Synopsis of the Thesis

Metabolism of Caffeine and its Analogues by a Mixed culture: a) A Novel Pathway for the Degradation of Caffeine b) Mixed culture as an Efficient Reagent for C-8 Oxidation of Substituted Xanthines c) Caffeine Oxidase-Purification and Characterization d) Immobilization : (i) Mixed Culture (ii) Caffeine Oxidase

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Exploration of microbes in carrying out transformations of natural products has been an important and fascinating method in the synthetic organic chemistry. Microbes offer the opportunity to effect selective transformations – a feature of great utility in organic syntheses, that otherwise would be difficult to carry out by conventional methods. Considerable progress has been made in the microbial transformations of various classes of organic compounds such as steroids, carbohydrates and aromatic compounds. Although alkaloids are one of the widely distributed class of natural products, progress in the field of microbial transformations of these compounds lagged behind considerably as compared to the advances made with regard to other classes of compounds.

Caffeine, a purine alkaloid, most of the population of the world is exposed to it to a greater or lesser extent since it occurs in a number of plants used in the preparation of widely consumed drinks, and has in addition a limited therapeutic use. The metabolism of caffeine (1,3,7-trimethylxanthine) and related methylated xanthines has been investigated both in microbes and mammals. Caffeine is generally toxic to bacteria although the concentration required is often relatively high. In microbial system degradation of caffeine is initiated by partial or complete N-demethylation followed by oxidation at C-8 position to produce the corresponding uric acids. In fact caffeine is N-demethylated in two parallel ways via theobromine (3,7-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine), the former appears to be the major pathway for degradation. Uric acid is degraded to allantoin and then to allantoic acid. Further degradation of allantoic acid results in formation of urea and glyoxalic acids as the end products of caffeine metabolism. Mammals metabolize caffeine via two common metabolic reactions, the N-demethylation and direct

oxidation of caffeine, yielding 1,3,7-trimethyluric acid, which further gets oxidized to 3,6,8-trimethylallantoin by cleavage of 6-membered ring or to substituted diaminouracil derivative by cleavage of 5-membered ring.

We were interested to find a microbial system, which will mimic the mammalian degradation of caffeine and related compounds. In our efforts to find such a system, we have isolated a mixed culture consortium consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* capable of utilizing caffeine as the sole source of carbon and nitrogen. In fact there has not been any study carried out so far on the degradation of caffeine by a mixed culture.

The main objective of the present investigation:

1. To establish the pathway for the degradation of caffeine by the bacterial consortium and to characterize some of the enzymes involved in the degradation.
2. Preliminary studies indicated that the mixed culture does not carry out N-demethylation but instead initiate the degradation by C-8 oxidation. Since 8-oxo derivatives of alkyl and substituted xanthines are biologically active and have numerous applications in the formulations of drugs and cosmetics, we were interested to find out whether the bacterial consortium can be used as a reagent to convert various substituted xanthines to their respective 8-oxo compounds. Chemical methods to prepare such compounds are tedious, not suitable for water soluble xanthine analogues and the yields not are satisfactory.
3. Although several caffeine analogues such as pentoxyfylline, lisofylline, enprofylline etc., are used as drugs, the corresponding 8-oxo derivatives are not available for biological evaluation since such compounds are hitherto not known. We wanted to explore the possibility of using mixed culture to prepare such compounds.
4. If the bacterial consortium carries out efficiently C-8 oxidation reaction of various xanthine analogues, then we would like to immobilize the mixed culture since normally immobilization stabilizes the microbial system for a longer period so that such a system can be commercially exploited particularly for decaffeination of tea/ coffee extract and for the synthesis of useful 8-oxo derivatives of xanthine analogues.

