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Study of the Influence of Substrates on Kojic Acid Production by Estuarine *Aspergillus oryzae* RMS2 Isolate Under Solid State Fermentation Using Sugar Cane Bagasse as an Inert Substrate

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Authors' contributions

This work was carried out in collaboration between both authors. Author CRK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SJ managed the analyses of the study. Author CRK managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: In the present study, sugarcane bagasse was evaluated as an inert support for the production of kojic acid under solid state fermentation using two different production medium as moistening agents for the maximum production of kojic acid. Different parameters such as fermentation time, carrier size, moisture content, pH, temperature and inoculum size were optimized using One-Factor-at-a-Time approach (OFAT).
Study Design: Two fermentation mixed media were designed with 1:6:3 concentrations, where 1% is carrier, 6% carbon substrate and 3% nitrogen substrate. Such as medium I containing (g/100 g) sugar cane bagasse (carrier), 10; rice bran (carbon substrate), 60; ground nut oil cake (nitrogen substrate) 30; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; NaCl, 0.25. Medium II containing (g/100 g) sugar cane bagasse (carrier), 10; tea waste (carbon substrate), 60; sunflower oil cake (nitrogen substrate), 30; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; NaCl, 0.25.

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Place and Duration of Study: Sample: Soil samples were collected from Vellar Estuary, Portonovo (Lat. 11°29' N; Long. 79°46'), South East co at of India, between January 2015 and July 2016.

Methodology: Soil samples were serially diluted and inoculated in to a Potato Dextrose agar plate (Hi-media) supplemented with 0.10 g of $FeCl_3$ and incubated for 7 days at 35±2°C when the kojic acid producer strain was grown on the medium then the kojic acid reacts with ferric ions the color of the medium turned red at around 1 week. It was observed on reverse side of petri plate. Kojic acid estimated according to colorimetric method of Bently (1957).

Results: It was observed that sugarcane bagasse impregnated with medium I, pH 5.0, Temperature 30°C. at 80% moisture content (v/w), carr ier size 0.5 mm and inoculum size of 30 ml/100 g support produced 109 g/kg of Kojic acid after 14 days as compared to 81 g/kg before optimization.

Conclusion: The combination of rich carbohydrates and rich protein substrates produced higher production (M1, 109 g/kg and M2, 106 g/kg) as compared to un-optimized conditions (81 g/kg and 79 g/kg, respectively). In addition, the yield (M1, 0.109 g/g substrate and M2, 0.106 g/g substrate) and productivity (M1, 0.324 g/kg.h and M2, 0.315 g/kg.h) obtained from the optimization was higher than that obtained from the un-optimized conditions (0.081 g/g substrate and 0.079 g/kg.h, respectively).

Keywords: Screening; Aspergillus oryzae RMS2; kojic acid crystals; carbon and protein substrates; solid state fermentation.

1. INTRODUCTION

Solid-state fermentation (SSF) process can be defined as the growth of microorganisms on moist solid substrates in the absence of free-flowing water. They have considerable economical potential in producing products for the food, feed, pharmaceutical, and agricultural industries [1]. There are two types of SSF systems distinguished on the basis of the nature of the solid phase used. The first and most commonly used system involve use of natural materials that serve both as a support and a nutrient source; the second system, involves cultivation on an inert support impregnated with a liquid medium [2]. SSF using inert supports impregnated with chemically defined liquid medium has several potential applications in both scientific studies and in the industrial production of high-value products such as antibiotics and enzymes [3]. The advantages of SSF on inert support in comparison to SSF on natural solid substrates include [4]; (1) enhancing the homogeneous aerobic conditions, (2) improving process control and monitoring, (3) the inert carrier has less physical structure changes or can even be constant during fermentation, (4) improved control of heat and mass transfer, (5) higher evaporation rates and thus better control of temperature, (6) good control of water activity, (7) shrinkage and channelling are avoidable, (8) less complicated and easy product recovery, (9) easy to extract extracellular products with fewer impurities, (10) the inert carrier allows precise modification of production liquid media, (11) easy and possible process modelling and process control because the production media are known and can be analysed, (12) biomass can be measured directly, (13) suitable to grow any microorganism based on defined media and (14) the inert carrier can be reused. Some natural solid substrates such as sugarcane bagasse and rice hulls can be used as inert carrier due to their low nutrients but high porosity and ability to provide a very good support in terms of controlling mass and heat transfer [5].

