International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10, No.1, pp 550-559, 2017

Optimization study for the High Production of Kojic Acid Crystals by Estuarine *Aspergillus oryzae* RMS2 Isolate

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Abstract : The present study describes the optimal conditions for the high production of kojic acid crystals by *Aspergillus oryzae* RMS2 isolate. Total eight physico-chemical factors such as incubation time, pH, temperature, carbon sources, nitrogen sources, minerals, NaCl concentration and fed-batch fermentation, which significantly influence the production were screened using the one factor at a time method. The results concluded that the organism produces kojic acid with a maximum yield (64 g/l) with fed-batch fermentation and biomass dry weight 15 g/l at the 12th day of incubation, pH 5.0, temperature 30^{o}c, KH_2PO_4 (2 g/l), $MgSO_4$ (0.5 g/l), NaCl 2.5%, glucose and yeast extract in concentrations of 100 g/l and 5 g/l, respectively were the favorable factors for kojic acid production. On the other hand, total three agro-food waste substrates such as rice bran, wheat bran, ragi bran, wereused and those all substrates were subjected to acid hydrolysis. Rice bran given maximum production of 56 g/l kojic acid production followed by wheat bran 53 g/l and ragi bran 51 g/l.
Key words; *Aspergillus oryzae* RMS2, kojic acid crystals, carbon and nitrogen sources, fed-batch fermentation, agro-food wastes.

Introduction:

The name ‘’Kojic acid’’ was derived from the word ‘’Koji’’, a fungus used as a starter inoculums in oriental fermented food products in japan. This crystalline substance was firstly isolated by Saito in 1907[1], from the mycelia of *Aspergillus oryzae* grown on steamed rice. The chemical structure was determined as 5-hydroxymethyl-δ-pyrone by Yabuta in 1942[2]. Kojic acid crystallizes in form colorless and prismatic needles[11]. In food industry, kojic acid is used as an agent to prevent undesirable melanosis (blackening) of agricultural products such as vegetables, fruits and crustaceans during storage by inhibiting the action of polyphenol oxidase (PPO) enzyme[24, 25]. Apart from that, it is also used as an ‘antispeck’ agent in raw noodles during production processes. This is to avoid the color changes and black spot formation on noodles by inhibiting the tyrosinase enzyme[4].

In the chemical industry, kojic acid can be used as an analytical tool for iron determination since the reaction of kojic acid with the trace of ferric ion can form deep red complex[5]. Kojic acid also has been used as a substrate for chemicals synthesis of comenic acid and 2-methyl-4-pyrone. Comenic acid is an important intermediate for the synthesis of maltol and its derivative, while 2-methyle-4-pyrone is a compound which is normally associated with natural pigments[6, 23]. In the medical field, kojic acid and some of its derivatives are used in cosmetic preparations to achieve a skin-lightening effect by inhibiting melanin formation and through a UV light protective action[7,19].Kojic acid is used as a pain killer and anti-inflammation drug[8, 22]. In addition, kojic acid is used as an anti-bacterial and anti-fungal agent[9].

Materials and Methods

Isolation of Strain:

Soil samples were collected from Vellar Estuary, Portonovo (Lat. 11°29' N; Long. 79°46'), South East coat of India. Soil samples were serially diluted and inoculated into a Potato Dextrose agar plate (Hi-media) and incubated for 5 days at 37°C.Based on predominance and distinct morphological properties fungal isolate was selected and purified by repeated subculturing and streak plating.

Medium and fermentations:

The medium proposed by Madihah et al. (1992)[10]was used for inoculums preparation and kojic acid fermentation with slight modification. The medium consisted of (g/l) glucose, 100; yeast extract, 5; KH_2PO_4, 1; MgSO4. $7H_20$ 0.5 and NaCl 0.5 %; pH 6.0, temperature at 37°C. All submerged batch fermentation were carried out using 250ml Erlenmeyer flasks containing100 ml medium. After inoculation with spores (A standard inoculum of 1 ml of spore suspension approximately 1×10^6 spores /ml was used in all experiment), the flasks were incubated on a rotary shaker agitated at 100 rpm.

