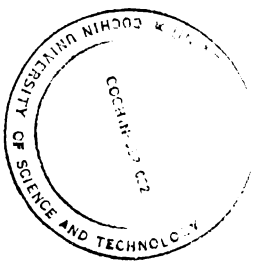
STUDIES ON THE MICROBIAL TRANSFORMATION OF ALKALOIDS: STRYCHNINE AND 2-NITROSTRYCHNINE

A THESIS SUBMITTED TO
THE COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE FACULTY OF SCIENCE



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MARCH - 1988

CERTIFICATE

Certified that this thesis is based on the work done by Mr. N. Ramachandra Sharma under my guidance in the Department of Applied Chemistry, Cochin University of Science and Technology, Cochin-22 and no part of this has been presented by him for any other degree.

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DECLARATION

Certified that the work presented in this thesis is based on the original work done by me under the guidance of Dr. P. Madhavan Pillai, Professor, Department of Applied Chemistry, Cochin University of Science and Technology, Cochin-22 and has not been included in any other thesis submitted for the award of any degree.

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my appreciation of the help and cooperation rendered by my colleagues. The financial support extended by the Department of Science and Technology, Government of India is gratefully acknowledged.

I am extremely indebted to my beloved parents for lending their moral support and encouragement. Finally, I record my sincere appreciation of the able assistance rendered by my wife, Suseela in typing this thesis, without whose catalytic influence and untiring enthusiasm this work would not have blossomed in this form.

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CHAPTER I

INTRODUCTION

Transformations of organic compounds by the application of microorganisms is gaining importance as a useful synthetic tool. Today microorganisms may be regarded as a class of chiral organic reagents which can bring about stereospecific and regiospecific chemical conversions on a large number of substrates.

The utilization of microbes to bring about desired chemical changes have a number of advantages when compared with chemical methods. Microorganisms possess a series of inducible enzymes that may be raised to very high levels in the presence of suitable substrates. In general, micro-organisms transform only one of several functional groups within a molecule, and more than one reaction may be carried out by a single organism thus cutting down on the number of steps. As these conversions are catalysed by microbial enzymes, they usually take place under mild reaction conditions with an unusually high degree of substrate and product specificity. Also, these conversions are usually stereospecific and the yields of the products are generally high. These reactions take place in aqueous medium and most often at room temperature. Therefore the use of expensive solvents and heating requirements can be avoided which are often

necessary in the case of chemical reactions.

Microbial processes also have some disadvantages. While chemical methods are, as a rule, easier to handle and require less complicated equipment, the fermentation technique is quite expensive. Since most microbial enzymes have rigid requirements for substrate binding, a large number of micro-organisms will have to be evaluated in order to find a suitable system for a desired reaction. The discovery of a new organism suitable to carry out the desired transformation can be a long and tedious process. Most microbial enzymes possess product specificity, but a microbial cell is most often endowed with many different types of enzymes which may give rise to a number of different products all of which will not be useful.

It is not yet possible to formulate a definitive set of rules because only a few systematic studies have been carried out on the action of microorganisms on various classes of organic compounds. However some useful generalizations have been arrived at. Usually, yeasts are most suitable for catalysing the reduction of compounds containing carbonyl groups, whereas fungi are capable of introducing hydroxyl groups into a wide variety of organic compounds. Bacteria,

generally, have the ability to oxidise organic substrates completely to carbondioxide and water.

Although the history of the use of microbial systems to achieve chemical reactions can be traced back to 3000 B.C., the significance and applications of microbial transformations have been mostly limited to carbohydrates and steroids, until recently. Practically very little work has been carried out in the microbial conversion of alkaloids as they are generally more difficult to be transformed compared to alicyclic compounds because they possess heterocyclic structures. Also, many microorganisms will not grow on alkaloids as they may be toxic to these organisms and inhibit their growth. However, since this class of substances include compounds with highly complex structures and have only limited potential for chemical transformations, the studies on their microbial transformations and degradations are important both from the fundamental theoretical aspects as well as from the possibility of producing less toxic compounds with desirable physiological properties.

Strychnine is the major alkaloid present in the seeds of Strychnos nuxvomica tree which grow naturally in this area. Strychnine has a very complex chemical structure and is known to stimulate all portions of the central nervous

system with preference to the spinal cord. However, it is a powerful convulsant and death results from asphyxia. Consequently strychnine has no therapeutic application in the western system of medicine at present. The objective of this work, therefore, was to convert strychnine by microbial transformation into a product having more desirable pharmacological properties so that this locally available natural product may find some use in the preparation of a therapeutic agent.

As the microorganisms obtained from the National Collections of Industrial Microorganisms (NCIM), National Chemical Laboratory, Poona were found to be ineffective in transforming strychnine into any useful derivative, organisms that grow on strychnine were isolated from the local soil using the elective culture technique. The isolated organism was purified and identified upto the generic level and was shown to belong to Arthrobacter species. Although other organisms belonging to Arthrobacter species have been reported to grow on strychnine, the new organism has been conclusively established as being different from the previously isolated organisms. The transformation product, which was isolated in about 10% yield was identified as C_{16}-Hanssen-acid from its physical properties and chemical reactions.

In addition to the microbial transformation of strychnine into C_{16}-Hanssen acid, the microbial conversion of some of its derivatives which may have practical applications were also attempted. Thus the conversion of 2-nitrostrychnine into 2-aminostrychnine was carried out by another organism isolated from the local environment. This organism was also identified to the generic level and was shown to belong to Pseudomonas species, which apparently contains a nitro-arylreductase enzyme system as the same culture reduced other aromatic nitro compounds also to the corresponding amines. The fact that 2-aminostrychnine was obtained in low yields indicates that the amine formed was further degraded by the same organism.

This work, thus has led to the isolation and characterisation of new microorganisms for the degradation of strychnine into C_{16}-Hanssen acid and for the microbial reduction of 2-nitrostrychnine into 2-aminostrychnine.

CHAPTER II

HISTORICAL REVIEW

2.1 Introduction

The potential of microorganisms to perform selected chemical transformations is almost unlimited[1-4]. In fact the application of microorganisms to achieve desired changes represents one of the most fascinating aspects of man's scientific and technological development. If we trace the history of the use of microorganisms, we can see that ancient civilizations have made use of yeasts even as early as 3000 B.C. to convert the sugar moiety of certain plant materials into ethyl alcohol in the production of intoxicating liquors[5]. The scientific explanation of these processes had to wait until 1857 when Pasteur published his paper on the nature of fermentations[6].

Following Pasteur's publication, experiments to obtain desirable changes in substrate molecules were attempted by many investigators. Thus Boutroux in 1880 succeeded in the conversion of glucose into gluconic acid[7]. Brown obtained propionic acid from n-propanol and fructose from mannitol[8]. Later, Bertrand discovered the conversion of sorbitol into sorbose[9] and the formation of dihydroxyacetone from glycerol[10]. Some of these conversions are still used for preparation of these materials on an industrial scale.