The mixed culture consisting of strains belonging to the genera *Klebsiella* and *Rhodococcus* utilize caffeine as the sole source of carbon and nitrogen. However, to

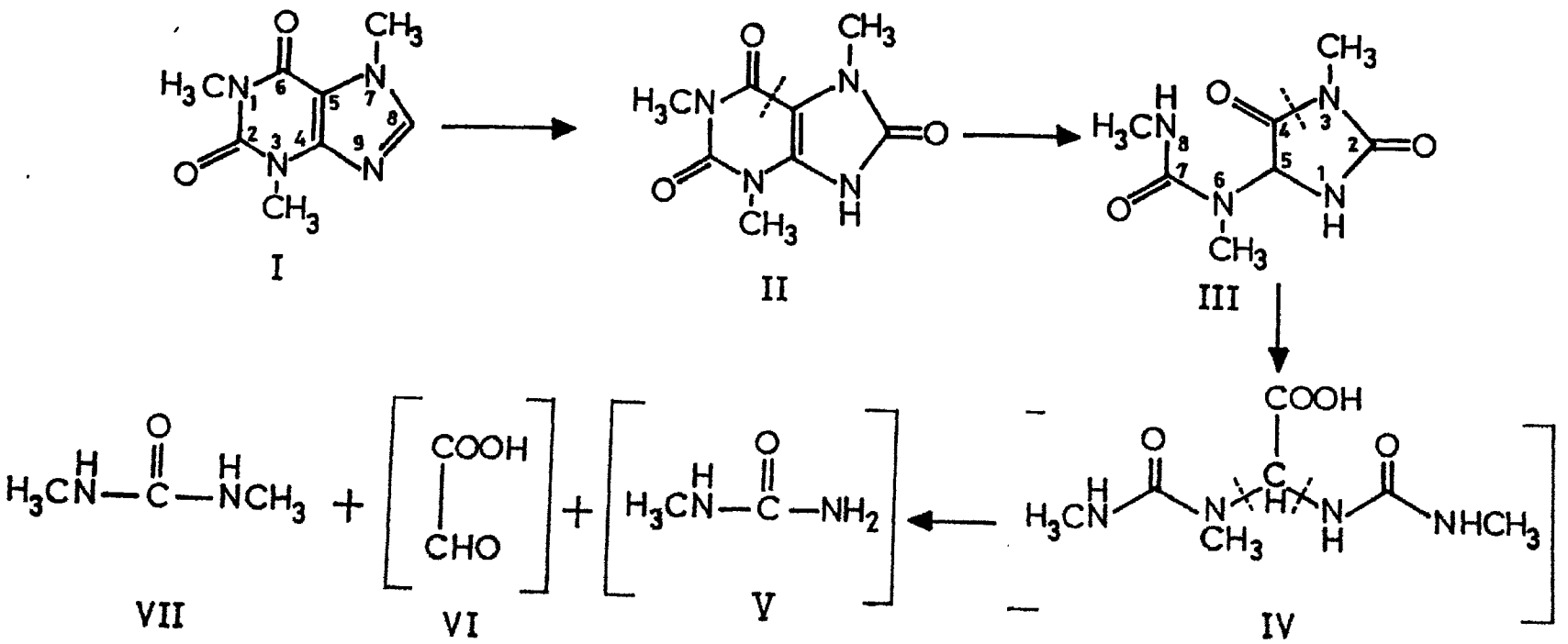

Fig:1 A Novel Pathway for the Degradation of Caffeine by Mixed Culture

enhance the growth rate 0.1% of glucose was added to the medium. Time course studies on the fermentation of caffeine (**I**) indicated that the growth reached a log phase in 18 h and **I** was completely metabolized in 36 h. The degradation of caffeine is initiated through C-8 oxidation resulting in the formation of 1,3,7-trimethyluric acid. In fact the conversion of caffeine into 1,3,7-trimethyluric acid has never been demonstrated earlier in the microbial system. It is also interesting to note that the mixed culture does not carry out N-demethylation reaction. This is supported by the fact that dimethylxanthines such as theophylline, theobromine and paraxanthine are not accepted by the mixed culture as carbon source. Even monomethylxanthines are not accepted as carbon source. Fermentation of caffeine by mixed culture does not accumulate 1,3,7-trimethyluric acid. However, incubation carried out in the presence of N-methylmaleimide results in the accumulation of 1,3,7-trimethyluric acid in the medium.

