

A NOVEL BIOCHEMICAL METHOD FOR PRODUCTION OF AN ANTIBACTERIAL DRUG TRIMETHOPRIM FROM INDUSTRIAL WASTES

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ABSTRACT

Objectives: Production of an antibacterial drug trimethoprim from industrial wastes by using novel biochemical methods.

Methods: A few reports are available in the literature on the production of Trimethoprim through biotechnological routes. A novel route for the synthesis of trimethoprim using the principles of fermentation technology and organic and biochemistry. Methylation, enzymatic reduction, condensation, guanidine interaction steps involved in this novel route of production are enzymatic conversion of natural tannins to gallic acid and enzymatic reduction of 3,4,5, trimethoxy benzoic acid to 3,4,5 trimethoxy benzaldehyde. Tannin from cashew was fermented in a shake flask by *Aspergillus niger* for gallic acid production. In this case of enzymatic reduction, 3,4,5 trimethoxybenzaldehyde is formed by the reduction of 3,4,5 trimethoxybenzoic acid using NAD oxidase and benzaldehyde NADP oxidoreductase enzymes from *pseudomonas fluorescens*-MTCC 103.

Results and Conclusion: Conventionally gallic acid is produced by acid hydrolysis of tannin rich substrate but this technology has several disadvantages regarding cost, yield and purity of the product. 3,4,5-trimethoxybenzoic acid was observed at 10 min at the TLC plate. RF value was found to be 0.32. This enzymatic reduction method is less complex, non polluting and does not involve formation of byproducts. On the whole, the various confirmatory tests on trimethoprim provided good yield. This novel biocatalytic approach can add new dimensions to the conventional process.

Keywords: Tannins, Trimethoprim, Gallic acid, Oxidoreduction, Guanidine interaction, *Pseudomonas fluorescens*.

INTRODUCTION

The chemical designation of trimethoprim is 5-[(3, 4, 5-trimethoxyphenyl) methyl]-2, 4- diaminopyrimidine. It is a white to yellowish compound with bitter taste. The trade names of the combined product are Bactrim and Spectra (Bean DC *et al.* 2005). Septrin (Burroughs Wellcome & Co), a new, widely used antibacterial drug, is a combination of trimethoprim and sulphamethoxazole. Trimethoprim is seldom if ever used alone. It acts as a bacteriostatic agent by competitively inhibiting bacterial folate reductase activity (Jane Sive *et al.* 1972). Gallic acid, both in conventional and novel methods, is methylated. Any suitable methylation agent can be used. Suitable methylation agents include, for example dimethyl sulphate, methyl p-toluene sulfonate, methyl iodide, diazomethane etc. Dimethyl sulphate is preferred, since according to literature. Dimethyl sulphate is been said to be a best methylating agent of gallic acid. Methylation of

gallic acid produces 3,4,5-trimethoxybenzoic acid which is then reduced to aldehyde. This process is common for both conventional and novel processes.

Conventionally the compound 3,4,5 trimethoxybenzaldehyde is produced by chemical methods like reduction of 3,4,5-trimethoxybenzoic acid by modified rosemund reaction and by the application of reissert reaction from vanillin by hydrogenation followed by treatment with DMF, with calcium and methylation with dimethyl sulphate, from gallic acid and syringaldehyde in the same manner as from vanillin. But these processes generate not only environment but also processing problems. Handling of pollution is the major constraint and also a number of side reactions were involved. Hence an enzymatic reduction method is proposed which is simpler, requires less number of stages and is a non polluting process. *Pseudomonas fluorescens* was found to reduce effectively 3,4,5-trimethoxybenzoic acid to 3,4,5-trimethoxybenzaldehyde.