Most of the early microbial transformations have, however, been largely confined to reactions involving carbohydrates[11], simple aliphatic and aromatic compounds[12-15] and steroids[16-19].

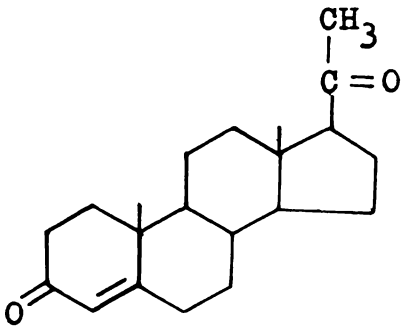
There were few known examples of transformations of

compounds of other structural classes, until 1959. Subsequently, however, a rapid development took place in this area of investigation following the classification of microbial transformations by Stodola which encouraged more fundamental studies involving different classes and types of substrates[20]. Most of the microbial conversions of organic compounds have been confined to the pharmaceutically and commercially important natural products such as steroids and terpenoids. Microbial transformation of alkaloids is a fairly recent field of investigation. We will limit our survey of previous work to these areas of natural products for the sake of brevity.

2.2 Microbial transformations of steroids

The oxidation and reduction of steroidal alcohols and ketones by yeasts and bacteria were first observed by Mamoli and Vercellone[21,22]. Murray and Peterson succeeded in their efforts to introduce oxygen functions into steroids using microbial techniques. They used a strain of the fungus, Rhizopus arrhizus, to convert progesterone (1) into 11 α-hydroxyprogesterone (2) in 10% yield, from which the naturally occurring adrenal hormone, cortisone (3) could be synthesised[23]. However, 6β,11α-dihydroxyprogesterone (4) and 11α-hydroxy-5α-pregnane-3,20-dione (5) were also formed in this conversion. Generally, the oxygenation at C-11 of steroid systems makes them pharmacologically active as antiinflammatory agents. This

microbial process is all the more important because the introduction of a hydroxyl group at 11 position is chemically very difficult. Further studies showed that a closely related fungus Rhizopus nigricans could effect this conversion in almost



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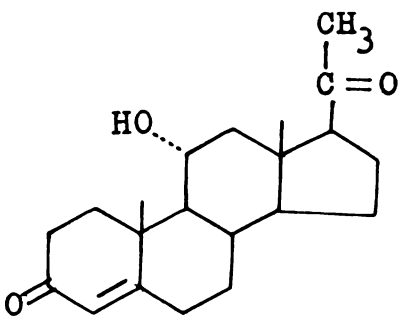

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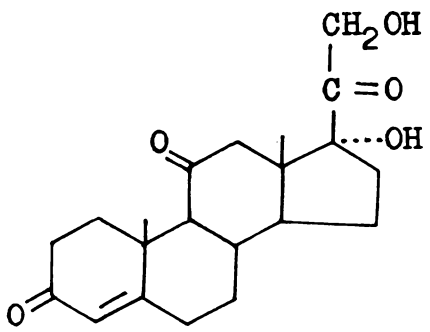

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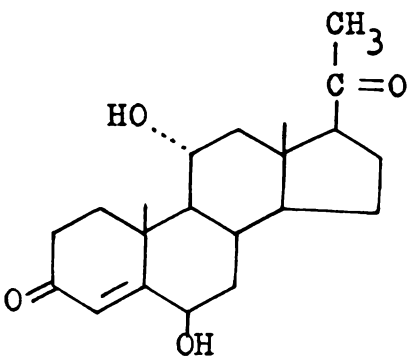

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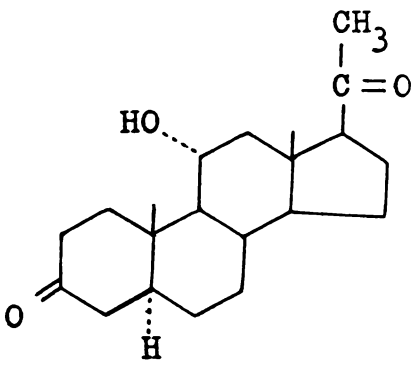

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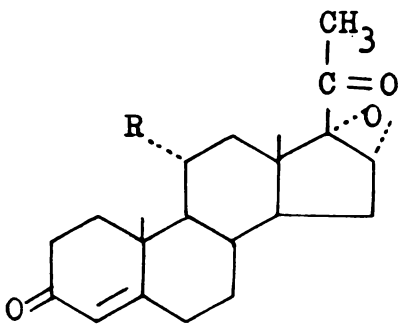
quantitative yield[24].

The microbial enzymes display a formidable, widespread and remarkable ability to hydroxylate steroids. It has been possible to introduce, microbiologically, a hydroxyl group at nearly every unsubstituted position of the steroid molecule[1]. Hydroxylations giving the 11 α and 6 β hydroxy derivatives are most common and 11 α hydroxylations of many steroidal systems have been scaled up. The conversion of 16α,17α-oxido-4-pregnene-3,20-dione (6) to 11 α-hydroxy-16 α,17 α-oxido-4-pregnene-3,20-dione (7) by a strain of Rhizopus species was scaled up and it was found that the fermentation went faster than when done on a laboratory scale[25].



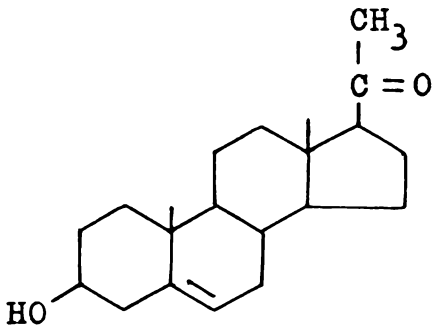
6, R = H

7, R = OH

An Aspergillus ochraceus strain (NRRL 405) converted pregnenolone (8) to progesterone (1), 11 α-hydroxyprogesterone (2) and 6β,11α-dihydroxyprogesterone (4)[26].

Colingsworth et al. showed that Cunninghamella

blakesleeana could produce cortisone (3) and cortisol (9) from Reichstein's compound-S (10)[27]. Both of these bio-oxidations viz 11 α -hydroxylation of progesterone (1) and 11 β -hydroxyla-



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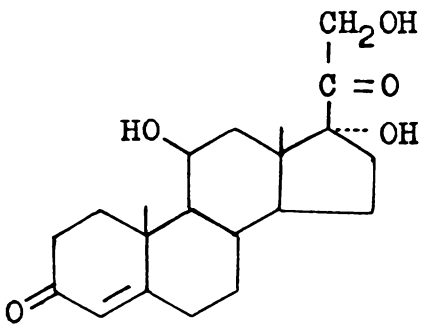

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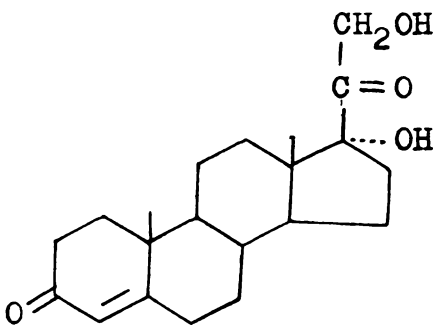

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tion of Reichstein's Compound-S (10) have been scaled up as a means of making adrenal hormones commercially.