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 [6], from the mycelia of *Aspergillus oryzae* grown on steamed rice. The chemical structure was determined as 5-hydroxymethyl-δ-pyrone by Yabuta in 1942 [7]. Kojic acid crystallizes in form colorless and prismatic needles. 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 [8]. Apart from that, it is also used as an 'antispeck' agent in raw noodles during production processes [9]. This is to avoid the color changes and black spot formation on noodles by inhibiting the tyrosinase enzyme.

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 [10]. 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 [11]. 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 [12]. Kojic acid is used as a pain killer and anti-inflammation drug [13]. In addition, kojic acid is used as an anti-bacterial and anti-fungal agent [14].

2. MATERIALS AND METHODS

2.1 Screening and 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 in to a Potato Dextrose agar plate (Hi-media) supplemented with 0.10 g of $FeCl_3$ and incubated for 7 days at 35± 2℃.

2.2 Raw Materials

There has been an increased exploitation of organic residues from various sectors of agriculture and industries over the past few decades. Crop residues such as bran, bagasse, oil cakes and fruit waste are utilised as potential raw material in bioprocesses as they provide an excellent substratum for the growth of microorganism supplying the essential nutrients to them. The Rich Carbohydrate substrates and the rich protein substrates are collected from local markets of parangipettai, Tamilnadu, India.

2.2.1 Rich carbohydrate substrates

Rice bran is one of the by-product obtained through paddy milling process. The nutritional value of rice bran per 100 g is protein 16.5 g, fat 21.3 g, minerals 8.3 g, crude fiber 11.4 g, carbohydrate 49.4 g, starch 24.1 g, free sugar 50 g and also contains vitamins. The energy value is 359 kcal. After making tea, that powder become waste material. Tea waste contains a large number of potentially bioactive chemicals, including flavinoids, amino acids, vitamins, caffeine, carbohydrates (monosaccharides 5%, polysaccharides 22%, pectins 6%, lignins 6%, cellulose and hemicellulose 7%,), crude fibre 11.70%, protein 17%, lipids 5%, mineral nutrients found in tea are selenium, and zinc (www.tocklai.org/activities/tea-chemistry/).

2.2.2 Rich protein substrates

Oil cakes are by-products obtained after oil extraction from the seeds. Oil cakes are of two types, edible and non-edible. Edible oil cakes have protein content ranging from 15% to 50% (www.seaofindia.com). Due to their rich protein content, they are used as animal feed. Groundnut oil cake consist 92.6% dry weight, 49.5% crude protein, 5.3% crude fibre, 0.11% calicium, 0.74% phosphorous. Sun flower oil cake has 34.1% protein content, 13.2% crude fibre, 0.30% calcium, 1.30% phosphorous.

2.2.3 Preparation of mixed substrate medium

Two fermentation media were prepared, with 1:6:3 concentrations, where 1% is carrier, 6% carbon substrate and 3% nitrogen substrate. Such as Medium I (M1) containing (g/100g) sugar cane bagasse (carrier), 10; rice bran (carbon substrate), 60; groundnut oil cake (notrogen substrate), 30; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; NaCl, 0.25. Medium II (M2) containing (g/100 g) sugar cane bagasse, 10; tea waste (carbon substrate), 60; sunflower oil cake (nitrogen substrate), 30; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; NaCl, 0.25. Sugar cane bagasse used as the basic inert substrate for SSF, was milled into 0.1 mm particle and KH_2PO_4, $MgSO_4.7H_2O$, NaCl, inoculum and distilled water used as moistening agents. The carbon substrates and protein substrates were ground and dried at 60℃ for 6 hours. The powders thus obtained were analysed for fermentable sugars and adjusted to pH 6.0. Medium I, 100 grams; medium II, 100 grams samples thus prepared were taken out separately into 500 ml Erlenmeyer flasks. The flasks rehydrated to required moisture content with initially 60% (v/w) relative humidity by adding moistening agents and the cotton plugged flasks were autoclaved at 121℃ at 15 psi for 15 mi then cooled to room temperature about 35±2℃. The contents of flasks were inoculated initially with 1 ml of inoculum/10 g

carrier (1×10^6 spores/ml) prepared with 0.1% Tween 80. Then the flasks were mixed thoroughly by gently beating on the palm of the hands and incubated in a slanting position in an incubator.

2.3 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 DNA Analyzer (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).

2.4 Isolation of Kojic Acid

To The culture 200 ml of water was added and the flasks kept in shaker for vigorous shaking at 180rpm for 2hours and filtered through two layers of cheese cloth into another flask and maintained under refrigeration at 5℃. After one night of storage, the precipitated crystals were separated by filtration. The crystals were collected (Fig. 1), dried at 80℃ for 24 h and weighed [15]. 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.