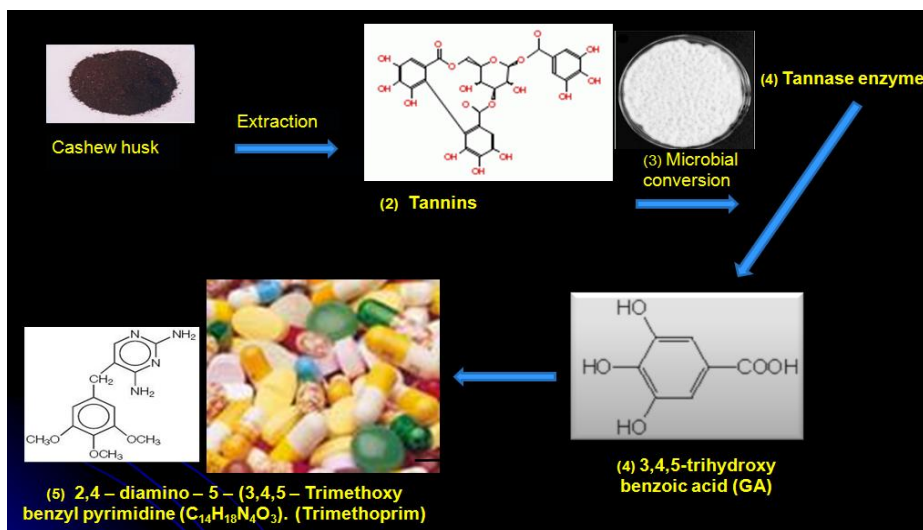


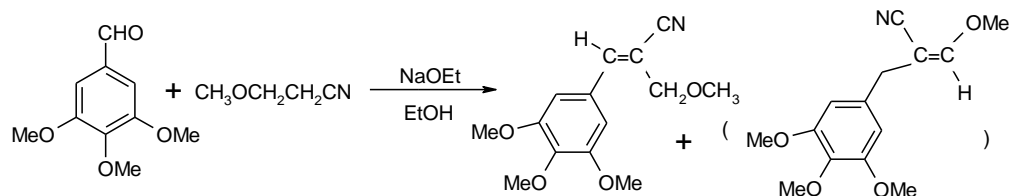
Fig. 1: Biosynthetic pathway of Trimethoprim from cashew husk tannin

The enzymatic method is advantageous compared to chemical methods, in one method; vanillin was first brominated in the presence of methanol, to give 95% bromovanillin. The bromine group from this compound is methylated by DMF, with Ca in the presence of CaCl_2 and NaHCO_3 to give syringaldehyde which on methylation with dimethyl sulphate in the presence of Na_2CO_3 to give 3,4,5-TMBA in 91% yield. The drawback of this method is that the product obtained contains sulphur as a pollutant and also by products include ester and ether. Hence this process needs number of steps to purify the product.

On the other hand, in the modified Rosemund reaction TMB was first converted into an acid chloride and then reduced to 3,4,5-TMBA. In this process, it was convenient to run the experiment in the laboratory scale, which is associated with catalyst poisoning. In the preparation from syringaldehyde, the syringaldehyde was prepared by oxidation of eucalypt lignin with nitrobenzene and alkali. For handling large volumes the apparatus costs are prohibitively expensive. Apart from that the product must be fractionally crystallized. In contrast enzymatic method requires no large scale experimental set up, and the product obtained is almost pure. Conventionally the drug trimethoprim was produced from synthetic Gallic acid but this process has generated a lot of environmental and processing problems, so a novel method of production of drug trimethoprim was developed which uses Gallic acid obtained from natural sources, this method has been proved to be effective in reducing problems related to environmental pollution and processing problems. Hence this process is more economical and pollution free.

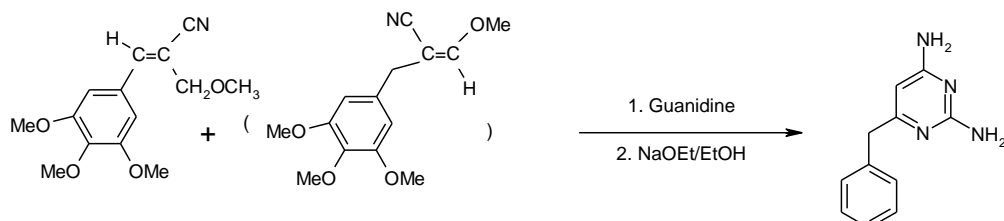
MATERIALS AND METHODS

Process development for the production of Trimethoxybenzoic acid from pure Gallic acid involves the usage of reagents Gallic acid, NaOH and Dimethylsulphate. Reduction of 3, 4, 5-trimethoxybenzoic acid to 3, 4, 5-trimethoxybenzaldehyde is done with *Pseudomonas fluorescens* MTCC-103 through the process of bioconversion. The media used for *Pseudomonas fluorescens* is nutrient agar, it contains 0.1gm of beef extract, 2g of yeast extract, 0.5g peptone, 0.5g NaCl and 1.5g of agar in 100 ml of water. The enzyme extraction followed a procedure of resuspension of culture in sterile distilled water. The spores were inoculated to nutrient medium which is made of 0.1gm of beef extract a, 2g of yeast extract, 0.5g peptone, 2g of glucose and 0.5g NaCl in 100 ml of water and incubated at 37 °C at 160 rpm for 48 hours in an orbital shaker incubator. The biomass was separated by refrigerated centrifugation at 4°C at 8000 rpm for 70 minutes then the supernatant obtained contains extra cellular enzyme. The other method done to obtain extra cellular enzyme was by filtration



Guanidine interaction

A solution of guanidine hydrochloride (12 g, 0.126 mol) in methanol was neutralized by a methanolic solution of sodium (3 g, 0.130 mol). The mixture of benzal nitriles (10.4 g, 0.04 mol) was dissolved in the guanidine solution, and the resulting solution was



using whatmann number 1 filter paper. The filtrate obtained after filtration contains extra cellular enzyme.

The separated biomass was homogenized to extract intracellular enzymes, with phosphate buffer at pH-7. The biomass was then centrifuged at 10,000 rpm at 4°C for 1 hour to separate the solution containing enzymes i.e., intracellular enzymes. Condensation and guanidine interaction involves the usage of reagents, sodium (2 g), methanol (50 ml), 3,4,5-trimethoxybenzaldehyde (9.8 g, 0.05 mol), β -methoxypropionitrile (4.25 g, 0.05 mol) and guanidine hydrochloride (12 g, 0.126 mol).