Immobilised cells of Curvularia lunata, a 11 β -hydroxylator, hydroxylated Reichstein's Compound-S (10) to

cortisol (9) in a reaction medium containing 2.5% dimethylsulphoxide[28].

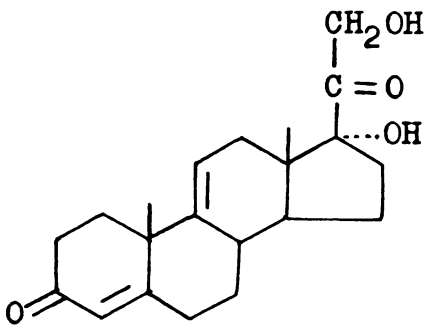
Studies by Dulaney and coworkers showed that the genus Aspergillus possessed a rather extensive ability to carry out 11α-hydroxylation besides hydroxylations at the 6β and 17 α positions, whereas most of the strains of Penicillium favoured 15-hydroxylation[29].

Bloom and Shull first observed the epoxidation of an isolated double bond using microorganisms. Thus Cunninghamella blakesleeana or Curvularia lunata or other 11β hydroxylating organisms converted 9(11)-dehydro Reichstein's Compound-S (11) to the 9β, 11β-oxido derivative, 12, and 9β, 11β-oxido-20 β-hydroxy derivative, 13[30]. Their work led to the theory that "a microorganism capable of introducing an axial hydroxyl function at C_n of a saturated steroid also effected the introduction of an epoxide grouping axial at C_n in the corresponding unsaturated substrate"[30].

Aromatization of steroids was observed with several microorganisms. 19-Nortestosterone (14) was converted by Septomyxa affinis to estrone (15) and estradiol (16)[31]. A strain of Corynebacterium simplex converted 19-hydroxymethyl-epidehydroandrosterone (17) to estrone (15) in 74% yield[32]. Estradiol (16) was transformed to the 2-hydroxy and 4-hydroxy derivatives, 18 and 19 respectively by a strain of Aspergillus

alliaceus[33].

The anti-inflammatory activity of well-known steroids



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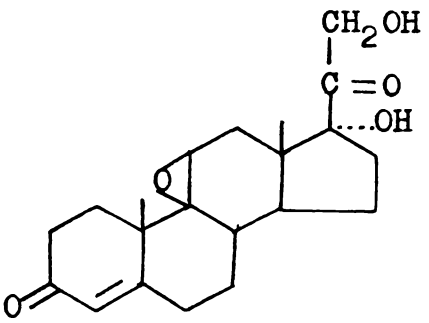

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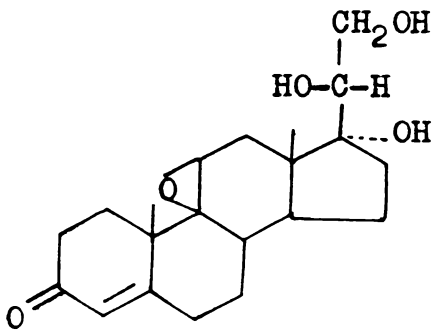

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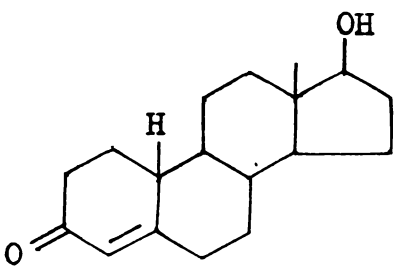

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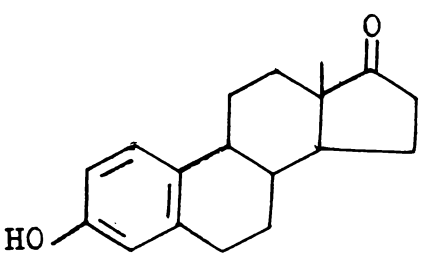

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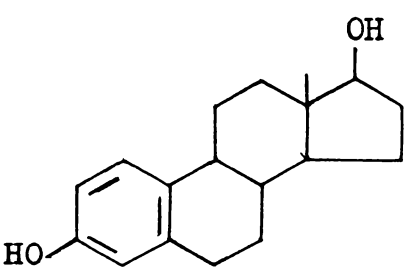

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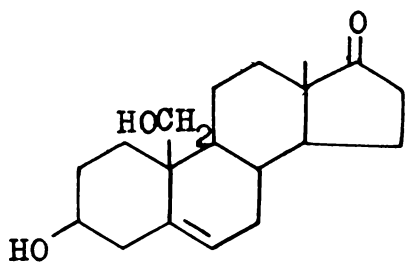
requires 11 β -hydroxy or 11-keto functions in pregnen-structures and is increased by a further Δ^1 double bond[3]. Therefore, this type of bioconversion is very important after the 11-hydroxylation or oxidation process. Nobile et al. reported the conversion of cortisol (9) to prednisolone (20), a compound with greater anti-inflammatory effects and less salt retention



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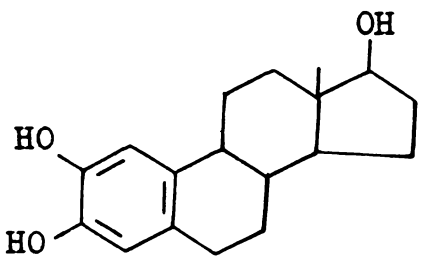

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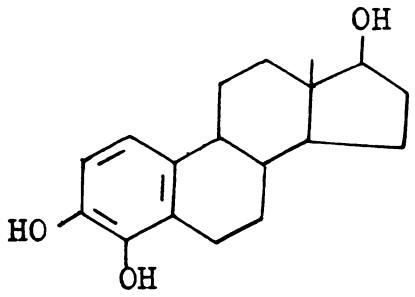

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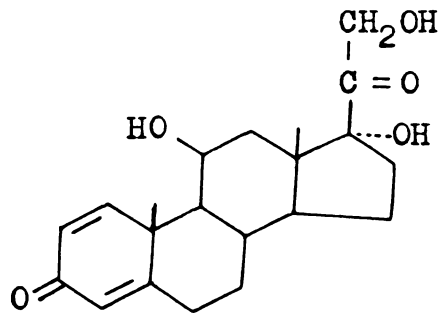

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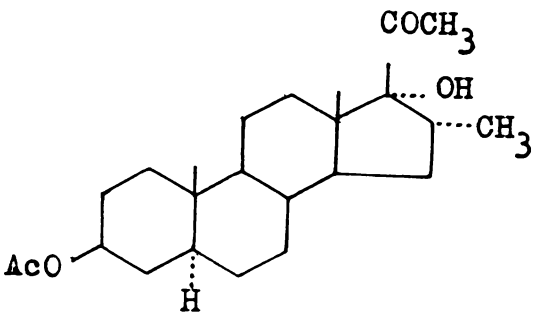
properties than cortisol, by strains of Corynebacterium simplex and many other microorganisms[34]. Recent reports show that use of immobilised viable cells of Arthrobacter simplex[35,36] and Arthrobacter globiformis[37] enhanced the yield and efficiency of this conversion.

