely used for the production of organic acids such as citric acid, gluconic acid, KA at industrial level. These organic acids are used in food, soap, cream and in pharmaceutical as well. In course of survey different fungal species were used for its synthesis but *Aspergillus* species show fairly high yield of KA. They give deep red color with ferric chloride and upon continuous extraction produce crystals of KA (Brian, 1951).

According to 2014 Global marketing report, cosmetics business was 460 billion US dollar in 2014 and it is expected to reach to 675 billion US dollar in 2020. Hydroquinone used in skin care product has been replaced by *Kojic acid* (Emami et al., 2007). Currently, *Kojic acid* is frequently used as a basic ingredient in skin lightening products because of its ability to block the formation of melanin, a pigment responsible for dark skin. (Masse et al. ,2001).

The main objective of this experiment was to investigate the highest potential strains for the production of KA among *A.flavus* and *A. oryzae* isolates through submerged fermentations techniques. For that fermentation procedure was optimized by varying condition as static and non-static, temperature and pH of the media was also investigated.

MATERIALS AND METHODS

Experimental Lay Out

Experiment was arranged by providing varying pH and Temperature in Static and Non-static conditions to attain Optimized conditions of fermentation Procedures for the highest yield of Kojic acid.

Collection and Maintenance of Fungus

In order to carried out this experimental analysis two species of *Aspergillus* (*A. flavus* and *A. oryzae*) were purchased from First Culture Bank of Pakistan, University of the Punjab Lahore. For the enhancement of the growth of mother culture, Sub-cultured by using potato dextrose agar (PDA) as the best source for the production of Micro-Organisms. Formation of Potato Dextrose Agar (PDA) media 3.9 g of PDA was dissolved in 100 ml of water in a 250 ml of Flask and were placed on hot plate for mixing until thoroughly dissolved in water. Subsequently, the flask containing the solution were cotton plugged and wrapped by aluminum foil. The flask was autoclaved for 1h, then poured into tubes and placed for ten min. After solidification of PDA solution, a loop of mother culture

was taken and streaked on the Slants. These Slants were covered tightly and placed into incubator for 7 days at 30°C. The whole procedure of Slant formation was conducted in a Laminar Flow to avoid contamination hence enhancing the number or growth of mother culture (Syarifuddin and Saidi, 2013).

Preparation of Culture Media

One liter of culture media was prepared by using 100 g of glucose (as a carbon source), 5 g yeast (as a nitrogen source), 1 g KH₂PO₄, 0.5 g MgSO₄ and 10 ml methanol. Aforementioned media being the high productive media was utilized by all referred Scientists (El-Aziz, 2013). To avoid the production of Aflatoxins, Methanol was used in the media solution (Madiah et al. 1998).

Maintenance of pH and Temperature

150 ml of Culture media was poured in 250 ml flask and twelve such numbers of flasks were prepared. pH of these flasks were adjusted to 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 by using H₂SO₄ and NaOH. 2 such flasks were prepared for each pH, 01 for Static and the other for Non-static (shaking) procedure. All flasks were labeled with (pH and target procedure)-wise and inoculated with *A. flavus* and *A. oryzae* separately. Then 06 flasks containing the solution ranging from 3.5 to 8.5 pH were placed on shaker at 30 °C and likewise 6 others in an incubator at 30 °C for 20 days.

150 ml of Culture media was poured in 8 prepared 250 ml flasks varying temperatures under 2 different procedures i.e. Static and Non-static (shaking). All flasks were labeled with temperature of 25, 30, 35 and 40 °C and target procedure wise and inoculated with *A. flavus* and *A. oryzae* separately in static and non-static conditions for about 20 days.

Wet and Dry Mycelia Weight

The flasks were removed from shaker and incubators and Mycelia mass was separated from culture and weighed (Weighing balance Model No. JJ224BC). The same mycelial mass was placed in oven to dry for 24 h and weighed again.

