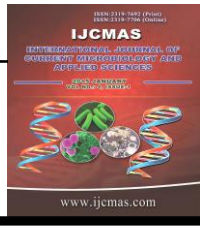
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 3 (2015) pp. 980-987**
http://www.ijcmas.com



Original Research Article

Microbial Production of Shikimic Acid, a Precursor of Neuraminidase Inhibitor- Antiviral Drug

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Keywords

Shikimic acid, Mutants, Coliforms, Periodate

A B S T R A C T

Shikimic acid (SA) is a high valued compound used in the synthesis of antiviral drugs. It is a key starting material utilized in the synthesis of neuraminidase inhibitor oseltamivir-phosphate, which is developed under the name of Tami flu for the treatment of antiviral infections. Shikimic acid is obtained via tedious multi-step isolation procedures from plants. The extraction yields of shikimic acid from plants are low therefore; it would be desirable to provide an alternate method to produce large quantities of shikimic acid. The main alternative to this process is fermentation using microorganisms producing shikimic acid. In the present work, 20 coliforms isolates were tested for its ability to produce shikimic acid. To increase the production of shikimic acid, 8 isolates producing shikimic acid more than 8 ug per ml were exposed to U.V.rays for different intervals of time. Two mutants in which the production was increased by 20% and 27% were selected further for optimization of fermentation condition.

Introduction

Shikimic acid (SA) is a high valued compound used in the synthesis of antiviral drugs. It is a key starting material utilized in the synthesis of neuraminidase inhibitor oseltamivir-phosphate, which is developed under the name of Tami flu for the treatment of antiviral infections. (Kramer et al, 2003,Ahn et al, 2008)

Shikimic acid is an attractive chiral synthon with its highly functionalized; six-membered carbocyclic ring and multiple asymmetric centers. A metabolic intermediate of aromatic amino acid biosynthesis, shikimic acid has emerged as an essential chiral starting material in the synthesis of neuraminidase inhibitors effective in the treatment of influenza. (Kim et al, 1997, Rohloff et al, 1998; Chandran et al ,2003). Chiral, as well as aromatic chemicals can also be synthesized from shikimic acid. For example acid catalyzed dehydration of shikimic acid produces p-hydroxybenzoic acid. P-Hydroxybenzoic acid, which has an annual production of 7×10^6 kg,, is the key precursor to parabens and a monomer used in the synthesis of liquid crystal polymers. Shikimic acid has also recently been used

as the starting point for synthesis of a large combinatorial library of molecules. (Shirai et al , 2001; Escalante et al,2010).

Shikimic acid is obtained via tedious multi-step isolation procedures from plants. Currently shikimic acid is produced from the fruit of *Illicium anisatum* or star anise, a Chinese plant. (Haslem, E. 1993)Against the fear of spread of potential flu pandemic, making large quantities of drug is a challenge before the pharmaceutical industry .Star anise is harvested only from March to May. The extraction yields of shikimic acid from plants are low because most of the secondary metabolites accumulate at low levels in plant cells. (Raghavendra et al, 2009; Nakagawa, et al 2011) Therefore, it would be desirable to provide an alternate method to produce large quantities of shikimic acid.

The main alternative to this process is fermentation using microorganisms producing shikimic acid. According to a 2003 review, fermentation of shikimic acid could provide, in principle, an unlimited supply of the raw material. And researchers are hard at work to improve the fermentation process, making it even easier to generate the drug's key ingredient. Shikimic acid is a natural intermediate in the formation of microbial amino acids. It is accumulated in the small quantities in many Gram negative bacteria in which aromatic amino acid biosynthetic pathway is functional (Zhou et al, 2008; Chemler et al, 2008 ; Marner W. D. 2009).In this context, the present work is aimed to study the production of shikimic acid from the coliforms isolated from natural environment.

Materials and Methods

Isolation and identification of coliforms

As per literature reports shikimic acid is produced by coliform group of bacteria therefore in the present work coliform bacteria were used for production of shikimic acid. Coliforms were isolated by inoculating one ml water sample obtained from different sources like river, sewage and effluent in 5 ml of MacConkey's broth. Tubes were incubated at 37 ^{0}C for 24 hrs. A loopful from the tubes showing gas production was inoculated on MacConkey's agar plates. Plates were incubated at 37 ^{0}C for 24 hrs. Organisms producing dark pink colored colonies were selected as coliforms. Total 20 isolates were selected for these studies. The isolates were identified up to species level on the basis of cultural, morphological and biochemical characterization.