2.5 Deteremination of Kojic Acid and Glucose

Kojic acid estimated according to colorimetric method of Bently [15], where 1 ml of diluted sample was mixed with 1 ml of ferric chloride ($FeCl_3$) solution. FeCl3 solution can be prepared by dissolving 1 g of FeCl3.6H2O in 100 ml of 0.1 M HCL. The absorbance of the reaction mixture was measured using spectrophotometer at a wavelength of 500 nm. Glucose was determined by DNS method [16].

3. RESULTS AND DISCUSSION

3.1 Screening for Kojic Acid Production

When the kojic acid producer strain was grown on the medium then the kojic acid reacts with ferric ions 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, it was observed on reverse side of petri plate (Fig. 2), other strains don't show the red color. The positive strain was selected for phylogenetic analysis and production optimization.

3.2 Effect of Incubation Time on Kojic Acid Production

The production medium flasks were incubated 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 2 days interval for 18 days. Fig. 3 shows that the kojic acid production yield varied with incubation time, Kojic acid increased as fermentation progressed up to 14 days (M1, 81 and M2, 79 g/kg) and then decreased. 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 [17], resulting in the decrease of kojic acid production [18]. The optimal incubation time was used for subsequent experimental runs.

3.3 Effect of Carrier Size on Kojic Acid Production

The sugarcane bagasse of five different particle size was used to get the optimal size for the maximum production of kojc acid viz., o.1mm, 0.3 mm, 0.5 mm, 0.7 mm, 0.9 mm fermentation was carried out at initial 60% (v/w) relative humidity, for 14 days. The particle size properties of solid substrates will lead to the shape, accessible area, surface area and porosity of the solid substrates (Richard et al. [19]). Processes like chopping, grinding and cutting create a condition for microorganisms to be active at the initial stages of growth and increase the degradation and hydrolysis rate since the solid substrate is insoluble. Fig. 4 shows that the kojic acid production yield varied with carrier size and optimum production yield (M1, 87 g/kg and M2,

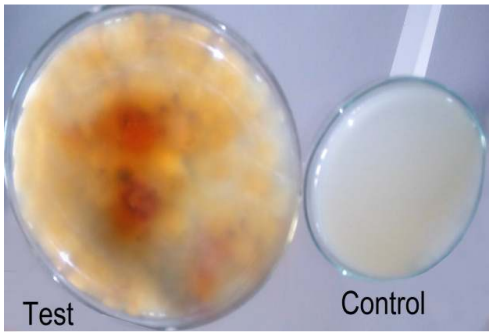
84 g/kg) was obtained for particle size of 0.5 mm, whereas the kojic acid yield was found to be reduced for larger particles (>0.5 mm) as well as for smaller particales (<0.5 mm). Smaller particle size would provide a larger surface area per volume and allow full contact of microorganism with the nutrients but the diffusion of oxygen would be affected and small particle size may be lead to clumping of substrates, resulting in reduced accessibility to nutrients leads to anaerobic cultre conditions with lower yield. Larger particle size provides small area per volume ratio and gives excellent diffusion of oxygen. The optimal carrier was used for subsequent experimental runs.

3.4 Effect of Moisture Content

Optimization of moisture content on kojic acid was carried out by incubating the cultures at 60%, 65%, 70%, 75%, 80% and 85% (v/w). The moisture content was adjusted by considering total volume that includes the distilled water, mineral salt solution and inoculum suspension. Initial moisture content is a critical factor for growth and metabolite production. Moisture content is intimately related to the definition of SSF, because it is necessary for new cell synthesis. Optimal moisture content depends on the nature of microorganism and the substrate used [20]. Fig. 5 shows the effect of initial moisture content on kojic acid production using sugarcane bagasse as an inert support. Initial moisture content of 80% supported maximum production of kojic acid (M1, 95 g/kg and M2, 93 g/kg). Further increase in moisture content did not increase the production; as well as the medium was found to be free flowing in flask. The optimum moisture content attained was applied for subsequent experimental runs.

3.5 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. Table 1 shows the effect of pH on kojic acid production, both M1 and M2 Medium achieved Maximum kojic acid production at pH 5.0 (98 and 96 g/kg, respectively) and the pH of the fermentation solutions was decreased to around 4.0 after 14 days of cultivation. The optimum level of pH 5.0 obtained was maintained for the following experimental runs.