Screening for kojic acid production:

The isolate was screened using 100 ml of production media broth with 0.10g of $Fecl_3$ supplemented and inoculated 1 ml ofspore suspension; the flasks were incubated on a rotary shaker agitated at 100 rpm.

Phylogenetic analysis:

The strain showing a strong red color was picked and the fungal DNA was isolated using Nucleospin plant II kit (Macherey-Nagel). The DNA of the fungal isolate was amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700,Applied Biosystms).Purified PCR product was sequenced in ABI 3500 DNAAnalyzer(Applied Biosystems).The fungal isolate was identified based on sequence homology with fungal sequences obtained from the GenBank DNA database hosted by NCBI (http://blast.ncbi.nlm.nih.gov), using the BLAST search tool. The ITS sequence data was deposited into GenBank under the following accession number:KX756390 (*Aspergillus oryzae* RMS2).

Isolation of kojic acid:

The cultre broth was filtered through two layers of cheese cloth into another flask and maintained under refrigeration at 5°C. After one night of storage,the precipitated crystals were separated by filtration. The crystals were collected, dried at 80°C for 24 h and weighed[11].For further kojic acid extraction,the filtrate was then mixed with ethyl acetate and kojic acid crystals were recovered by evaporation and weighed. They were combined and purified by repeated crystallization from a mixture of acetone and water[11].

Deteremination of kojic acid and glucose:

Culture media were decanted, the mycelium was washed several times with distilled water and oven dried at 80°C for 24 hrs to get mycelia dry weight. The supernatant was used for kojic acid estimation according to colorimetric method of Bently (1957)[12]. Glucose was determined byDNS method[13].

Agro-Food Waste Substrates and acid Hydrolysis:

A total of three agro-food wastes viz. Rice bran, wheat bran, ragi bran were used and those substrates procured from the local markets of Parangipettai. Acid hydrolysis of hemicelluloses hydrolysate was carried out to cleave the xylooligosaccharies into monomeric sugars by autoclaving at 121°C with concentration of HCl varied from 2% v/v for min[14].

Results and Discussion

Screening for kojic acid production: when the kojic acid producer strain was grown on the medium, the color of the medium turned red at around 1 week. Kojic acid forms a chelated compound with ferric ions and subsequently generates a red color, which indicates presence of kojic acid.

Figure1.A) Growth of *Aspergillusoryzae* RMS2 showing Red color B) Control.

Figure 2.Kojic acid crystals

Effect of Incubation Time on kojic acid production:

The production medium flasks wereincubated in different incubation time ranging from 2 to 18 days, the effect of incubation time on kojic acid fermentation was determined by sampling the cultures 2days interval for 18 days. Kojic acid and biomass dry weight increased as fermentation progressed up to 12 days (49 and 14 g/l, respectively) and then decreased. The growth normally reached maximum after 6days and the production of kojic acid would start after about 2 days, whereby the production continued almost linearly until the exhaustion of glucose. After all supplies of glucose had been consumed, kojic acid accumulated in the culture may be utilised by microorganism to produce other substances such as oxalic and other acids[15], resulting in the decrease of kojic acid production[20]. The optimal incubation time was used for subsequent experimental runs.