Production of shikimic acid

Inoculum preparation

A loopful of 24 hours growth of each coliform isolate from nutrient agar slant was inoculated in 10 ml of inoculum medium having composition per liter, Bacto-Tryptone (10 gms), Bacto-Yeast Extract (5 gm), NaCl (5 gms), pH 7. All the tubes were incubated at 37 ^{0}C for 24 hrs.

Production of shikimic acid

Five percent inoculum was transferred in the production medium having composition per liter, Glucose (50.00 gms), Monopotassium Phosphate (1 gm), Ammonium Sulphate (25 gms), Magnesium Sulphate - 7 H_2O (00.40 gm), L – Tyrosine (00.10 gm), L – Tryptophan (00.10 gm), L – Phenylalanine (00.10 gm), P – Aminobenzoic Acid (01.00 mg), Ferrous Sulphate - 7 H_2O (09.90 mg), Manganese Sulphate - 4 H_2O (07.20 mg), Zinc Chloride (25.00 mg), Copper Sulphate - 7 H_2O (00.50 mg), Calcium Carbonate (20.00 gms), pH 7. All the flasks were incubated at 37 ^{0}C for 24

hours. After incubation 10 ml sample was removed, extracted and used for assay purpose.

Extraction of Shikimic acid

After incubation period of 24 hours, 10 ml of fermented broth was removed aseptically and centrifuged at 10,000 rpm for 30 minutes. Supernatant was collected and used as a product for the assay purpose.

Detection of Shikimic acid

Shikimic acid produced was detected by Periodate method (Gaitonde and Gordon, 1957). In this method shikimic acid is oxidized by periodic acid to give trans-aconitic acid and a dialdehyde. In alkaline condition it produces yellow colour which is measured UV spectrophotometrically.

Quantification of shikimic acid

To 3 ml of supernatant 0.5 ml of 1% periodic acid was added. After mixing, the mixture was incubated for 3 hours at 37 ^{0}C. Then 0.5 ml of 1N NaOH was added and immediately 0.3 ml of 0.1 M Glycine was added and within 10 minutes. Optical density was read at 380 nm in UV spectrophotometer. Concentration of Shikimic acid was determined by using standard shikimic acid (Hi Media Pvt. Ltd.) in 1ug to 10 ug per ml range.

Mutation of selected isolates:

To increase the yield of shikimic acid, mutation was carried by physical mutagenesis using UV irradiation. Seven Isolates (C1, C8, C9, C12, C16, C18 and C19) giving maximum production of shikimic acid were selected for the mutation. All selected isolates were streak inoculated on fresh sterile nutrient agar slants for the preparation of active culture. After obtaining active culture purity was checked and one loopful culture of each isolate was inoculated in 5 ml of sterile nutrient broth separately and incubated for 24 hours. After incubation serial dilutions of inoculum were prepared. 10^{-5} dilution of every culture was spread inoculated on sterile MacConkey's agar plates. These plates were exposed to UV rays for 5, 10, 15, 20 and 30 seconds respectively along with control. All plates were incubated at 37 ^{0}C for 24 hours. After incubation well isolated, dark pink coloured colonies were selected as mutants. All selected mutant colonies were tested for shikimic acid production. And results were compared with wild isolates.

Optimization of fermentation condition:

Various parameters that enhance the production of shikimic acid were investigated by optimizing the fermentation conditions. The parameters studied were incubation period (0 to 120 hrs), concentration of inoculum (1 to 7%), effect of different carbon and nitrogen sources and concentration of glucose (1 to 7%). Different carbon sources used were glucose, fructose, lactose, sucrose, maltose and glycerol while nitrogen sources studied were ammonium sulphate, ammonium nitrate, ammonium chloride, asparagine, urea and peptone.

Partial Purification of Shikimic acid

Partial purification of shikimic acid was carried out using ethanol. 5 ml of extracted sample and 5 ml of ethanol was mixed in a tube. After proper shaking precipitate formed was kept in deep fridge for overnight. Supernatant and precipitate was separated. Supernatant was kept for evaporation. 50% of evaporated sample was used for the thin layer chromatography.

Detection of Shikimic acid by thin layer chromatography

Thin layer chromatography was performed on silica gel TLC plates (Misra and Dey, 2013). Standard shikimic acid purchased from Hi Media Pvt. Ltd and partially purified sample obtained in the present work were manually spotted using a capillary. Plates were developed in Ethyl acetate : Methanol : Formic acid : Water (8:1:1:1) to the top and dried at 110^0C for 10 minutes before use. Dried plates were sprayed with 60% aqueous sulphuric acid and observed for development of brown charred spots. Rf values were calculated and compared with standard.