Analysis of Kojic acid (KA)

Colorimetric method was used for the quantification of *Kojic acid* (Bentley, 1957). 1 ml of diluted sample was mixed into 1 ml FeCl₃ solution (1g of FeCl₃. 6H₂O in 100 ml of 0.1N HCL) (Sikem, 2013). Sample gave a reddish purple color due to reaction between hydroxyl and phenolic group as KA makes a complex with FeCl₃ resultantly giving this purple color. Absorbance of the sample was measured at 500 nm by using UV- VIS Dual Split-Beam Spectrophotometer (Model No. UV2400PC) and equivalence was checked by *Kojic acid* standard curve (Crueger and Bentley, 2006).

Comparison of Kojic acid Concentration By Standard Curve

Comparison of Standard Curve of self-prepared KA and prepared from standard KA obtained from PCSIR, Lahore was drafted to evaluate the comparative yield. 10 different dilute solutions of Standard *Kojic acid* were prepared and placed in Spectrophotometer (Model No. UV2400PC) at 500 nm to measure absorbance of dilute solutions and plotted a graph between the

concentrations of multiple dilute solutions and absorbance (Figure 1). Later, need-based dilute solutions of self-prepared *Kojic acid* (culture media) were prepared and likewise placed in Spectrophotometer (Model No. UV2400PC) at 500 nm to measure absorbance of dilute solutions. Trend analysis w.r.t. absorbance of dilute solutions was noted after intervals of 5 days and obtained O.D. of Standard and self-prepared *Kojic acid* were compared to evaluate the comparative yield for both.

Extraction and crystallization of Kojic acid

Extraction of *Kojic acid* crystals was carried out by filtration of culture media by Watt man filter paper No. 1. The left mycelial mass was further used for wet and dry weight procedure as mentioned above. The left over solution was treated with ethyl Acetate and placed at 5°C in refrigerator (Model No. FYLC-400/FYLC-600) for 24 h. Crystals of KA were seen next day at the bottom of beaker, than filtration was carried out to attain them which were oven dried at 80°C for 01 hour (Figure 2 A and B) (Chaves, 2012).

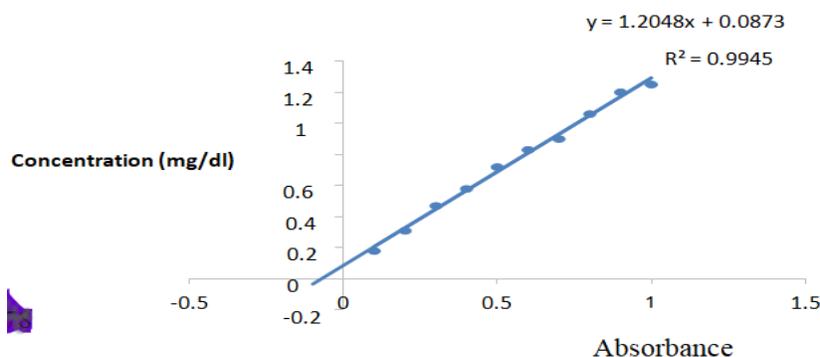


Figure 1 Standard Curve of Pure *Kojic acid*



Figure 2 A: extraction of *Kojic acid*; B: *Kojic acid* Crystals C: *Kojic acid* on TLC plate.
Thin Layer Chromatography (TLC)

It was carried out to authenticate the presence of *Kojic acid* in culture media. Three Dilute solutions; One for Standard *Kojic acid* and two for *A. flavus* and *A. oryzae* (self-prepared *Kojic acids*) were prepared (1 ml water should contained 1 mg of *Kojic acid*) in each dilute solution to further perform TLC procedure by using TLC plates coated with silica gel (Kisel gel 160F 254) and a line marked with A (standard *Kojic acid*), B (*Kojic acid* prepared with *A. flavus*) and C

(*Kojic acid* prepared with *A. oryzae*) (Fig 3 A). All 03 solutions were dropped on the plate and placed in mixture of water, acetic acid, acetone and ethyl acetate (1:1:3:5). After 15 minutes the plate was taken out and placed until dried. A solution of FeCl₃(1 g FeCl₃ with 850 μl of HCl and 100 ml of water) was sprayed on TLC plate to obtain observable spots of poured *Kojic acid* (Figure 2 C).