A mixed culture of Mycobacterium smegmatis and Arthrobacter simplex converted 16 α-methyl-3 β ,17 α -dihydroxy-5 α -pregnane-20-one-3 β -acetate (21) to 16 α -methyl-17 α -hydroxy-pregna-1,4-diene-3,20-dione (22) in about 63% yield[38]. Incubation of 22 with Absidia glauca gave the 11 α-hydroxy derivative, 23, in 55 % yield[38]. Dehydroisoandrosterone (24) on



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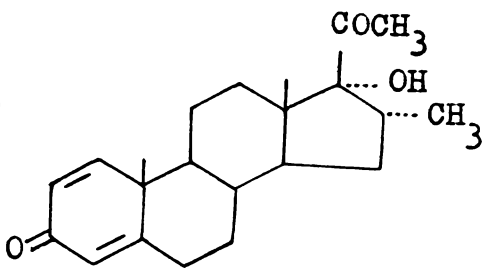

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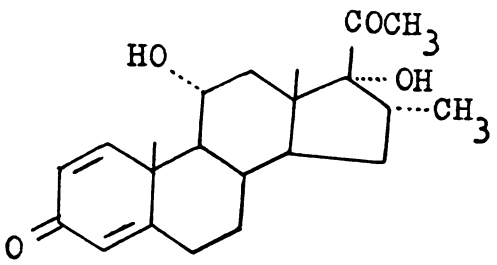

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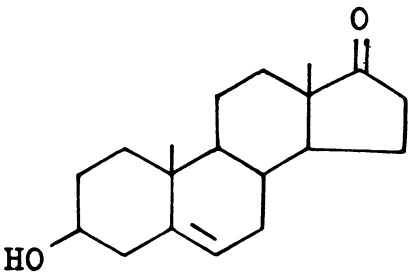
incubation with Fusarium oxysporum gave androsta-1,4-diene-3,17-dione (25) in 90% yield[39].



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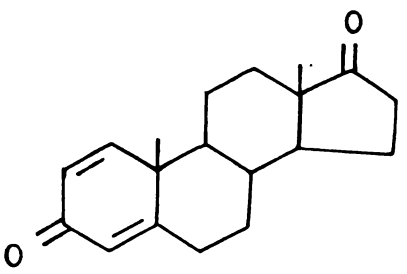

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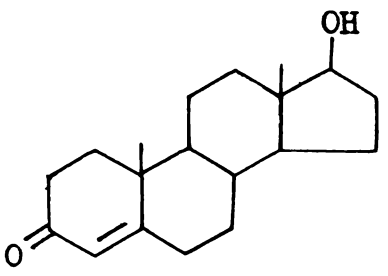
Testosterone (17 β -hydroxyandrost-4-en-3-one,26) was converted by Mycobacterium phlei to androsta-1,4-diene-3,17-dione (25)[40]. Bacillus sphaericus strains, (ATCC 7054 and 7055), on the other hand, transformed 26 to androst-4-ene-3,17-dione (27)[41]. Also, a strain of Streptomyces hydrogenans effected the same conversion[42]. A strain of Aspergillus fumigatus was reported to convert testosterone (26) to the 15 β-hydroxy derivative, 28[43].

Degradation of the side chain of cholesterol (29) by microorganisms had been known since 1913. Androsta-1,4-diene-3,17-dione (25) was isolated as a product, when cholesterol (29) was incubated with Mycobacterium phlei, in the presence of

chelating agents like 8-hydroxyquinoline[40]. Recent reports show that a mutant of Corynebacterium (chol.73) was able to convert cholesterol (29) to 20-carboxypregna-1,4-diene-3-one (30) in nearly quantitative yields[44]. Many phytosterols



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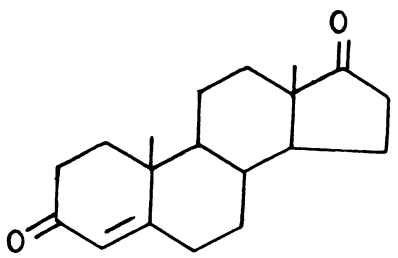

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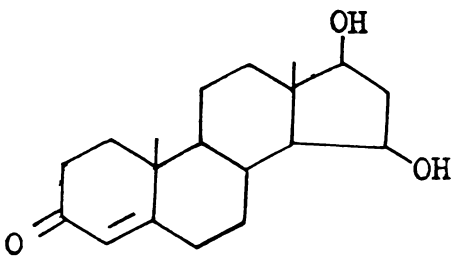

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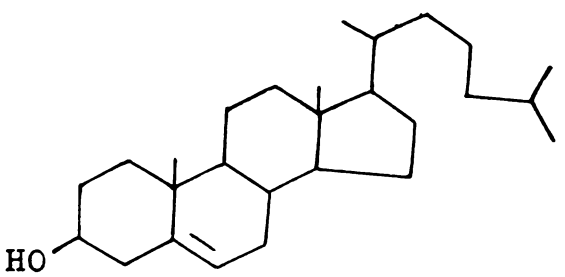

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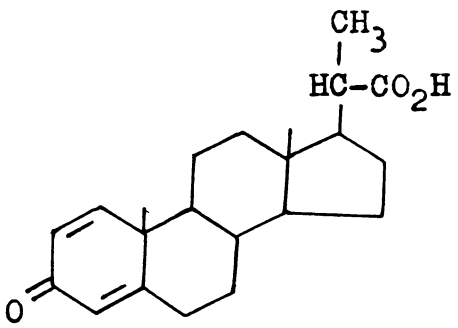

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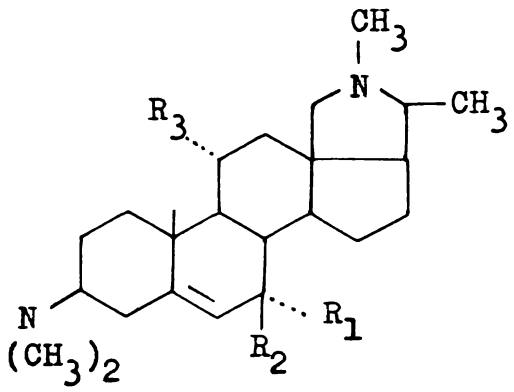
were also converted to androsta-1,4-diene-3,17-dione (25), using a strain of Arthrobacter simplex (ATCC 6946)[45].