Figs. 1 and 2. Kojic acid crystals; Growth of *Aspergillus oryzae* RMS2 showing red color

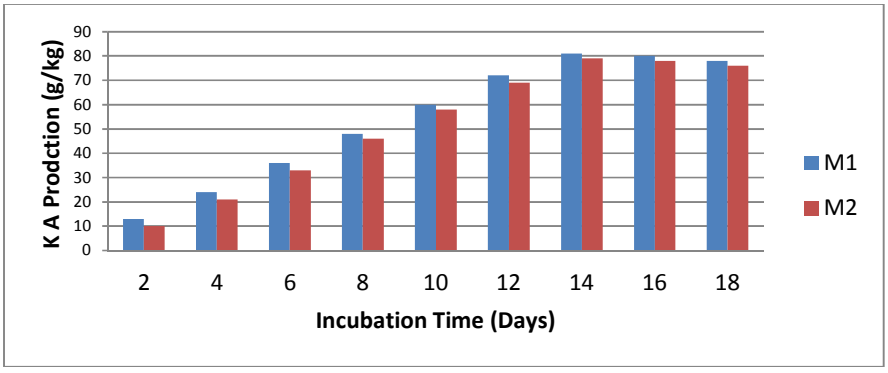


Fig. 3. Effect of incubation time on kojic acid production by *Aspergillus oryzae* RMS2

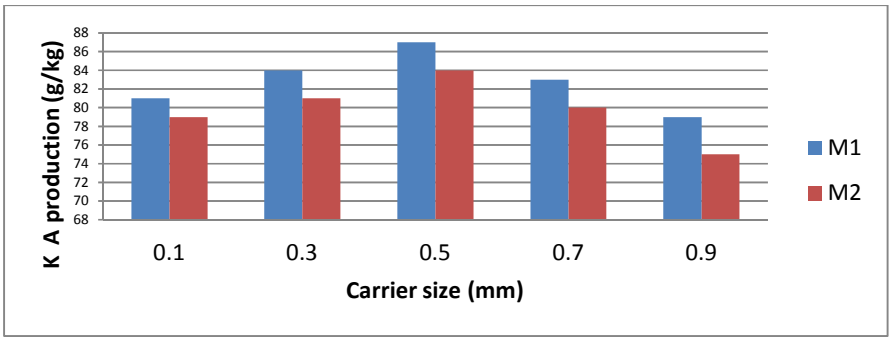


Fig. 4. Effect of carrier size on kojic acid production by *Aspergillus oryzae* RMS2

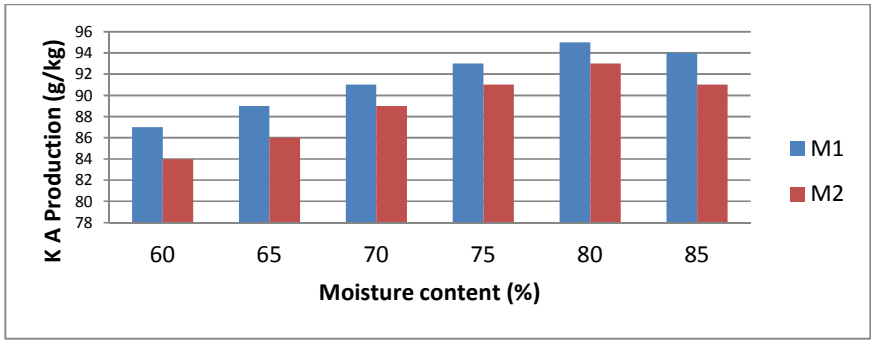


Fig. 5. Effect of moisture on kojic acid production by *Aspergillus oryzae* RMS2

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

Initial pH	Mixed medium production, P_m (g/kg)	Final pH
3.0	M1, 91; M2, 89	M1, 2.6; M2, 2.7
4.0	M1, 96; M2, 94	M1, 3.2; M2, 3.3
5.0	M1, 98; M2, 96	M1, 4.0; M2, 4.0
6.0	M1, 95; M2, 93	M1, 5.3; M2, 5.4
7.0	M1, 89; M2, 87	M1, 6.4; M2, 6.5
8.0	M1, 77; M2, 75	M1, 7.5; M2, 7.6

P_m, maximum kojic acid concentration obtained during fermentation

3.6 Effect of Temperature on Kojic Acid Production

Different temperature ranging from 20℃, 25℃, 30℃, 35℃, 40℃ were tested at pH 5 for 14 days. Temperature was found to have a decided effect on kojic acid production. The problem regarding temperature arises during the SSF process due to the heat generated from microbial activity and accumulated in the system [21]. The heat needs to be removed from the system to avoid overheating and thereby disturbing the growth of microorganisms and the formation of products. Fig. 6 shows the effect of temperature on kojic acid production, maximum kojic acid production was achieved at 30℃ (M1, 100 and M2 98 g/kg). The optimum temperature 30℃ obtained was maintained for the following experimental runs.