Fourier Transform Infrared Spectroscopy (FTIR)

To further authenticate the presence of *Kojic acid* in extracted crystals, Fourier Transform Infrared Spectroscopy (FTIR) at FTIR spectrometer (a Shimadzu Prestige 2, Apodization: Happ-Genzel spectrometer) had been performed by ACRC Department of PCSIR laboratories, Lahore, Punjab, Pakistan. Each spectrum was recorded from 4000 to 500 cm⁻¹ and 64 scans were accumulated for each spectrum.

High Performance Liquid Chromatography (HPLC)

It has been done as described by the protocol of Asma et al. (2018). Crystals of *Kojic acid* has been dissolved in 80 mL C₂H₅OH (80%), for one minute, filtered and stored at minus 20 °C till analysis. 5 mL aliquot of stored extract was dried under vacuum at room temperature and resuspended in one mL C₂H₅OH (80 %) and filtered through 0.45 um nylon 66 filter paper and injected 10 μL from this solution into the HPLC system. Perkin-Elmer model of HPLC with binary LC pump of 250, an LC 600 auto sampler, a UV/V with spectrometric UV detector (at 270 nm) of LC- 290, PV Nelson 900 series INTERFACE, Hewlett- HewlettPakkrad 3394 Integration and a Bondapak C-18 column (250*4.6 mm). Mobile phase was 0.5% orthophosphoric acid in CH₃ OH; 1 mL/ minute flow rate was set and Aglycone content was measured using Quercetin dihydrate (95%) as standard. Concentration was measured by using following formula

$$\text{Concentration of } Kojic \text{ acid} = \frac{\text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times \text{Concentration of Sample}$$

Patil et al. [13]. Twenty grams of onion sample, by removing dry leaves, was grounded with

RESULTS AND DISCUSSION

Effect of pH on *A.flavus*:

In the past, a number of research analysis on synthesis of *Kojic acid* lead to the conclusion that numerous *Aspergillus* species have ability to produce *Kojic acid*. During this work the impacts of varying pH was closely examined on the production of *Kojic acid* in submerge fermentation. It was found that at 20th day the maximum yield of *Kojic acid* crystals as g/L was obtained (13, 16,

9, 7, 5 and 4 under static culture condition and 8, 10, 6, 4 and 3) under non-static at pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 correspondingly. Moreover maximum amount of *Kojic acid* crystals formulated from *A. flavus* was obtained at pH 4.5 under Static and Non-static culture media. The highest yield of *Kojic acid* crystals at pH 4.5 under static and Non-static conditions was 16 g/L and 10 g/L respectively. Increase in pH of the culture media resulted negative impacts on quantitative concentration of *Kojic acid* and pH 4.5 was found to be most appropriate for the highest yield of *Kojic acid*. On the other hand, culture media of *A. flavus* at Non-static conditions gave low yield as compared to the ones treated at Static Conditions (Figure 3).

The subject research study results were found to be parallel to the results of Hazza *et al.*, (2013). Effect of high pH was significant on fungus metabolism. The metabolism of fungus shunted to some other pathways instead of *Kojic acid* production, so yield of *Kojic acid* reduced very much with high pH (Rosfarizan *et al.*, 2010). *A. flavus* showed highest yield of *Kojic acid* crystals at pH 4.0. With an increase in pH of the culture media the growth of fungus and yield of *Kojic acid* ceased (Kady *et al.*, 2014).

Effect of pH on *A. oryzae*

Yield of *Kojic acid* crystals significantly affected when high pH and conditions applied. At day 20, concentration of *Kojic acid* was 6, 4, 3, 2, 1 and 0.9 g/L at pH 3.5-8.5 respectively under Static conditions, whereas, under Non-static conditions the yield was 5, 3, 2, 1.6, 1.1 and 0.5 g/L at pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 respectively (Figure 5).