Result and Discussion

20 coliforms were isolated from water samples collected from different sources like sewage, river water and effluent. All these isolates were identified on the basis of cultural, morphological and biochemical characterization. The isolates identified were *Escherichia coli* (9), Citrobacter Spp. (6) and Enterobacter spp. (5) by referring Bergey's Manual of Systematic Bacteriology.

Ability of shikimic acid production of all 20 isolates was checked. Quantification of shikimic acid was done by using periodic acid assay method. Out of 20 isolates 7 isolates giving production of shikimic acid more than 8 ug/ml (C1, C8, C9, C12, C16, C18, and C19), were selected for the further studies. The yield of shikimic acid in ug/ml of these isolates were 11.4, 10, 12.2, 11.0, 8.2, 12.5, 8.3 respectively (Figure 1). To improve the production of shikimic acid, mutation of selected 7 isolates was done by physical mutagenesis using UV radiation. 11 mutants obtained on MacConkey's agar plate were selected for shikimic acid production. The production of shikimic acid was increased in M2 and M4 mutants by 20.17% and 27.19% compared with its wild strain (Figure 2).

While optimizing the fermentation condition, M2 and M4 mutants and its wild strain C1 *Escherichia coli* were selected. The maximum production of shikimic acid was obtained after 24 hours in all three strains (Figure 3). The production was decreased after 48 hours. 5% concentration of inoculum gave maximum production of shikimic acid (Figure 4).The effect of carbon sources on shikimic acid production showed, glucose and fructose as better carbon sources than lactose, sucrose and maltose (Figure 5). Among nitrogen sources, maximum production was observed with asparagine and ammonium sulphate.(Figure 6). The production was not increased in inorganic salts like ammonium chloride and ammonium nitrate or organic nitrogen source like urea and peptone. 5% concentration of glucose gave maximum production of shikimic acid in both the mutants and its wild strain, C1 (Figure 7). Higher yield of shikimic acid with 5%glucose and asparagine (4.5%) as nitrogen source was also reported by Tripathi et al (2013).

Partial purification of extract obtained by the fermentation was carried out by ethanol. The presence of shikimic acid in the extract was confirmed by TLC. Charred brown coloured spots comparable with standard having Rf of 0.58 lying very close to standard value of 0.59 were obtained. So the present work concludes that *Escherichia coli* isolated from natural environment and its mutants have a potential of shikimic acid production. As shikimic acid is a high valued intermediate in various antiviral drug preparations the present work will be of much significance against the fear of spread of swine flu.