Caffeine grown cells metabolize 1,3,7-trimethyluric acid to a polar metabolite, which was isolated from the medium by preparative TLC. This polar metabolite gave positive reaction to the Young-Conway test suggesting that the metabolite isolated might be a derivative of allantoin. The spectral analyses (NMR,MS) of this metabolite revealed that it was 3,6,8-trimethylallantoin, a compound never been isolated and characterized during the microbial degradation of caffeine. Incubation of 3,6,8-trimethylallantoin with caffeine grown cells yields dimethylurea as one of the metabolites. Oxygen uptake studies indicated that caffeine grown cells oxidized 1,3,7-trimethyluric acid, 3,6,8-trimethylallantoin, dimethylurea and monomethylurea, but monomethyl and dimethylxanthines were not oxidized. The cell-free extract prepared from caffeine grown cells readily convert caffeine into 1,3,7-trimethyluric acid in the presence of an electron acceptor, viz., phenazine ethosulfate. Based on the above results, a novel pathway for the degradation of caffeine has been proposed which was never been reported earlier [**Fig:1**].

Although mixed culture does not accept theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) as a carbon source, cells grown on caffeine quantitatively convert these dimethylxanthines to their corresponding dimethyluric acids. Interestingly, the dimethyluric acids formed do not get further metabolized. To ascertain the generality of the reaction, we have

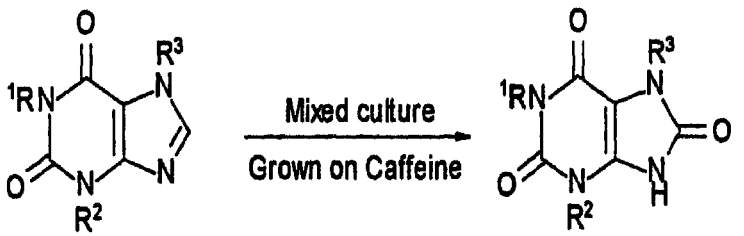

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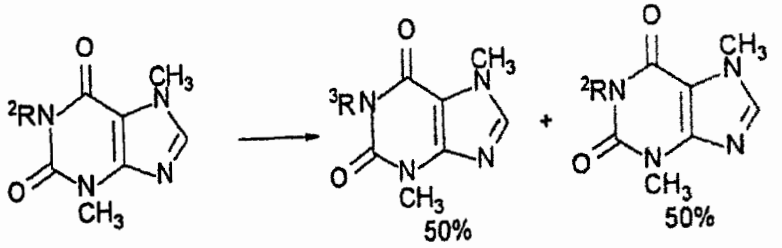
Fig:2 C-8 oxidation of substituted xanthines with substitution at the 1-,3-,and 7-positions using biocatalysts.

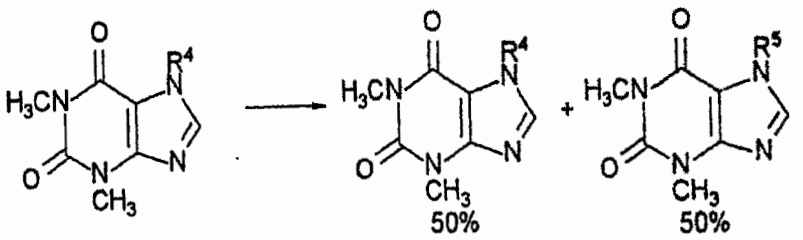
	R^1	R^2	R^3	Product*
1*	CH_3	CH_3	CH_3	21
2	CH_3	CH_3	H	22
3	H	CH_3	CH_3	23
4	CH_3	H	CH_3	24
5	CH_3CH_2	CH_3	CH_3	25
6	$CH_3CH_2CH_2$	CH_3	CH_3	26
7	$CH_3CH_2CH_2CH_2$	CH_3	CH_3	27
8	$CH_3CH_2CH_2CH_2CH_2$	CH_3	CH_3	28
9	CH_2CH_2OH	CH_3	CH_3	29
10	Ph-CH_2	CH_3	CH_3	30
11	$CH_3COCH_2CH_2CH_2CH_2$	CH_3	CH_3	31
12	CH_3COCH_2	CH_3	CH_3	32
13	CH_2=CH-CH_2	CH_3	CH_3	33
14	CH=C- CH_2	CH_3	CH_3	34
15	CH_2=CH-CH_2CH_2	CH_3	CH_3	35
16	H	$CH_3CH_2CH_2$	H	36
17	CH_3	$CH_3CH_2CH_2$	CH_3	37

* Isolated yields over 95-97%.



