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Research Article

PRODUCTION OF THEOBROMINE FROM *PSUEDOMONAS SP.*

Narendra Kumar.S*, Trilok Chandran B, Sophia Namratha, Bharath B R, Chandra Kumar H P, Varsha Kishore
Department of Biotechnology, R.V.College of Engineering, Bangalore-560059, Karnataka, India

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*Email: narendraks@rvce.edu.in

ABSTRACT

Theobromine is a colourless and odourless compound with a slightly bitter taste naturally present in the cacao tree and its seeds. It has a similar, but lesser, effect than caffeine in the human nervous system, making it a lesser homologue. Theobromine plays an important role in vasodilator (a blood vessel widener), a diuretic (urination aid) and heart stimulant and as a skin moisturant. Therefore it is preferred as a potential therapeutic supplement to many critical diseases besides being used as dietary supplement in the confectionary industries. The large number of clinical trails is being conducted to evaluate its potential for therapies in neurodegenerative diseases, cardiac diseases; cancer etc and overall results are very much positive and optimistic. This has driven the demand for Theobromine supply to meet the market requirements. Theobromine can be produced either by synthetic or natural route. The Theobromine production by natural route is preferred over synthetic method due to low production cost, safe environmental issue. At present there two methods of producing theobromine by natural route are extracting from the plants sources and microbial fermentation. Currently an attempt is made to develop a process for the production of this molecule from a microbial species. Bacterial culture, *Psuedomonas putida* was utilized for this study. In the present work, this strain was taken for production of Theobromine in shake flask fermentation. The optimization of media for better yield during shake flask fermentation and the results obtained are presented and discussed.

Key Words: *Psuedomonas putida,* Theobromine, Neutraceuticals, Fermentation and Thin Layer Chromatography.

INTRODUCTION

Theobromine: Theobromine is the primary alkaloid found in cocoa and chocolate. Cocoa powder can vary in the amount of theobromine content and widely used in "dark" than "milk" chocolate as dietary supplement and nutraceutical. Dietary Supplement Health and Education Act defines Theobromine as "dietary supplement" i.e. food that provides medical or health benefits, including the prevention and/or treatment of a disease. The term "Nutraceutical," was coined from two words, "nutrition" and "pharmaceutical". Thus, Theobromine is not only dietary supplements but also aid in the prevention and/or treatment of disease or disorder and also represented for use as a conventional food or as the sole item of meal or diet[1].

Structure and sources of Theobromine: Theobromine also known as xantheose is a bitter alkaloid of the cacao plant, with the chemical formula $C_7H_8N_4O_2$[2]. It has a two-dimensional molecular structure and is a naturally-occurring alkaloid. Theobromine is a slightly water-soluble, crystalline, bitter powder; the colour has been listed as either white or colourless. Despite its name, the compound contains no bromine and word theobromine is derived from Theobroma, the name of the genus of the cocoa tree, (which itself is made up of the Greek roots theo ("God") and brosi ("food"), meaning "food of the gods") with the suffix -ine given to alkaloids and other basic nitrogen-containing compounds[3]. Theobromine is the primary alkaloid found in cocoa. Cocoa beans naturally contain approximately 300-1200 mg/ounce theobromine. Compared with other alkaloids, such as nicotide and morphine, purine alkaloids, including theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-methylxanthine) are distributed widely throughout the plant and animal kingdom[4,5]. Higher quality chocolate tends to contain more theobromine than lower quality chocolate[6]. Theobromine is the dominant purine alkaloid in seeds of cacao, Theobroma cacao. The cotyledons of mature beans contain 2.2–2.7% on a dry weight basis and 0.6–0.8% caffeine, while shells contain 0.6–0.7% theobromine and 0.5–0.6% caffeine[7].

Commercial Importance of Theobromine: Theobromine affects humans similarly to caffeine, but on a much smaller scale. Theobromine has been used as a drug for its anti carcinogenic effect[8], as a skin moisturant[9], Vasodilator[10], treatment of Asthma[11], cardiovascular and as Antitussive[7]. Particularly in cases where cardiac failure has resulted in an accumulation of body fluid. It has been administered with digitalis in order to relieve dilatation. Because of its ability to dilate blood vessels, theobromine also has been used to treat high blood pressure.