Methylation

16g of sodium hydroxide was mixed in 100 ml of water, to this solution 10g of Gallic acid (3,4,5-trihydroxybenzoic acid) was added. The flask was immediately stoppered in order to avoid darkening of the solution due to the oxidation with atmospheric oxygen. The mixture was shaken occasionally. To the above mixture 13ml of Dimethylsulphate was added and the mixture was shaken without any external warming in order to ensure proper mixing. Methylation is usually a rapid process, in this process Gallic acid was converted to 3,4,5-TMB and the Dimethylsulphate was converted to methyl hydrogen sulphate which dissolves in alkali i.e. NaOH. The sample obtained after methylation was subjected to thin layer chromatography to confirm TMB.

Enzymatic reduction

0.5mg of 3,4,5-Trimethoxybenzoic acid was taken in 10ml of distilled water. To this 1ml of intra cellular and 1 ml of extracellular enzyme was added separately. The two samples were then maintained at 37°C for 3 hours. 3,4,5-trimethoxybenzaldehyde was produced. Absorbance was read after 3 hours at 287 nm using UV/visible spectrophotometer and ethanol as solvent. The formation of aldehyde was confirmed using 2,4-Dinitro phenone and tollens' reagent.

Condensation

To a solution of sodium (2 g) in methanol (50 ml) 3,4,5-trimethoxybenzaldehyde (9.8 g, 0.05 mol) and β -methoxypropionitrile (4.25 g, 0.05 mol) was added. After heating at reflux temperature for 7 h, the solution was cooled, the methanol was removed under reduced pressure, and the mixture of benzal nitriles was dissolved in ether. The ethereal solution was washed consecutively by water (2 X 100 ml), NaHSO_3 (2 X 100 ml), and water (2 X 50 ml) and dried overnight (sodium sulfate). After removal of the drying agent and the solvent, the brown oil was distilled in vacuum.

refluxed for 48 h. after cooling the solvent was reduced in volume, and a precipitate was collected. The precipitate was further purified by passage through an alumina column and elution with an appropriate solvent (2-5 % methanol in chloroform). The resulting solid is 2,4-Diamino-5-(3,4,5-trimethoxy benzyl) pyrimidine, Trimethoprim.

The formation of trimethoprim after guanidine interaction of methoxymethyl-3,4,5-triethoxycinnamionitrile with guanidine hydrochloride was confirmed by determining the antibiotic sensitivity of bacterial strains using cup-plate method.

RESULTS AND DISCUSSION

In the methylation process, methylation of Gallic acid with Dimethylsulphate in the presence of methyl alcohol leads to formation of 3, 4, 5-trimethoxybenzoic acid. 3,4,5-trimethoxybenzoic acid conformation was done by Thin layer Chromatography and compared the Rf values with that of the standard 3,4,5-trimethoxybenzoic acid. This indicated that the conversion has occurred in the broth was 3,4,5-trimethoxybenzoic acid. The chromatogram revealed that appeared as the hydrolysis progressed. 3,4,5-trimethoxybenzoic acid was observed at 10 min at the TLC plate. Rf value was found to be 0.32

In enzymatic reduction process, when 2,4 Dinitrophenone was added to final reaction product in the presence of methanol a red precipitate formed is due to the formation of 2,4Dinitrophenylhydrozone when 3, 4, 5-TMBA reacts with 2,4 DNP. When two drops of 3, 4, 5-Trimethoxybenzaldehyde was added to freshly prepared Tollen's reagent. Within 4 minutes the inside surface of the flask was coated with a reflective silver mirror indicating the presence of aldehyde.

The formation of trimethoprim after condensation and guanidine interaction was confirmed by determining the antibiotic sensitivity of bacterial strains using cup-plate method. After placing the cups containing the product over a lawn of culture of microorganism, and observing for 24 hours, a clear zone of inhibition is seen approximately 15mm around the cup for standard trimethoprim and approximately 12mm for the resultant product.

Table 1: Physical properties of the trimethoprim

Sample	Colour	Solubility	Molecular formula	Molecular weight	Melting point
Trimethoprim	Yellowish-white	Very slightly soluble in water. Slightly soluble in alcohol	C ₁₄ H ₁₈ N ₄ O ₃	290.3	201-03°C

Conventionally the compound 3,4,5 trimethoxybenzaldehyde is produced by chemical methods like reduction of 3,4,5-triethoxybenzoic acid by modified rosemund reaction and by the application of reissert reaction from vanillin by hydrogenation followed by treatment with DMF, with calcium and methylation with dimethyl sulphate, from gallic acid and syringaldehyde in the same manner as from vanillin. But these processes generate not only environment but also processing problems. Handling of pollution is the major constraint and also a number of side reactions were involved. However, it has been qualitatively confirmed that the resultant product obtained is an antibiotic *i.e.* Trimethoprim. This method requires no large scale experimental set up, and the product obtained is almost pure. Hence this process is more economical and pollution free.

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