2.3 Microbial transformations of steroidal alkaloids

Steroidal alkaloids are hydroxylated in the same way as steroids. Thus, conessine (31) underwent microbiological allylic oxidations to produce the 7 α -hydroxy and 7 β -hydroxy derivatives, 32 and 33, besides the 11α -hydroxy derivative, 34 in the presence of Gloeosporium fructigenum f. americana[46]. Compounds, 32 and 33 were also produced from 31 by Cunninghamella echinulata (NRRL A 11498)[47]. But a strain of Stachybotrys parvispora transformed conessine (31) to con-4-enin-3-one (35) and 11α -hydroxycon-4-enin-3-one (36)[46].



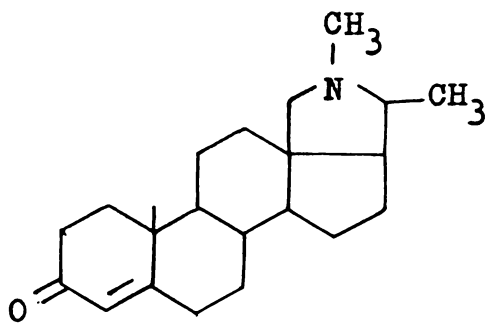
31, $R_1=R_2=R_3=H$

32, $R_2=R_3=H$; $R_1=OH$

33, $R_1=R_3=H$; $R_2=OH$

34, $R_1=R_2=H$; $R_3=OH$

Solasodine (37) similarly underwent allylic oxidations to give the 7 ∝ -hydroxy and 7 β -hydroxy derivatives, 38 and 39, alongwith the 9 ∝ -hydroxy and 11 ∝ -hydroxy derivatives, 40 and 41 in the presence of Helicostylum piriforme[48].



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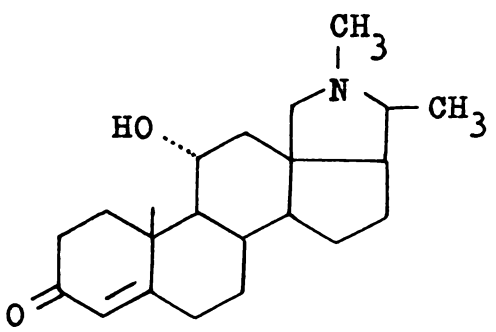

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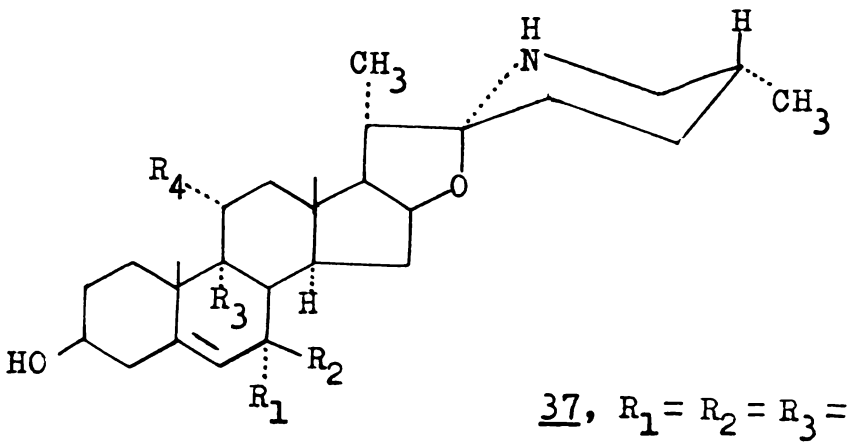


37, $R_1 = R_2 = R_3 = R_4 = H$

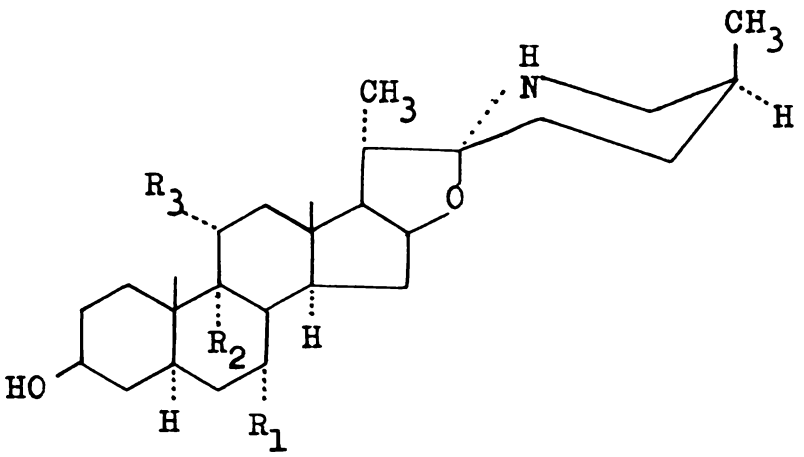
38, $R_2 = R_3 = R_4 = H$; $R_1 = OH$

39, $R_1 = R_3 = R_4 = H$; $R_2 = OH$

40, $R_1 = R_2 = R_4 = H$; $R_3 = OH$

41, $R_1 = R_2 = R_3 = H$; $R_4 = OH$

Tomatidine (42) was converted to the 7α-hydroxy, 9α-hydroxy and 7α,11α-dihydroxy derivatives, 43, 44 and 45 respectively by the same mould H. piriforme[49].



42, $R_1 = R_2 = R_3 = H$

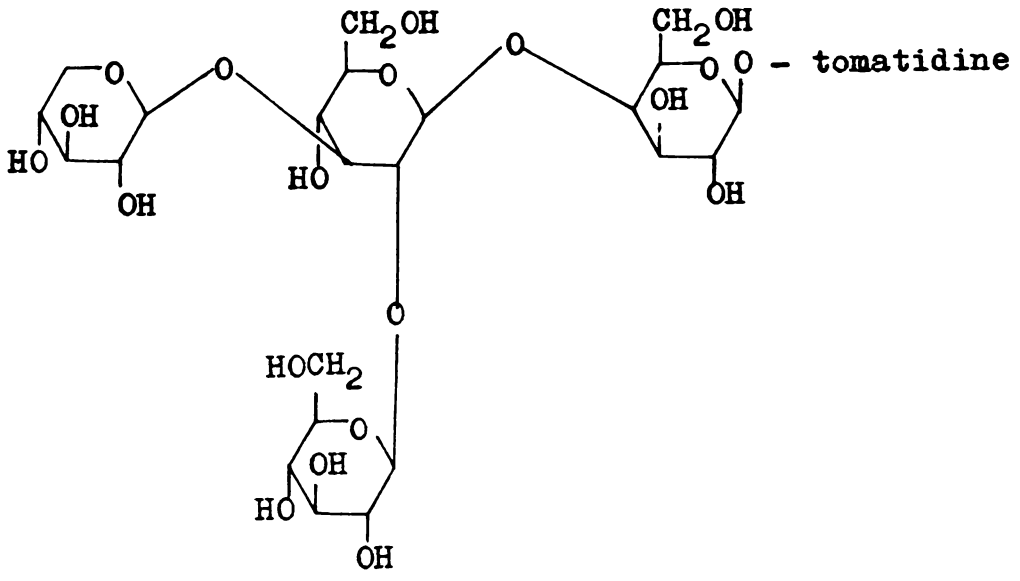
43, $R_2 = R_3 = H$; $R_1 = OH$

44, $R_1 = R_3 = H$; $R_2 = OH$

45, $R_2 = H$; $R_1 = R_3 = OH$

Tomatine (46) was reported to form a conjugate with

lactic acid on incubation with a strain of Nocardia restrictus. Such microbial conjugation of steroid alkaloids or their glycosides are rarely observed[50]. A strain of Aspergillus niger when grown in a malt agar medium at pH 4.5, in presence of the crude glycoalkaloids from Solanum khasianum, hydrolysed the same to the aglycone solasodine (37)[51].