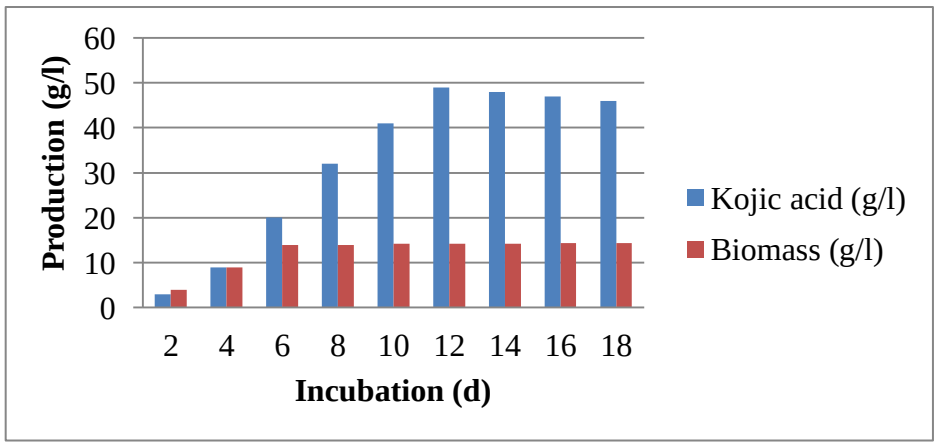

Fig.3. Effect of incubation time on kojic acid production by *Aspergillus oryzae* RMS2.

Effect of pH on kojic acid production:

Production medium with different pH 3.0,4.0,5.0,6.0,7.0,8.0 were tested, the pH of the medium was adjusted using 1 N NaOH or 1 N HCL. Maximum kojic acid production was achieved at pH 5.0 (51 g/l) and the pH of the fermentation solutions was decreased to around 3.9 after 12 days of cultivation. The growth rate of mycelia reached their maximum 14 g/l at pH 6.0. The optimum level of pH 5.0 obtained was maintained for the following experimental runs.

Table 1.Effet of pH on koji acid producton by *Apergillus oryzae* RMS2

Initial pH	P_m (g/l)	X_m (g/l)	Final pH
3.0	43	12.5	2.6
4.0	48	13	3.1
5.0	51	13.7	3.9
6.0	49	14	5
7.0	45	13.8	6.3
8.0	39	12.9	7.5

P_m, maximum kojic acid concentration; X_m, maximum cell concentration obtained during fermentation.

Effect of Temperature on kojic acid production:

Production medium with different temperature ranging from 20°C, 25°C, 30°C, 35°C, 40°C were tested at pH 5 for 12 days. Temperature was found to have a decided effect on kojic acid production and biomass yield. Maximum kojic acid production was achieved at 30°C (53 g/l). The highest biomass dry weight yield was appeared at 25°C (14 g/l). The optimum temperature 30°C obtained was maintained for the following experimental runs.

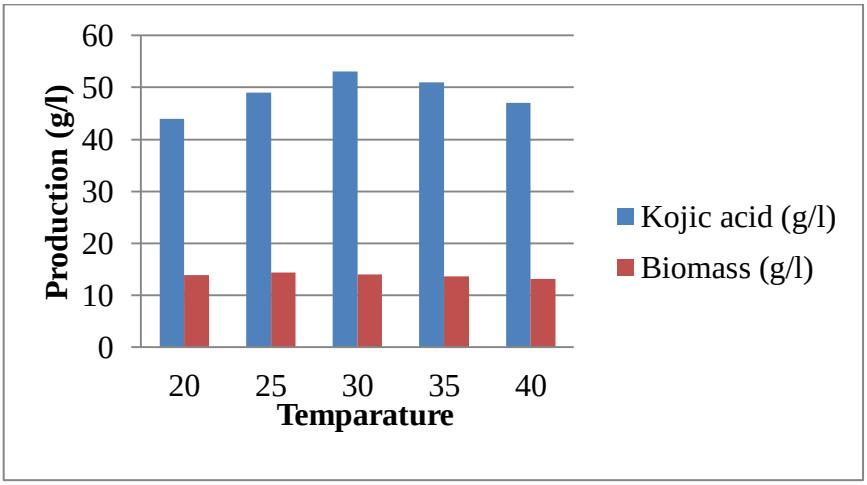

Fig.4. Effect of Temperature on kojic aid production by *Aspergillus oryzae* RMS2.