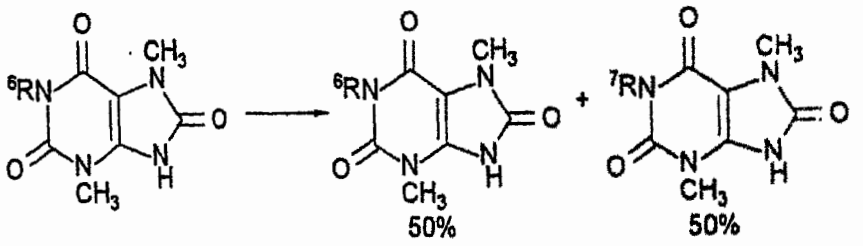
R^2=$CH_2CHOHCH_3$, R^3= CH_2COCH_3

R^2=-$(CH_2)_4CHOHCH_3$, R^3=-$(CH_2)_4COCH_3$



R^4=-$CH_2CHOHCH_3$, R^5=CH_2COCH_3

R^4=-$(CH_2)_4CHOHCH_3$, R^5=-$(CH_2)_4COCH_3$



R^6=-$CH_2CHOHCH_3$, R^7= -CH_2COCH_3

R^6=-$(CH_2)_4CHOHCH_3$, R^7=-$(CH_2)_4COCH_3$

Fig:3 Enantioselective oxidation of (±)hydroxyalkylxanthines by mixed culture grown on glucose.

synthesized various substituted xanthines with substitution at 1-, 3-, and 7- positions. It was observed that caffeine grown cells convert quantitatively analogues of theobromine with N-1-H replaced by various groups to their corresponding C-8 oxidized compounds **[Fig:2]**. In fact many of the C-8 oxidized compounds (uric acids) prepared are hitherto unknown. It is interesting to note that theophylline analogues prepared by replacing N-7-H with alkyl substitution higher than methyl were not accepted by the mixed culture. We have also demonstrated that the mixed culture can be used to carryout enantioselective oxidation of xanthines containing hydroxyalkyl substitution at N-1 or N-7 positions to their corresponding optically active alcohol. Thus using the mixed culture we have not only prepared the optically active lisofylline and its lower homologue but also the corresponding 8-oxo derivatives **[Fig:3]**.

The fourth chapter of the thesis deals with the immobilization of mixed culture in the removal of caffeine and its utility to bring about C-8 oxidation of substituted xanthines with substitution at 1-, 3- and 7- positions. We have shown that immobilization of whole cells has many advantages over the free cells. They have higher operational stability than free cells. We have carried out preliminary studies on immobilization of mixed culture by entrapment technique and we have used sodium alginate as a matrix since it is a widely studied method.

We have carried out the initial experiments to standardize parameters, such as pH, temperature, stability etc. All the above experiments were carried out with both free and immobilized cells. The pH and temperature optimum for free and immobilized cells were found to be the same. Stability studies have shown that immobilized cells are more stable than free cells and they do not loose much of its original activity even after using 3 times. The immobilized system has been shown to carryout very efficiently the C-8 oxidation of various substituted xanthines. Besides, a simple bioreactor designed using immobilized mixed culture was shown to efficiently carryout the decaffeination of tea extract.

The last chapter reports the purification, and partial characterization of caffeine oxidase- a novel enzyme from the mixed culture consortium. As far as we know this enzyme has never been reported so far. The enzyme catalyzes the oxidation of caffeine at the C-8 position to produce 1,3,7-trimethyluric acid. The enzyme was purified to

homogeneity by a combination of ion-exchange and hydrophobic column chromatographies. The subunit molecular mass of the protein was determined to be 85kDa. The spectral characteristics of the purified enzyme suggest that it is a flavoprotein containing non-heme iron. The enzyme was also immobilized and immobilized system was shown to be significantly more stable than the free enzyme. Some of the properties of the immobilized enzyme have also been studied and compared with the free enzyme.