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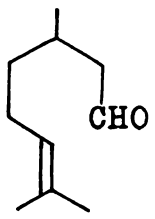
2.4 Microbial transformations of terpenoids

Most investigations on the microbial transformation of terpenes have been centered on the steroidal terpenes probably because of their commercial importance. The non steroidal terpenes remains more or less an unexplored area[52]. Microbial transformations of monoterpenes and sesquiterpenes have been recently reviewed elsewhere[53]. The vast majority of microbial transformations of terpenes involves the breakdown of these natural products. Also, they have been of little practical value for the preparation of metabolites because of the difficulties encountered in preventing their complete decompositions. These experiments are made more difficult by the fact that suitable strains have only been obtained by tedious adaptation tests on appropriate substrates and that the substrates as well as the end products are usually lost as volatile compounds by the aeration used in deep tank fermentations[4].

Microbial transformations of a number of acyclic terpenes, cyclic terpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes have been reported, as briefly reviewed here.

Mayer and Neuberg, in 1915, were the first to report the reduction of citronellal (47), an acyclic terpene, to citronellol (48) by yeast[54]. Also some strains of Acetobacter xylinum or Acetobacter ascendens caused transformation of citronellal (47) to citronellol (48) and citronellic acid (49)[55].

The microbial breakdown of acyclic terpenes was investigated in detail by Seuberg et al. using Pseudomonas citronellolis, isolated from soil samples by enrichment technique with citronellol (48) as the main carbon source. This strain was capable of degrading citronellal (47), citronellol (48), citronellic acid (49), geraniol (50), geranic acid (51) and a sesquiterpene, farnesol (52). The



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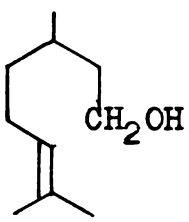

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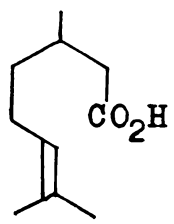

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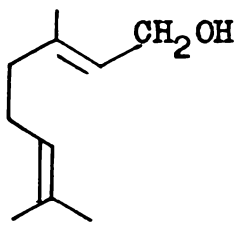

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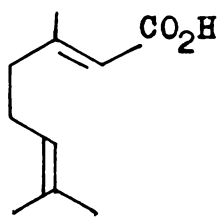

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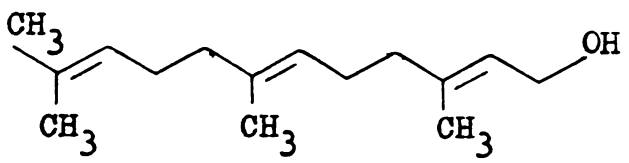

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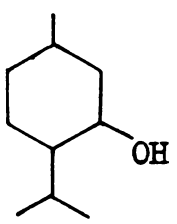
end products were acetic acid, acetoacetic acid and citric acid[56,57].

Joglekar and Dhavlikar were able to conclusively prove the cyclisation of acyclic terpene when they obtained menthol (53) in addition to citronellol (48), citronellic acid (49), dihydrocitronellol (54) and 2,8-dihydroxy-2,6-dimethyl octane (55) from citronellal (47) by the action of Pseudomonas aeruginosa[58,59].



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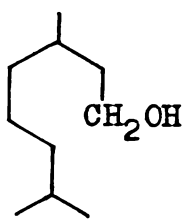

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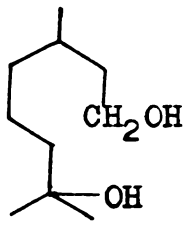

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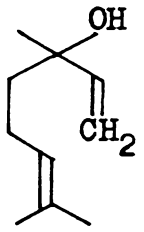
The cyclisation of linalool (56) to camphor (57) was reported by Mitzutani and others, using a newly isolated pseudomonad (strain A)[60]. 5-Methyl-5-vinyl-tetrahydro-2-furanone (58), 4-methyl-trans-3-hexenoic acid (59) and 2,6-dimethyl-6-hydroxy-trans-2,7-octadienoic acid (60) were also formed in this transformation.

Gunsalus and his students studied extensively the degradation of camphor (57) using Pseudomonas putida strain C_1 and an unidentified Corynebacterium, strain T_1. The strain C_1

oxidized (+) camphor (57) to 5-endo-hydroxy camphor (61), 5-exo-hydroxy camphor (62) and 5-oxocamphor (63)[61]. A different strain of Pseudomonas putida, C_5 also degraded 57 by another route,



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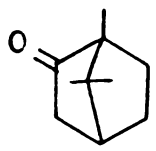

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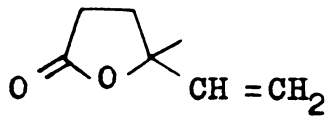

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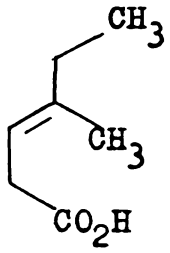

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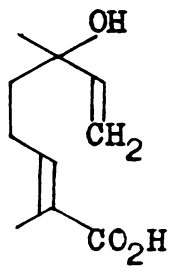

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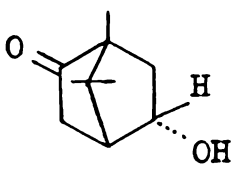
where the initally formed 1,2-campholide (64) was converted to the 5-exo-hydroxy derivative, 65[62]. However, both these strains gave 66 and 67 as the end products, by different pathways.