Effect of Carbon source on kojicacd production:

Production medium with different carbon sources such as 100 g/l of glucose, sucrose, xylose, fructose, starch, lactose, maltose and arabinose were used as carbon source.

Table .2. Effect of Carbon source on kojic acid production by *Aspergils oryzae* RMS2

Carbon source	P_m (g/l)	X_m (g/l)	$Y_{p/s}$ (g/g)	P (g/L.h)
Glucose	53	14	0.53	0.184
Sucrose	51	13.8	0.51	0.177
Xylose	49	13.6	0.49	0.170
Fructose	42	13.2	0.42	0.145
Starch	41	13.5	0.41	0.142
Lactose	33	12.9	0.33	0.114
Maltose	31	12.7	0.31	0.107
arabinose	21	12	0.21	0.072

P_m, maximum kojic acid concentration; X_m, maximum cell concentration obtained durng fermentation; Y_{ps}, Yield of kojic acid (g KA/g sugar); P, overall productivity after 288 hs.

The results showed that glucose was the best carbon source for kojic acid production followed by sucrose, xylose, fructose, starch, lactose, maltose and arabinose (53, 51, 49, 42, 41, 33, 31 and 21 g/l, respectively), in addition to biomass dry weight yield (14, 13.8, 13.6, 13.2, 13.5, 12.9, 12.7 and 12 g/l, respectively). Megalla et al.1986[16] suggested that , during the fermentation, kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments[22]. On the other hand, lactose and maltose are poor source of carbon for kojic acid production and very little when arabinose was used.

Effect of Glucose concentration on kojic acid production:

The results shown that glucose was found to be the best carbon source for kojic acid production, accordingly glucose was tested in production medium at different concentrations ranging from 25-150 g/l. At 25 and 50 g/l glucose, all supplied glucose was consumed for biomass built-up and kojic acid production was decreased. These results shown that 100 g/l glucose induced maximum kojic acid production (53 g/l), at 125 and 150 g/l glucose induced maximum biomass dry weight yield (15.5 and 16.2 g/l, respectively). The osmotic pressure apparently had an unfavourable effect since the production of the acid dropped off sharply, whereas, the biomass dry weight was increased[21].

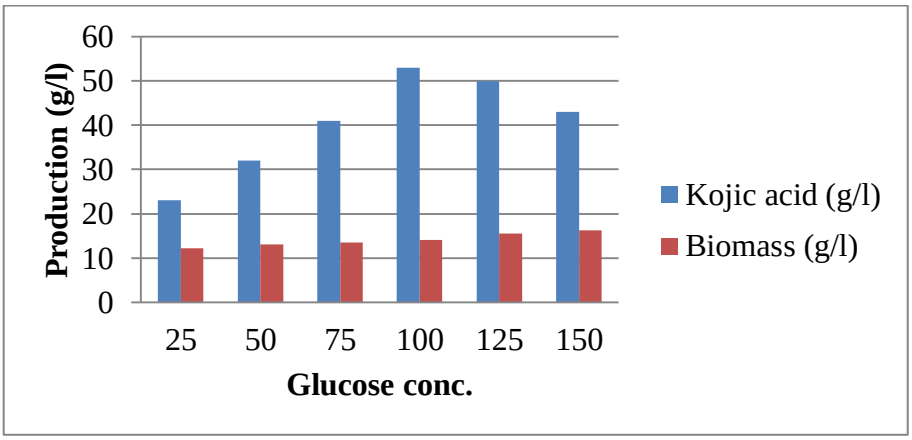

Fig.5. Effect of glucose concentration on kojic acid production by *Aspergillus oryzae* RMS2.