Figure.1 Production of Shikimic acid by selected isolates

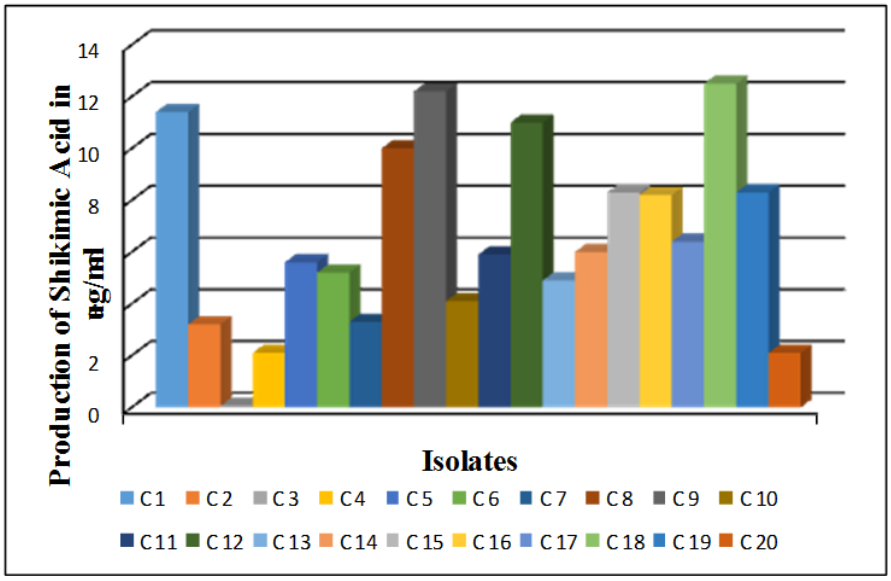


Figure.2 Production of Shikimic acid by selected mutants

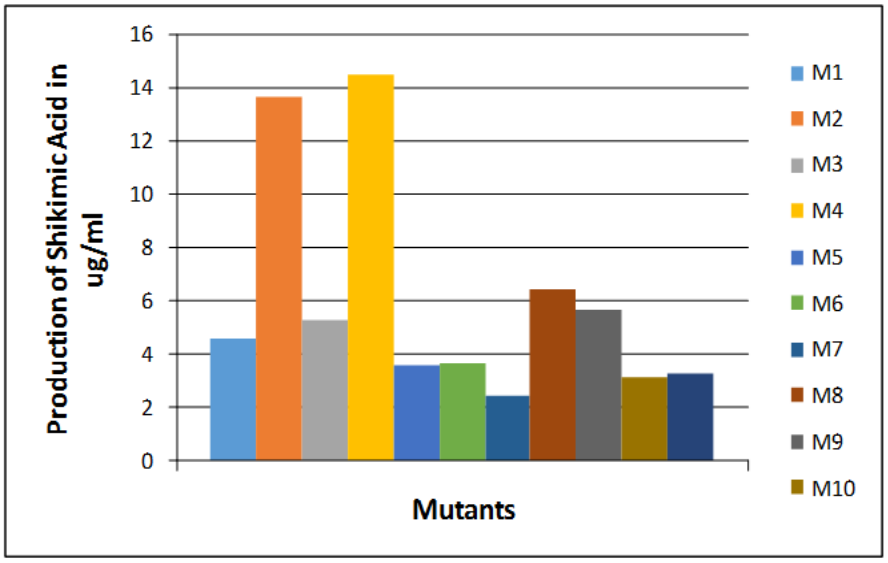


Figure.3 Effect of incubation period on production of Shikimic acid

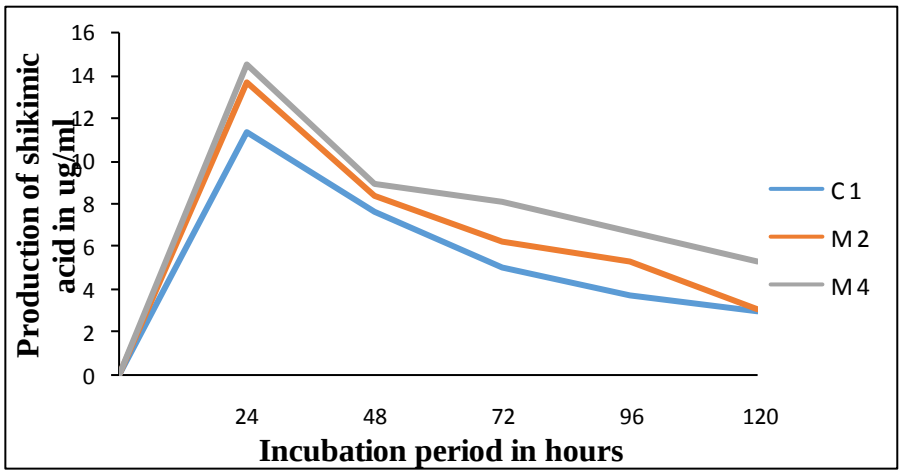


Figure.4 Effect of inoculum concentration on production of shikimic acid:

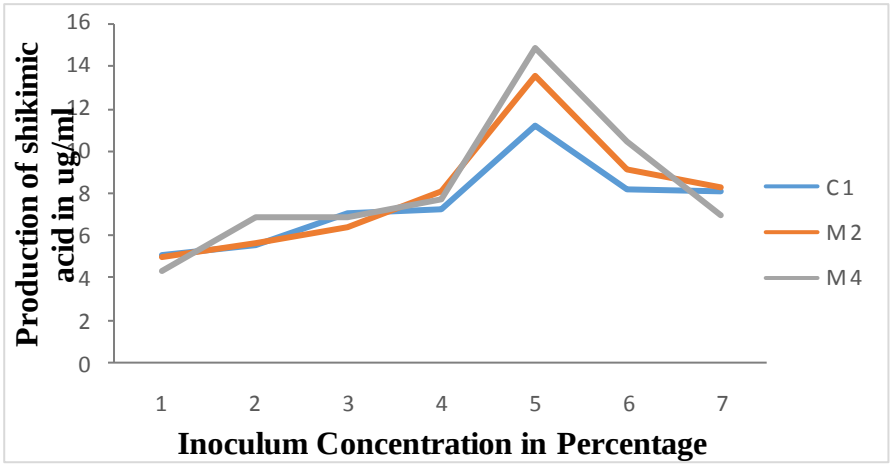


Figure.5 Effect of carbon source on production of shikimic acid

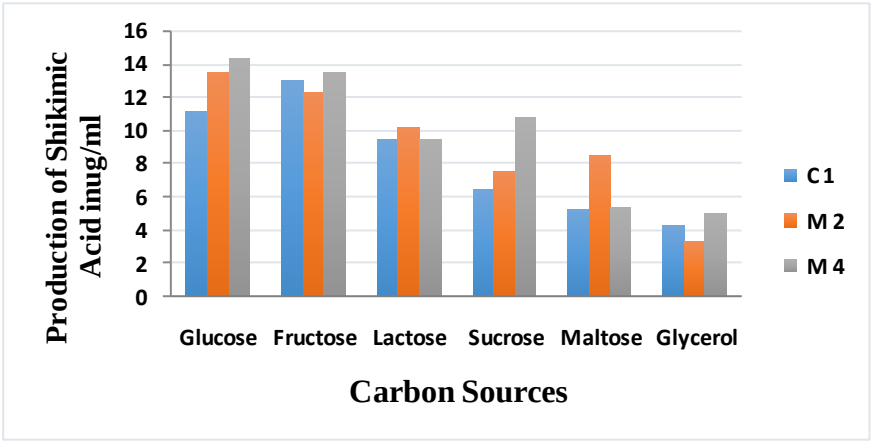


Figure.6 Effect of nitrogen source on production of shikimic acid

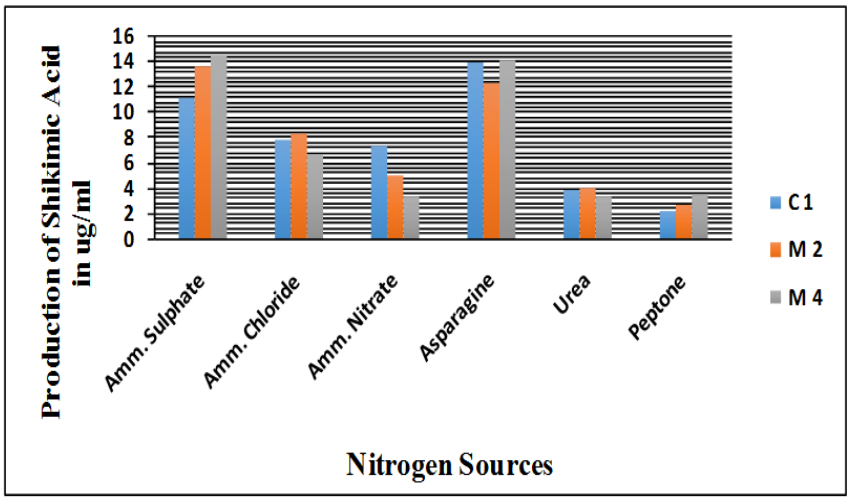
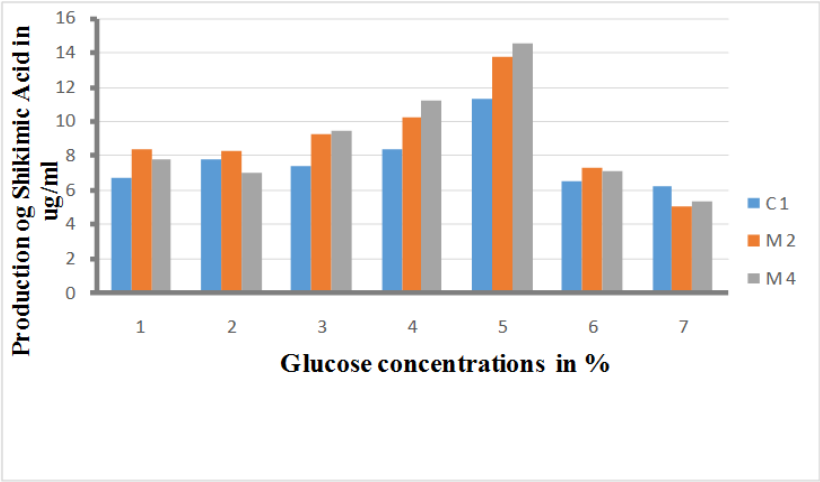


Figure.7 Effect of glucose concentration on production of shikimic acid



Acknowledgement

Authors are grateful to UGC, New Delhi, India, for providing financial support to carry out this work.

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