A large number of clinical trails are being conducted to evaluate its potential for therapies in neurodegenerative diseases, cardiac diseases; cancer etc and overall results are very much positive and optimistic[8]. Theobromine was found strong inhibitory effects of theobromine on the activity of the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1), which is implied in acute and chronic inflammatory diseases such as stroke, ischemia perfusion and diabetes, and implied in chronic obstructive pulmonary disease[12].

Production strategies: Theobromine can be produced by both synthetically and natural route[13]. For the chemical synthesis of Theobromine, it is important to develop a highly stereo selective process, due to the 3,7-dihydro-3,7-dimethyl-1H-purine-2,6-dione are highly stereospecific nature[14,15], it is difficult to synthesize by chemical route. But, these methods are environmentally unfavorable and suffer from low overall yields. Thus in early 19th century the plant extraction was in practice, but 1990 onwards several trails are conducted to produce by microbial system. At present there two methods of producing theobromine by natural route are extracting from the plants sources and microbial fermentation. Production of theobromine by microbial fermentation is preferred over synthetic and plant extraction methods due to low production cost, safe environmental issue[16]. It has been reported that certain Pseudomonas species and other bacteria degraded caffeine in the presence of a carbon source[17]. Enzymological aspects of caffeine degradation by Pseudomonas putida in detail[18]. In Pseudomonas sp. fructose and glucose were shown to enhance the degradation of caffeine to theobromine by Pseudomonas sp[19].

MATERIALS AND METHODS

Producing Organism: The strain *Pseudomonas putida* was used for producing Theobromine[19,20].

Growth Media: Culture Maintenance Medium-the Theobromine producing culture was maintained on Agar slant having a composition of 0.3 % yeast extract; 0.5 % of peptone, 1% Nacl and 2 % agar. The pH of the medium was 7; Production Medium-the production medium contains 1 % peptone, 0.2 % yeast extract, 0.25% $MgSO_4$, KH_2PO_4 and Na_2HPO_4. The pH of the medium was 7.2[21].

Chemicals: The list of chemical (analytical grade) includes Yeast Extract, peptone, $MgSO_4.7H_2O$, KH_2PO_4, Na_2HPO_4, Glucose, EDTA, Tris HCL and Agar. Solvents: Absolute ethanol, 70% Ethanol, Butanol, Chloroform, Acetic Acid, Methanol, Benzene, Caffeine, Theobromine.

Instruments and Apparatus: Autoclave, Incubator, Centrifuge, Eppendorf tubes, controlled Orbital shaker, Laminar air flow, pH meter, Micro pipettes, Sterile tooth pick, Para film, Test tubes, Spreader, Petri plates, Conical flask, Beaker, measuring cylinder, Capillary tubes and Thin Layer Chromatography UV Chamber.

Shake Flasks Kinetics: The seed culture was grown in a 500 ml Erlenmeyer flask containing 150ml of Nutrient Broth medium under agitation of 150 rpm at 37˚C for 24 hrs on a rotary shaking incubator. The shake flask kinetics were performed in a six Erlenmeyer flasks (l Litre) containing 300 ml of the production medium at 37 ˚C, 150 rpm for 48 hrs after inoculating with 5% (v/v) of the seed culture[22]. The samples are aseptically withdrawn at different time interval from each flask for analysis of pH and OD[23,27].

Downstream Processing: The total broth was pooled and transferred into sterile centrifuged tubes and centrifuged at 6,000 rpm for 6 minutes at 4^0C[24,25]. The biomass was recovered in the form pellet and transferred to a Vial. The resulting pellet was treated with solution (50mM Glucose, 25mM Tris-Hcl, 10mM EDTA) for cell lysis. The vial was vortexed gently to get homogeneous suspension. The vial was kept in ice for 10 minutes. 2 ml of 0.2 N NaoH, 1% SDS was added to the vial and mixed well. The tubes were incubated for 10 minutes at room temperature and shifted to ice for 5 minutes. The cell suspension obtained was centrifuged 8000 rpm using 2-5% methanol[26]. The resulting supernatant was analyzed for the presence of Theobromine using TLC with Mobile Phase system chloroform, benzene, methanol, acetic acid in the ratio of 50:35:10:5[24].