Effect of Nitrogen Source on kojic acid production:

Production media with Different nitrogen sources such as 5g/l of ammonium sulphate, ammonium nitrate, yeast extract, peptone, sodium nitrate were used as nitrogen source. The types of nitrogen source used greatly influenced both growth and kojic acid production. These results showed that the use of 5g/l yeast extract or peptone resulted in the highest kojic acid production (53 and 51g/l,respectively) compared with the other nitrogen sources. Yeast extract probably contain higher levels of other essential components required for

growth and fermentation, such as vitamins, amino acids and oligoelements[17]. The use of NH4+ ions as on inorganic nitrogen source may repress enzymes associated with kojic acid synthesis[3].

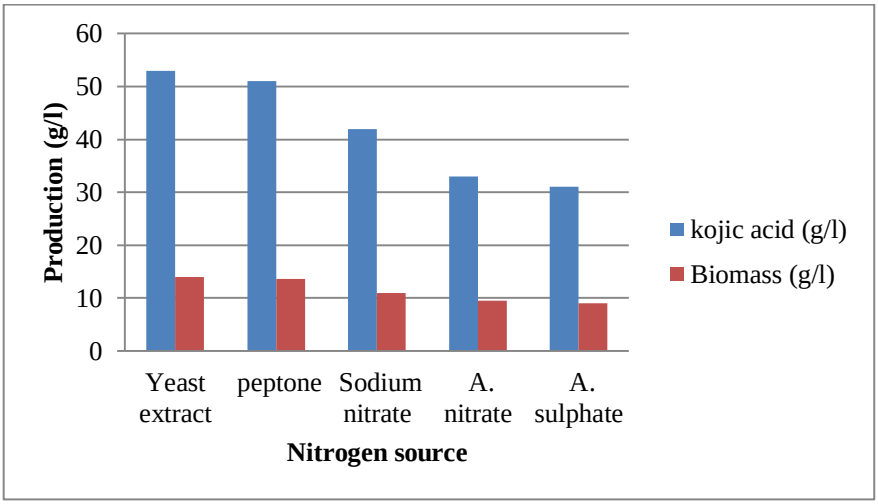

Fig.7. Effect of nitrogen source on kojic acid production by *Aspergills oryzae* RMS2.

Effect of yeast extract concentration on kojic acid production:

The results indicated that yeast extract was found to be the best nitrogen source for kojic acid production, accordingly yeast extract was tested in production medium at different concentrations (g/l) 1, 3, 5, 7, 10 while maintaining other parameters (incubation time, pH, temperature, and glucose) at their optimum levels. Biomass dry weight (4, 9, 14, 19, 24 g/l) increased proportionally with yeast extract concentration. The optimum production (53 g/l) was obtained at 5 g/l, at low yeast extract concentration 1 and 3 (g/l), slow mycelia growth was appeared and kojic acid (21 and 42 g/l, respectively) recorded very low. At high concentration of yeast extract 7 and 10 (g/l) in a medium, kojic acid production decreased (51 and 47 g/l, respectively), due to the presence of high yeast extract concentration, glucose cannot be converted to kojic acid but it was utilised for cell growth instead. Limitation of nitrogen supply is required to limit the growth so that more glucose can be converted to kojic acid.