3.7 Effect of Inoculum on Kojic Acid Production

Optimization of inoculum size was carried out by adding 1 ml, 2 ml, 3 ml, 4 ml and 5 ml to the production medium. Inoculum can be described as a preparation containing high number of viable cells, which may be added to bring about desirable changes in the solid substrate [22]. Fig. 7 shows the effect of Inoculum on kojic acid production, inoculum of 3 ml/ 10 g of inert support gave maximum production (M1, 109 g/kg and M2, 106 g/kg). Further increase in inoculum size did not increase the yields further, we observed at 4 ml and 5 ml inoculum causes over growth of mycelium and it utilised the substrates for mycelium growth and not enhanced the kojic acid production.

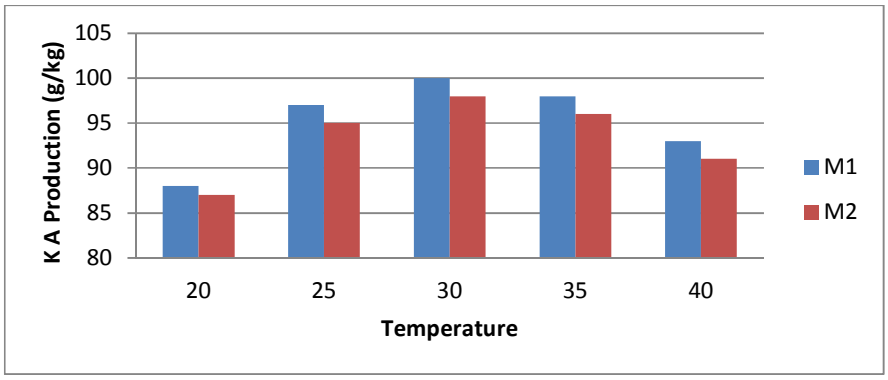


Fig. 6. Effect of temperature on kojic acid production by *Aspergillus oryzae* RMS2