At 20th day, the best yield of crystals of *Kojic acid* has been obtained from *A. oryzae* at pH 3.5 (6 g/L under static and 5 g/L under non-static conditions). Moreover, Static condition has shown good results of yield as compared to that of Non-static. Acquired results are similar to the results of Hazza, (2013) and Hassan *et al.*, (2014). Same results was explained by Kady *et al.*, (2014), *A. oryzae* and its strains with an increase in pH give low yield of acid and growth of fungus. *A. oryzae* show high growth on low pH i.e. 2 while high *Kojic acid* production is obtained at pH 3.5. As explained by Basappa *et al.*, (1970), yield of *Kojic acid* severely reduced with a small deviation in pH and *A. oryzae* showed highest yield at pH 3.0 and increased pH decreased both yield of acid and fungal growth.

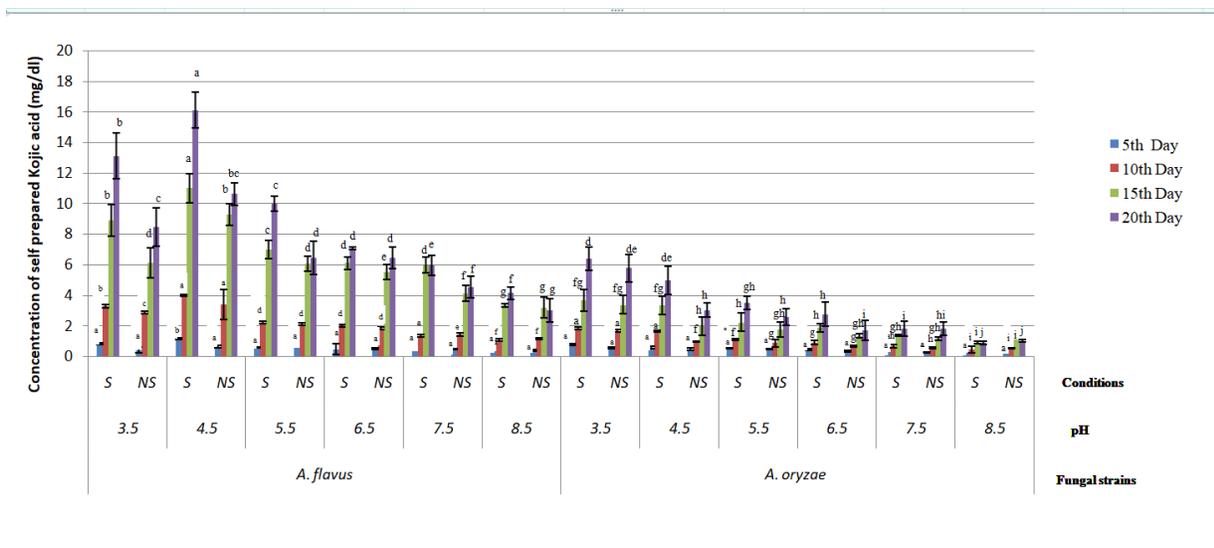


Figure 3 Concentration of self-prepared Kojic acid from *A. flavus* and *A. oryzae* in static and in non-static conditions on different days at different pH

S= static, NS= non-static, a= Maximum amount of *Kojic acid* (as per statistical analysis), j= Least amount of *Kojic acid* (as per statistical analysis)

Effect of temperature on *A. flavus*

Yield of *Kojic acid* crystals was strangely different at different temperature(s) (Figure 4) after 5, 10, 15 and 20 days. The growth of fungus was good at 25°C to 30°C and extraordinarily high at 30°C and unusually low at 40°C. At day 20 the obtained yield of *Kojic acid* crystals was 6, 12, 5 and 3 g/L at 25 °C, 30°C, 35°C and 40°C respectively. Highest yield of *Kojic acid* was 12 g/L at 30°C (Gqalen *et al.*, 1997 ; Ito, 2001).