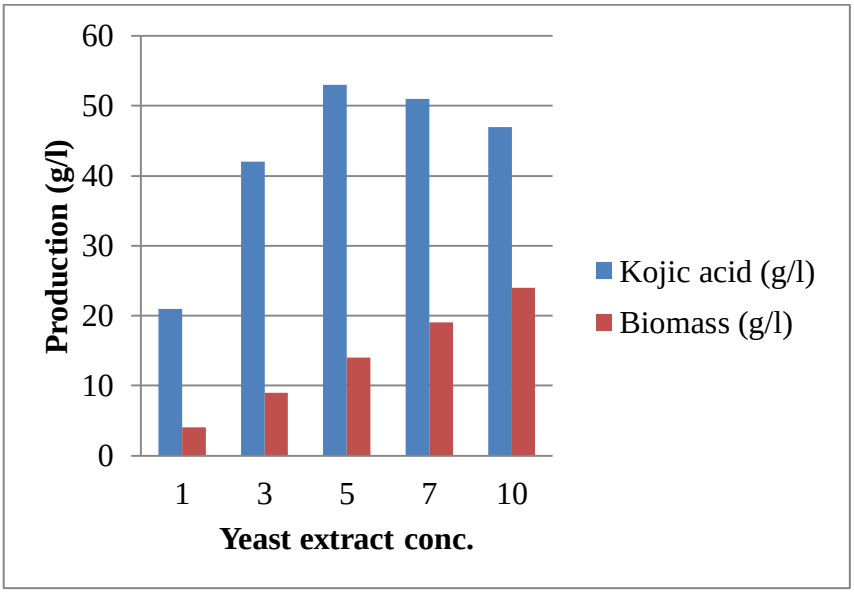

Fig.6. Effect of yeast extract concentration on kojic acid production by *Aspergillus oryzae* RMS2.

Effect of Minerals concentration on kojic acid production:

Different concentrations of KH_2PO_4 (0.5-2.5 g/l) and $MgSO_4$ (0.1-0.9 g/l) were added to production medium. Phosphate is an essential nutrient for the growth of most kojic acid producing fungi. It incorporates bio-molecules such as nucleic acids, phospholipids plays an important role in energy metabolism. The proper concentrations of phosphate KH_2PO_4 (2 g/l) in the culture broth gives a significant improvement on kojic acid production (55 g/l). On the contrary, at lower concentrations of phosphate (0.5 g/l), the rate of kojic acid was decreased (51 g/l). However, that the variation in $MgSO_4$ concentrations ranging from (0.1-0.9 g/l) gave no effect on kojic acid production by *Aspergillus oryzae* RMS2. The optimum mineral concentration 2 g/l obtained was maintained for the following experimental runs.

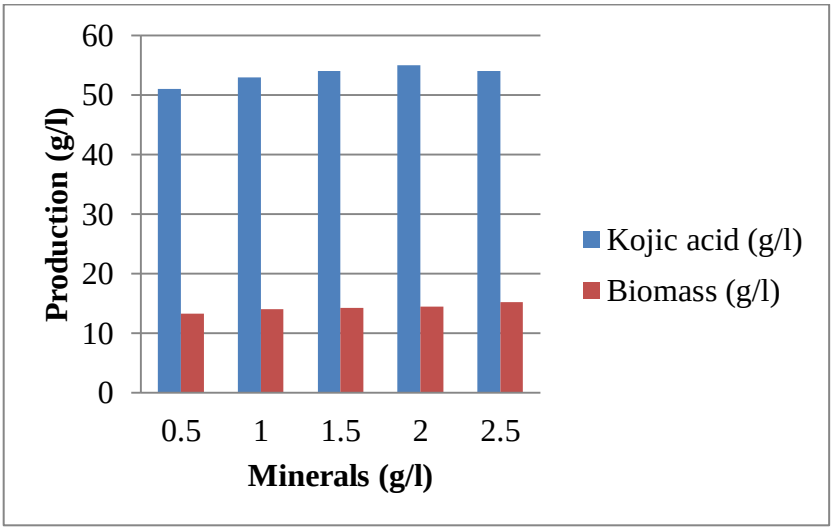

Fig.8. Effect of minerals on kojic acid production by *Aspergillus oryzae* RMS2.

Effect of NaCl on kojic acid production:

Different concentrations of NaCl (0.5%, 0.1%, 1.5%, 2%, 2.5%, 3%) were added to the production medium. At 2.5% of NaCl given maximum production 58 g/l and mycelia dry weight 15 g/l, above this concentration kojic acid production decreased 57 g/l.