Corynebacterium strain T_1 degraded 57 by first producing 6-endo-hydroxycamphor (68) and 6-oxocamphor (69)[63]. The degradation of both enantiomers of camphor by Corynebacterium strain T_1 gave the common intermediate, 69, and the further



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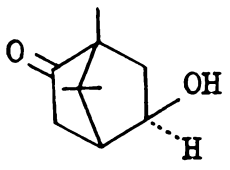

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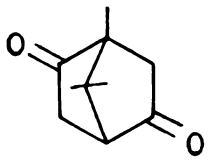

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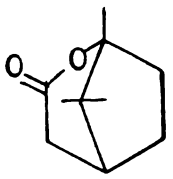

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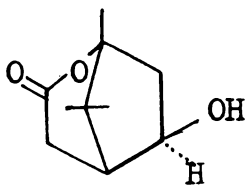

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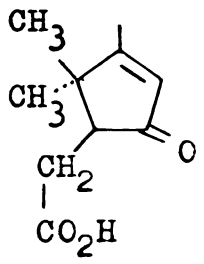

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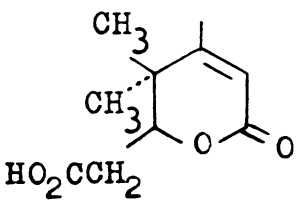

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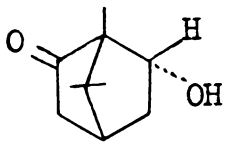

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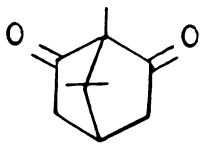
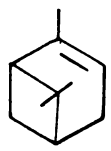


69

degradation proceeded as a single pathway[64]. In the case of Pseudomonas putida both the enantiomers are oxidised by separate paths via the optical antipodes[65]. The microbial transformation of 57 giving 64 and 66 giving 67 represent reactions analogous to Baeyer-Villiger oxidations of cyclic ketones by peracids.

The studies by Bhattacharyya et al.[66] and Prema and Bhattacharyya[67] showed that the fungus Aspergillus niger (NCIM 612) was able to utilize the bicyclic mono-terpene d-α-pinene (70). Three oxidised products, d-verbenone (71), d-cis-verbenol (72) and d-trans-sobrerol (73) were formed in a stereospecific manner. β-Pinene (74) was transformed by Aspergillus niger (NCIM 612)



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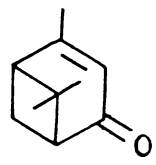

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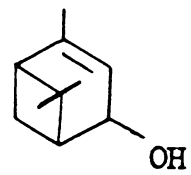

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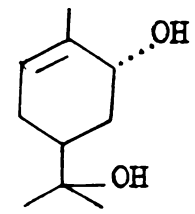

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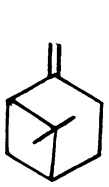
to (-) pinocarveol (75), (-) pinocarvone (76) and (-) myrtenol (77)[68]. Limonene (78) was converted by the same mould to

cis-carveol (79), carvone (80), α-terpineol (81) and 2,8-p-menthadien-1-ol (82)[69]. From these observations Bhattacharyya and Ganapathy concluded that Aspergillus niger introduced the hydr-



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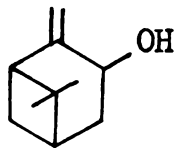

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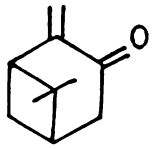

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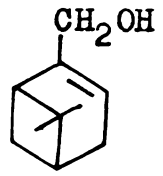

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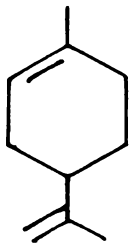

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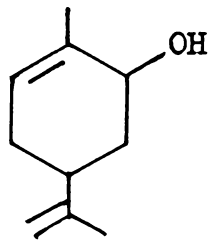

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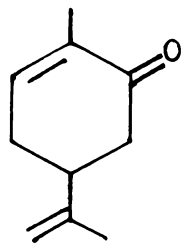

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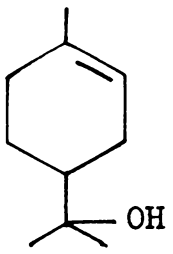

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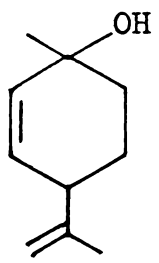

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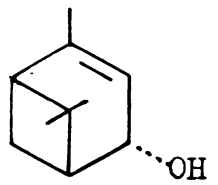


83

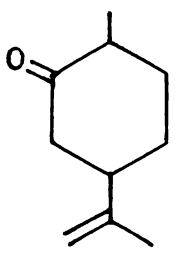
oxyl groups into the substrate molecules in three different ways- a) hydration of a double bond b) oxygenation at a position allylic to a double bond and c) oxygenation of a double bond followed by secondary reactions[70].

A strain of Serratia marcescens, isolated from sewage sludge, could effect the allylic oxidation of d-α-pinene (70) to d-trans-verbenol (83) as major product, alongwith d-verbenone (71) and d-trans-sobrerol (73) as minor products[71]. Allylic oxygenation of α and β-pinene was reported to be the most characteristic reaction of a strain of the honey fungus Armillariella mellea[72].

The microbial degradation of limonene (78) by a soil pseudomonad yielded a variety of neutral and acidic products- cis-carveol (79), carvone (80), dihydrocarvone (84), 1,2-cis-dihydroxy-8-p-menthene (85), 1-hydroxy-2-oxo-8-p-menthene (86), 1,2-trans-dihydroxy-8-p-menthene (87), 6,9-dihydroxy-1-p-menthene (88), perillic acid (89), 3-isopropenyl-heptanedioic acid (90), 2-hydroxy-8-p-menthen-7-oic acid (91) and 4,9-dihydroxy-1,8(10)-p-menthadien-7-oic acid (92). Three different pathways of limonene catabolism were also proposed[73]. A major part of limonene, however, was completely metabolised to carbondioxide and water.

Co-oxidation of limonene (78) in a medium containing a hydrocarbon, with Corynebacterium hydrocarboclastus, depre-

ssed further oxidation of the substrate and yielded carvone (80) which was isolated in good yields[74]. This process is commercially viable for the production of carvone (80).



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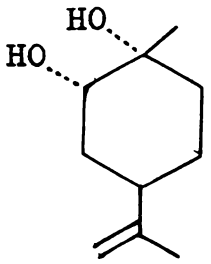

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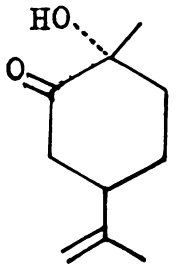

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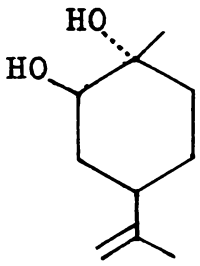

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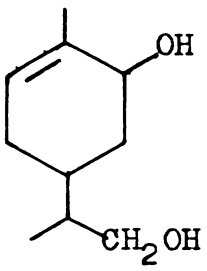

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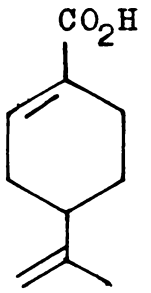

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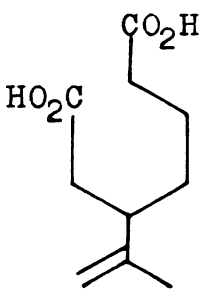