Table 1: Growth Curve Analysis

Time (Hrs)	Media 1		Media 2		Media 3		Media 4		Media 5		Media 6	
	OD	pH	OD	pH	OD	pH	OD	pH	OD	pH	OD	pH
0	0.00	7.25	0.00	7.50	0.00	7.20	0.00	7.15	0.00	7.20	0.00	6.80
8	0.07	6.50	0.04	7.05	0.05	6.72	0.10	7.12	0.02	6.60	0.08	6.82
16	0.08	6.92	0.09	7.20	0.09	7.02	0.30	7.19	0.05	6.65	0.14	7.12
24	0.11	7.25	0.12	7.90	0.18	7.45	0.43	7.27	0.06	6.82	0.16	7.24
32	0.15	7.76	0.15	8.12	0.18	7.96	0.43	7.31	0.07	7.50	0.15	7.63
40	0.15	8.10	0.16	8.32	0.19	7.96	0.46	7.35	0.07	7.52	0.17	7.61

Table 2: Media Composition for optimal growth of producing microorganism

Ingredients	Media 1 (in %)	Media 2 (in %)	Media 3 (in %)	Media 4 (in %)	Media 5 (in %)	Media 6 (in %)
Peptone	2.00	2.00	1.00	1.00	1.00	0.50
Yeast Extract	0.20	0.20	0.20	0.20	0.10	0.50
$MgSO_4.7H_2O$	0.25	0.02	0.02	0.02	0.01	0.02
KH_2PO_4	0.50	0.50	0.05	0.02	0.01	0.02
Na_2HPO_4	0.25	0.25	0.25	0.12	0.01	0.25

Table 3: pH and OD profile for Producing Microorganism in Media IV

Time (Hrs)	pH	OD
0	7.15	0
8	7.12	0.1
16	7.19	0.3
24	7.27	0.43
32	7.31	0.43
40	7.35	0.46

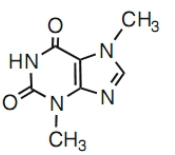

Figure 1: Structure of Theobromine

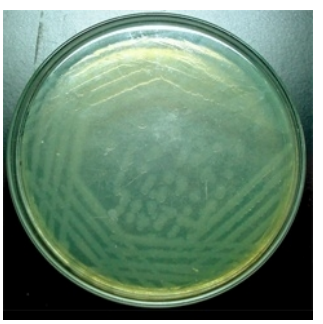

Figure 2: Producing organism (*Pseudomonas putida*)