Effect of temperature on *A. oryzae*

Temperature adversely affects the yield of *Kojic acid* as well growth of *A. oryzae*. High growth of fungus was seen when temperature is 25 °C to 30 °C (Figure 6). At 30 °C the yield of *Kojic acid* was amazingly high and at 40 °C it was extremely low. The yield obtained at day 20 was 2, 4, 1.0 and 0.2 g/L at 25 °C, 30°C, 35°C and 40°C correspondingly. Highest obtained yield was 4 g/L at 30°C and lowest as 0.2 g/L at 40 °C. A decrease in *Kojic acid* concentration with increase in temperature was observed, whereas, 28°C was found to be the best temperature by El-Aasar (2006) in *A. oryzae*. Same results are reported by Devi *et al.* (2014) and Rosfarizan *et al.* (2010) that an increase in temperature shows significant effect on yield of *Kojic acid*.

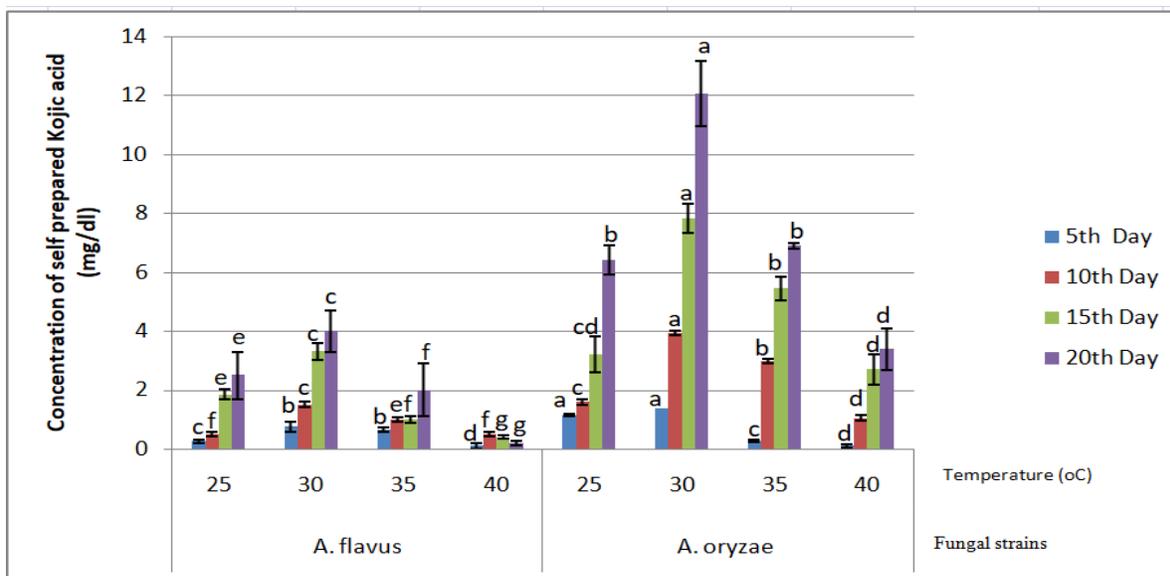
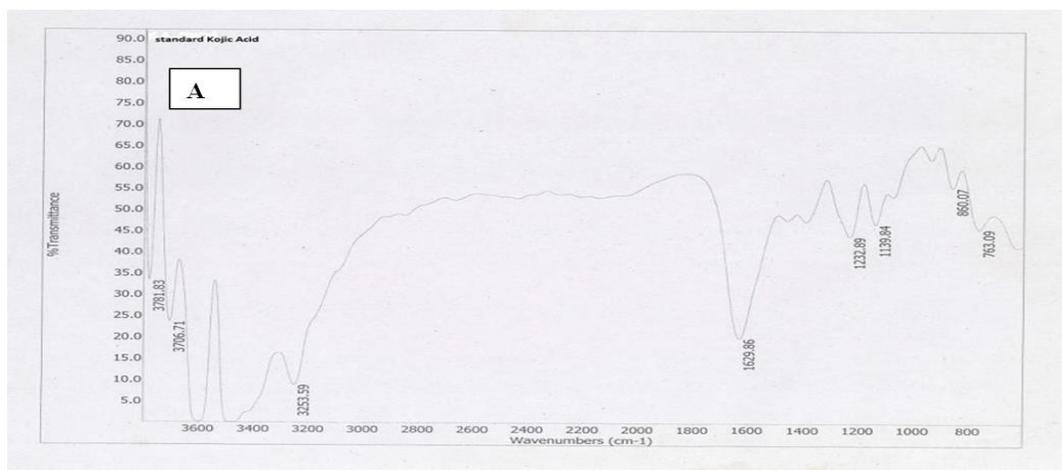