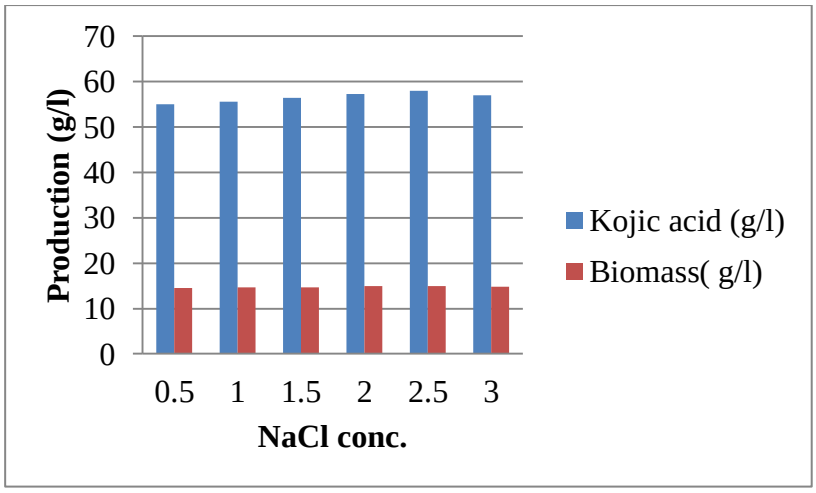

Fig.9. Effect of Nacl concentration on kojic acid production by *Aspergillus oryzae* RMS2.

Effect of Fed-Batch fermentation on kojic acid production:

Fed-Batch fermentation was carried out using 1000 ml of production medium with optimized conditions (Incubation 12 days: pH 5.0; Temp-30C; KH_2PO_4, 2g/l; NaCl, 2.5%). Initially 50g of glucose was

added to the production medium and another 50g of sterilized powdered glucose was added intermittently on 6th day to the initial batch fermentation after glucose supply was exhausted. In kojic acid fermentation, the fungi initially utilize the carbon source for growth and then synthesize the kojic acid in subsequent declining phase and early stationary phase[18]. In this technique of fermentation, the production of kojic acid was increased almost linearly up to about 64 g/l after 288 h. In addition, the yield (0.64 g kojc acid/g glucose) and productivity (0.222 g/L.h) obtained from the fed-batch fermentation were higher than that obtained from the batch fermentation (0.58 g kojic acid/g glucose and 0.201 g/L.h, respectively).

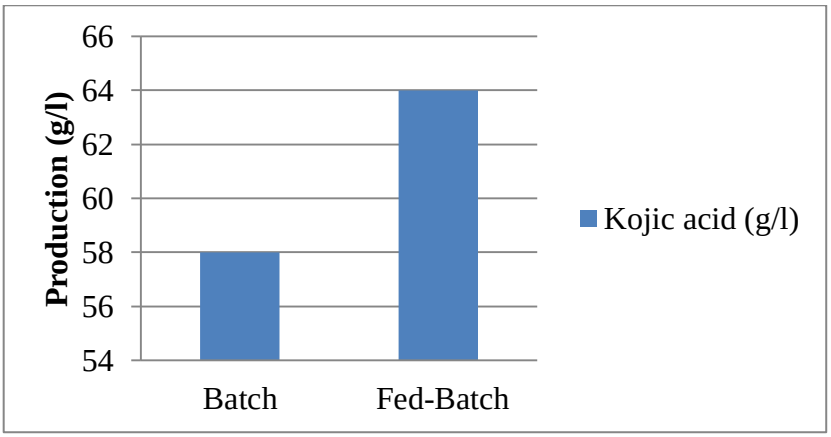

Fig.10. Effect of Fed-Batch fermentation on kojic acid production by *Aspergillus oryzae* RMS2.

Mass scale cultures for kojic acid production:

Based on the optimization results, the mass scale cultures 1000ml of kojic acid optimized production medium were prepared. Instead of glucose 100g of acid treated rice bran, wheat bran, ragi bran substrates were added to the production medium. After sterilization, 1ml of spore suspension inoculated and incubated for 12 days. Rice bran given maximum production of 56 g/l kojic acid followed by wheat bran 53 g/l and ragi bran 51 g/l.