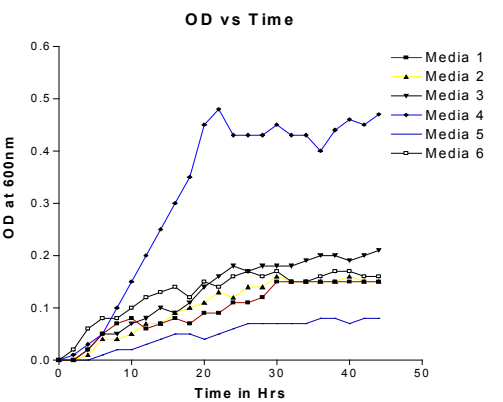

Figure 3: Time verses OD of Shake Flask Kinetics

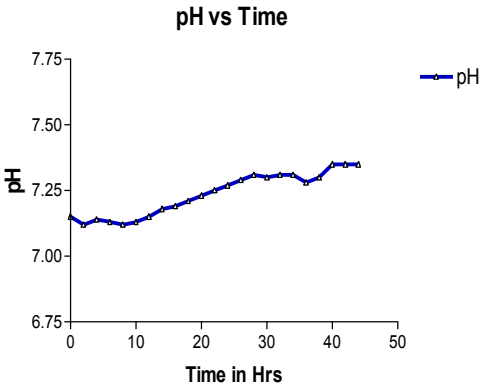

Figure 4: Time verses pH of Shake Flask Kinetics for media IV

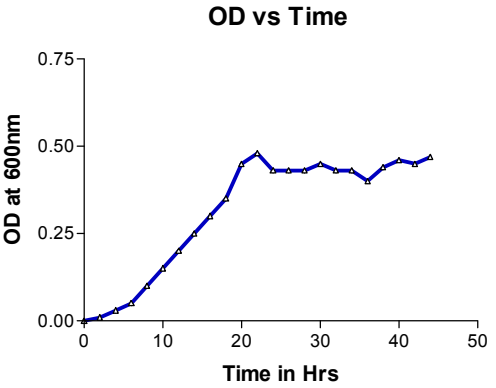

Figure 5: Time verses OD of Shake Flask Kinetics for media IV

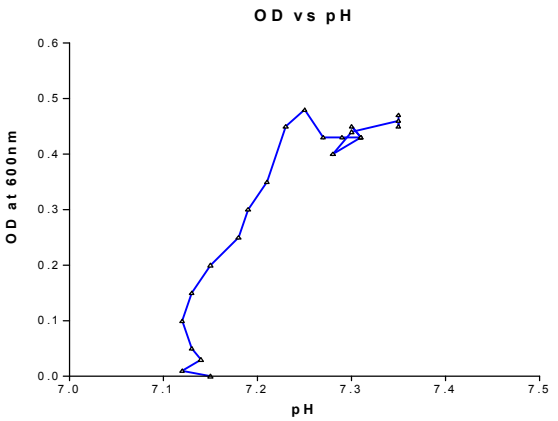

Figure 6: pH verses OD of Shake Flask Kinetics for media IV

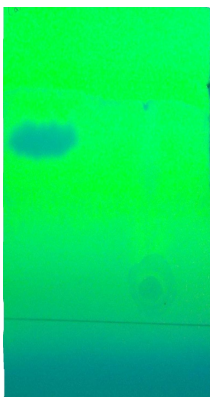

Figure 7: TLC Analysis of samples for identification.

RESULTS

Microorganism: Theobromine producing culture *Pseudomonas putida* was grown in NB media and the stock culture was maintained at 4 ˚C. The purity of the culture was checked regularly and morphological observation done under microscope.

Shake Flask Kinetics: The growth kinetics of *Pseudomonas putida* was carried out in 1 L Erlenmeyer flask with 200 ml of production media (varied Concentrations) broth on a shaker run at 100 rpm at 37 ˚C for 44 hrs. Periodical sampling was carried out for every 2 hours to check pH and OD. The data obtained was presented in the Table 1 and its respective media compositions are given in table 2.

pH and OD profile for *Pseudomonas putida* in Media IV: The growth kinetics of producing Microorganism for optimized media was conducted under similar condition to identify its pH and Maximum growth.

TLC Analysis: The crude extract obtained from cell biomass was purified by microfiltration and solvent extraction as described in above downstream processing steps. The solvent fractions containing desired compound was primarily tested on commercially available silica gel plates as stationary phase.

DISCUSSION

The culture appears as cream colour in the plate. The *Pseudomonas putida* is gram negative and rod shaped bacterium observed under microscope as shown in figure 2.

Time verses OD values of Shake Flask Kinetics was plotted and it observed that the maximum growth of microorganism is obtained for Media design IV at 100 rpm, 37^{o} C and at above 22 hours as shown in the figure 3.

Time verses OD, pH values for media IV was plotted and it observed that the maximum growth occurs between 20-40 log hrs for media IV and during this log period pH shift was in the range 7.15-7.35 indicating that pH control can be achieved through media components (buffering capacity) as shown in the figure 4 & 5. Further maximum growth was observed at pH 7.25 as shown in the figure 6. The results found to be optimistic and help to understand the growth kinetics of *Pseudomonas putida.*

TLC chromatogram developed was shown in the figure 7, spots on the plate indicate the presence of desired compound when compared to standard; however for confirmational analysis the samples are to be analyzed by using sophisticated instruments like HPLC or GC.

CONCLUSION

Overall, an attempt has been made to develop a process for Theobromine production using a *Pseudomonas putida*. The same culture was used in process studies for media optimization. It was found that a maximum growth of microorganism is obtained for Media design IV at 100 rpm. The optimal pH for the maximum growth was found to be 7.25. Also, an attempt was made to identify and purify the compound from the fermentation broth. Further studies have to be continued in terms of process development in fermentor and also to optimize its purification process.

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