Figure 4 Concentration of self-prepared *Kojic acid* from *A. flavus* and *A. oryzae* at different temperature on different days.

a= Maximum amount of *Kojic acid* (as per statistical analysis), j= Least amount of *Kojic acid* (as per statistical analysis)

FTIR analysis of *Kojic acid*:

FTIR Spectrum analysis of the sample showed the presence of various active functional groups, which had been indicated by the peaks which presents the presence of Amines N-H stretch (at 3253.59 cm^{-1} and 3179.41 cm^{-1}) of Crystals. Samples also showed the presence of alkenes at 1629.86 cm^{-1} (Cyclic-C=C). Furthermore these samples showed the presence of alcoholic compound (O-H stretch) at $3600\text{ wavelength cm}^{-1}$ (Figure 5).



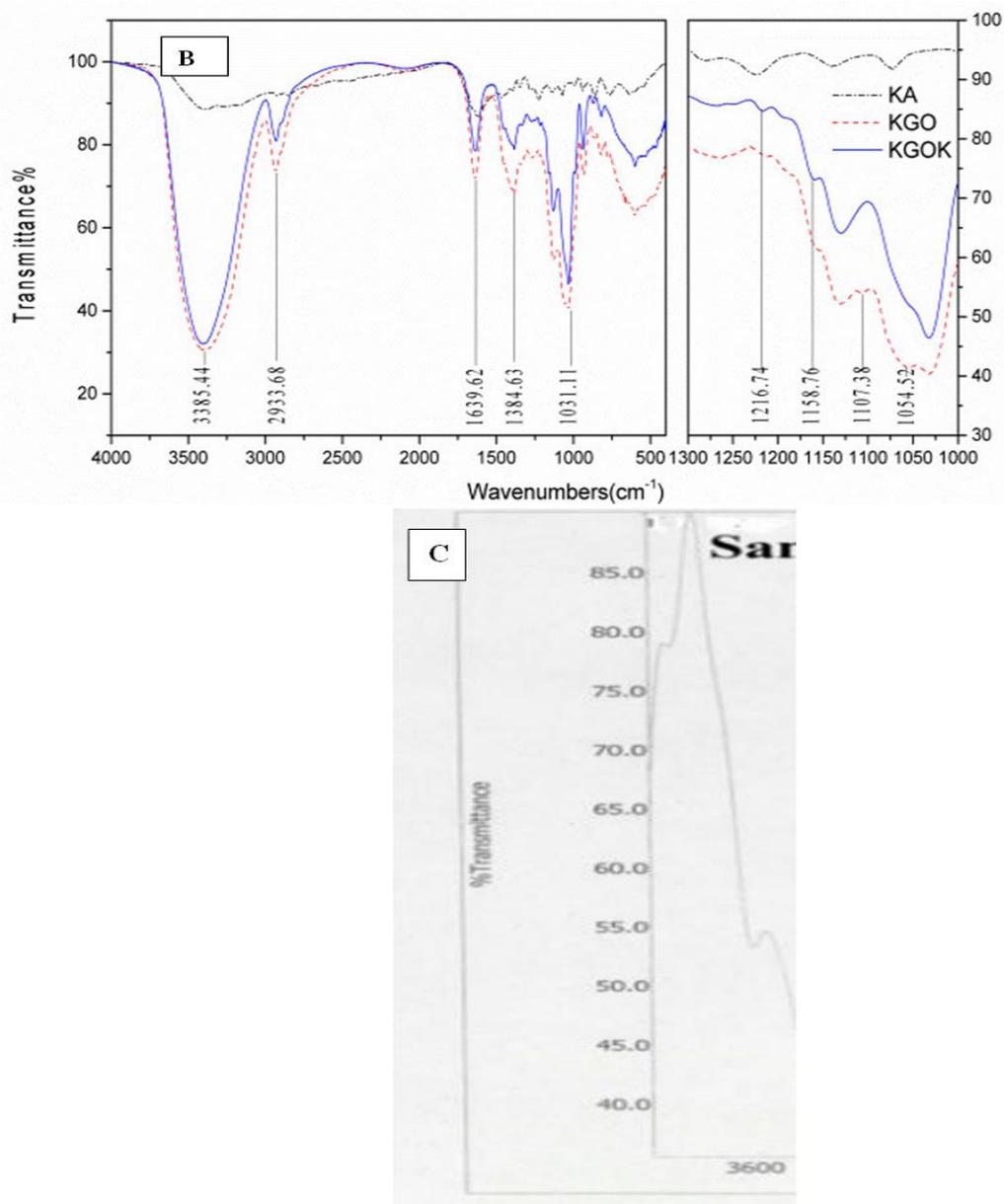


Figure 5 FTIR Spectrum of *Kojic acid* (KA) in the wavelength region 4000-400 cm⁻¹ from (A) *A. flavus* (B) *A. oryzae* (C) Standard *Kojic acid* crystals

4.6 HPLC of *Kojic acid*:

The best results obtained by HPLC of *A. flavus* and *oryzae* contained the compound with the same retention time as that of *Kojic acid* standard (Figure 6). All the experimental analysis (HPLC, TLC and FTIR) indicated that *Kojic acid* was produced by both fungus.