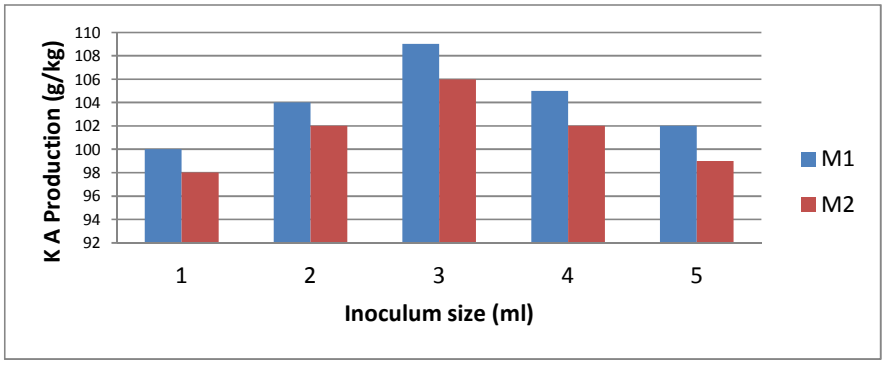


Fig. 7. Effect of inoculum on kojic acid production by *Aspergils oryzae* RMS2

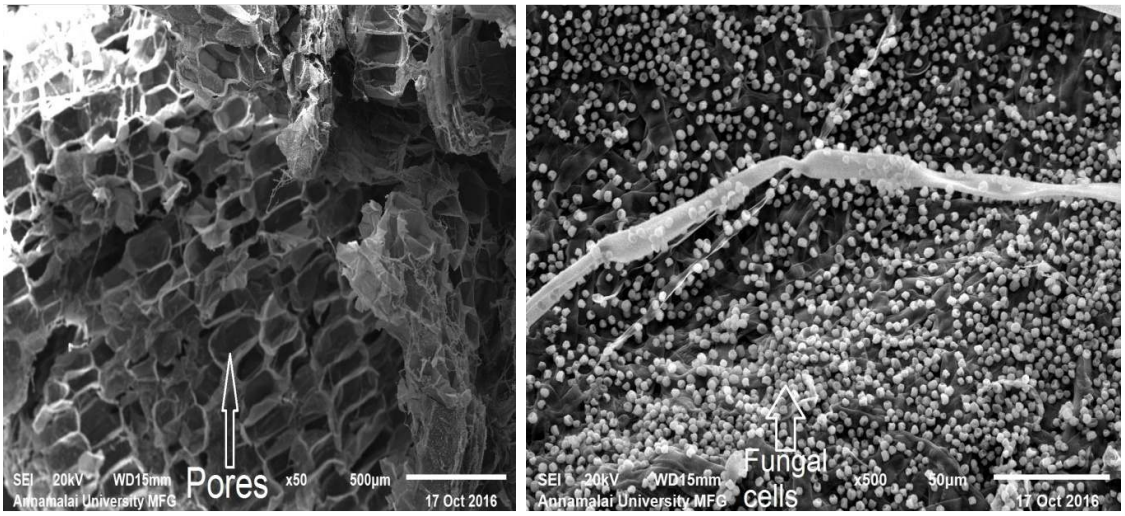


Fig. 8. Scanning electron microscopic images

3.8 Electron Microscopic Scanning

The dried sample were fixed on a specimen holder with tape and then sputtered with platinum in sputter-coater under high vacuum condition. Each sample was examined at 50x and 500x-fold magnification.

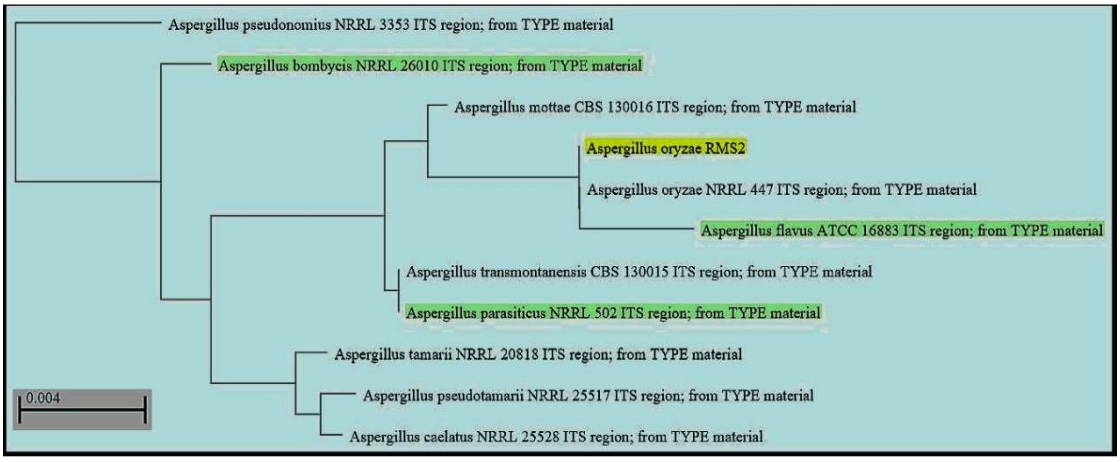


Fig. 9. Phylogenetic position of fungal isolate based on internal transcribed spacer region (ITS) from fungi type and reference material. Strains used in this study are indicated yellow

4. CONCLUSION

Scale-up was carried out using 1kg substrates (M1 and M2) with optimized conditions (Incubation, 14 days; carrier size, 0.5 mm; moisture content, 80%; pH, 5.0; temperature, 30℃; inoculum, 30 ml/100 g carrier) in 5000 ml flasks and kojic acid was isolated by using 2000 ml of water then by shaking the flasks at 180 rpm for 2 hours. The combination of rich carbohydrates and rich nitrogen substrates produced higher production of kojic acid (M1, 109 g/kg and M2, 106 g/kg) as compared to un-optimized conditions (81 g/kg and 79 g/kg, respectively). In addition, the yield (M1, 0.109 g/g substrate and M2, 0.106 g/g substrate) and productivity (M1, 0.324 g/kg.h and M2, 0.315 g/kg.h) obtained from the optimization was higher than that obtained from the un-optimized conditions (0.081 g kojic acid/g substrate and 0.079 g kojic aid/kg.h, respectively). Sugar cane bagasse was found to be an efficient solid support for kojic acd production, pores and as a low nutrient substrate enhances the productivity, very cost-effective and eco-friendly rather than using synthetic or semi synthetic solid supports.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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