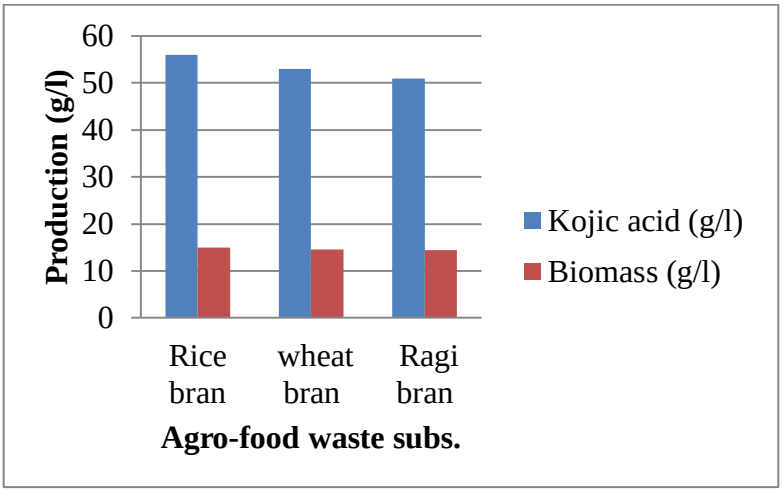

Fig.11. Mass scale kojic production using agro-food waste substrates by *Aspergillus oryzae* RMS2.

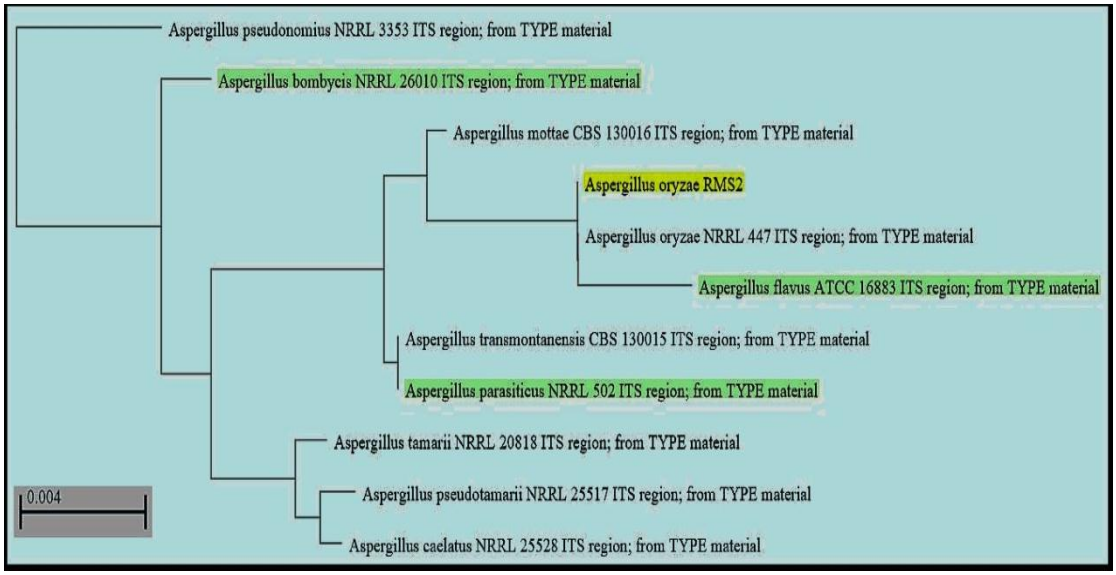

Fig.12. Phylogenetic position of fungal isolate based on internal transcribed spacer region (ITS) from fungi type and reference material. Strains used in this study are indicated yellow.

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