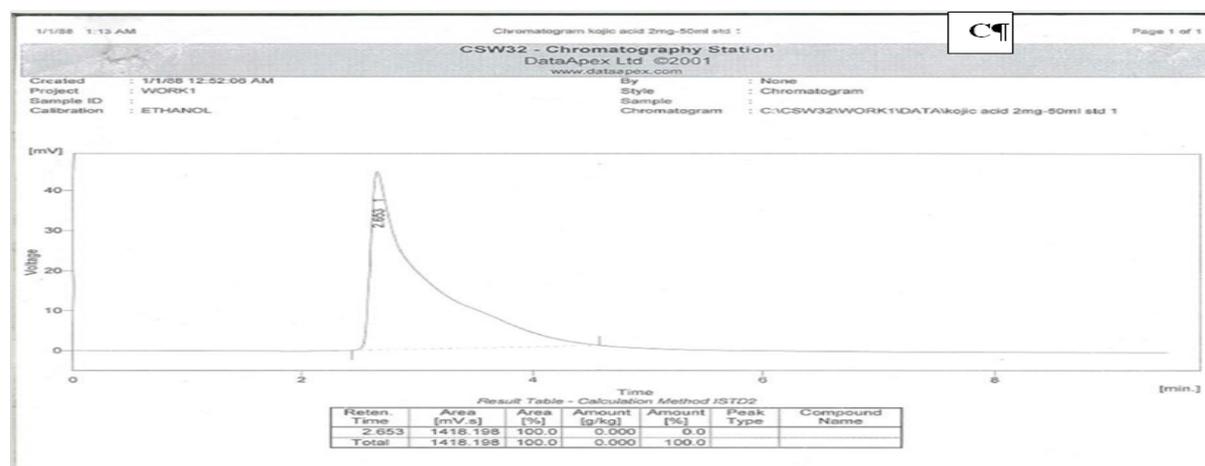
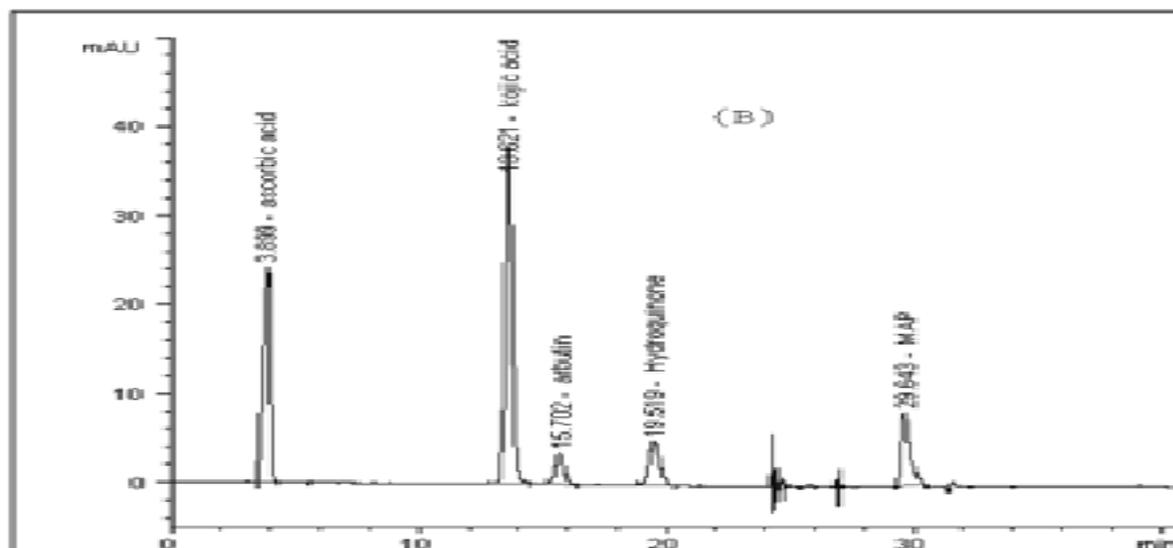
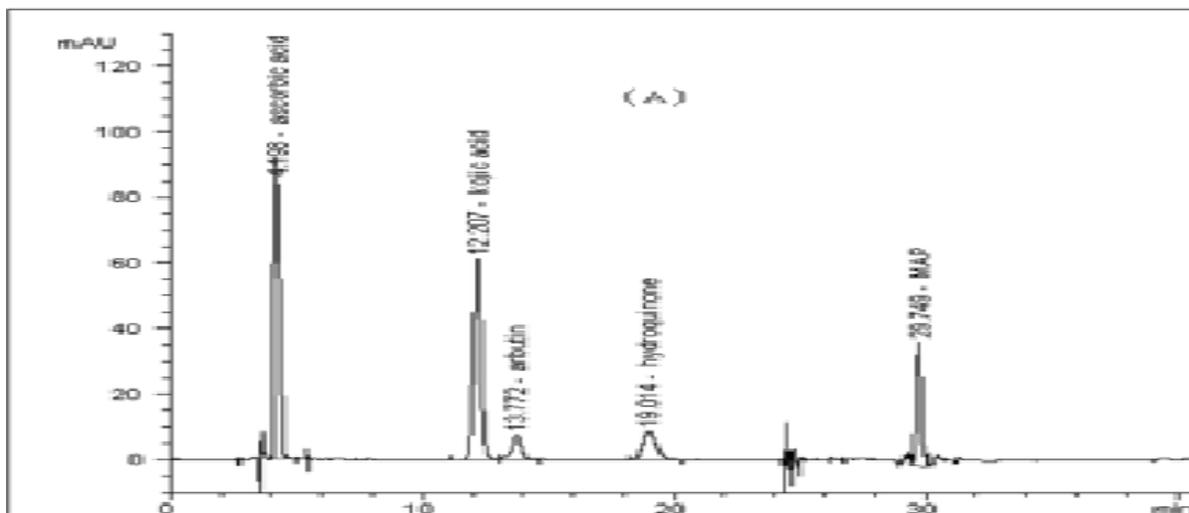


Figure 6 HPLC of standard *Kojic acid* shows *Kojic acid* peak from (A) *A. flavus* (B) *A. oryzae* (C) Standard

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