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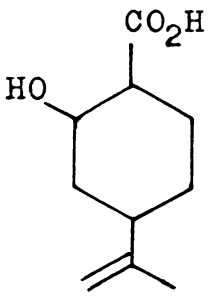

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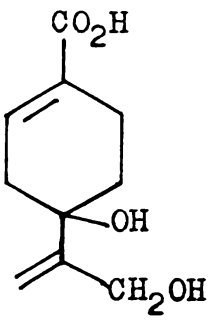
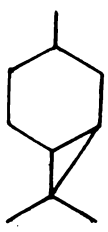


92

Prema and Bhattacharyya also studied the action of Aspergillus niger on the monoterpenic hydrocarbons, carane (93) and 3-carene (94) and the sesquiterpenic hydrocarbons, humulene (95) and α-santalene (96)[68]. Carane (93), 3-carene (94) and humulene (95) resisted oxidation, although 3-carene (94) was converted to an unidentified hydroxy ketone $C_{10}H_{14}O_2$ in poor yields after prolonged fermentation. α-Santalene (96) was converted to tere-santalic acid (97), tere-santalol (98) in low yields and to a tertiary alcohol $C_{15}H_{24}O$, which was not completely characterised.



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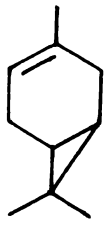

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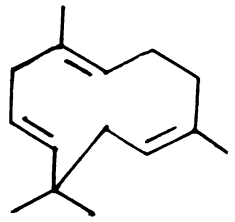

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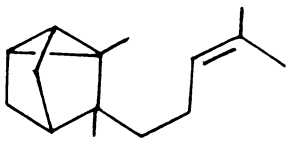

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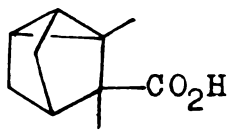

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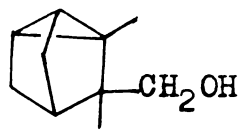
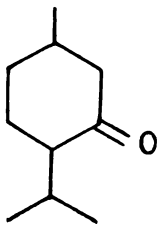


98

A bacterium isolated from the sewage by enrichment techniques was found to utilise (-) menthol (53) as the sole source of carbon producing menthone (99), 3,7-dimethyl-6-hydroxyoctanoic acid (100) and 3,7-dimethyl-6-oxo-octanoic acid (101)[75].



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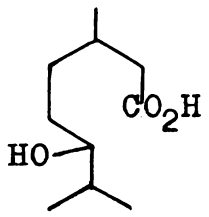

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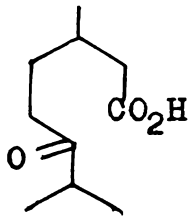
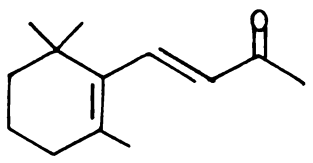


101

The pregrown mycelia of the fungus, Lasiodiplodia theobromae (ATCC 28570) was able to transform β-ionone (102) into a number of metabolites by mainly degrading the side chain of the molecule by a C_2-unit. The enzyme system responsible for this degradation was proposed to be an oxygenase. β-Cyclohomogeraniol (103) was isolated as the main product[76]. α-Kessyl alcohol (104), a constituent of various species of Japanese Valerian, was hydroxylated by several microorganisms (Cunninghamella blakesleeana, Corticium centrifugum, Corticium sasakii, Streptomyces aureofaciens) to kessyl glycol (105), kessane-

2 β,7-diol (106) and a minor product kessan-2-one-7-ol (107)[77]. Needless to say that it is very difficult to bring about these hydroxylations chemically.



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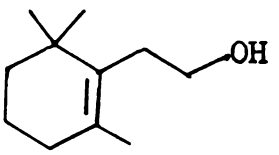

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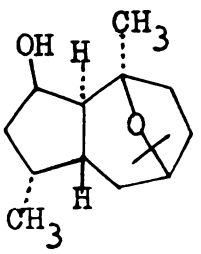

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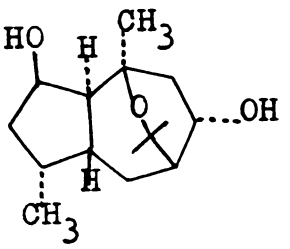

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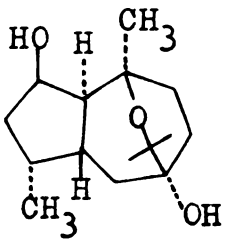

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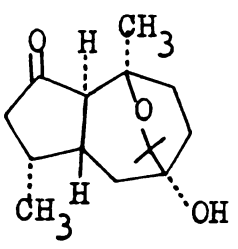
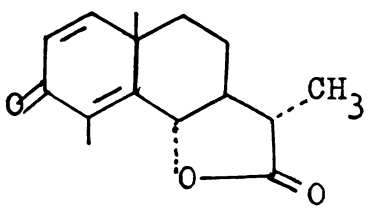


107

α-Santonin (108) on incubation with Cunninghamella blakesleeana or Streptomyces aureofaciens yielded 1,2-dihydro-α-santonin (109) as the main metabolite[78]. The same conversion was effected with a strain of Streptomyces cinereocrocatus (NRRL 3443)[79]. The soil microorganism, Pseudomonas cichorii strain S, isolated by enrichment techniques utilised α-santonin (108) as the sole carbon source, producing 1,2-dihydro-α-santonin (109) and lumisantonin (110)[80,81]. Later studies showed that α-santonin reduction was catalysed by a NADH or NADPH dependant oxidoreductase enzyme. It was also postulated that the enzymatic reduction of α-santonin was taking place via the



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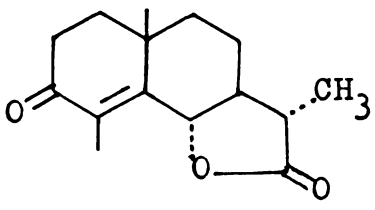

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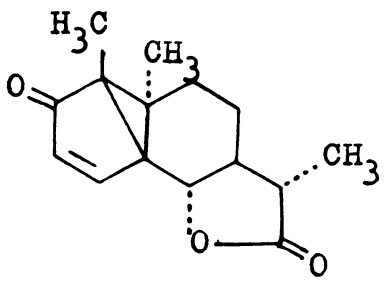
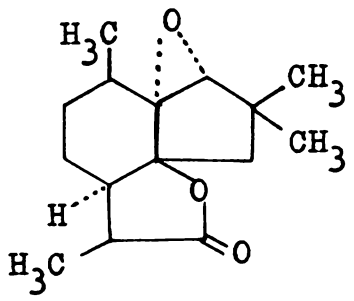


110

formation of a transient zwitterionic intermediate, which underwent nonenzymatic 1,4-sigmatropic rearrangement to yield lumisantonin (110) during the solvent extraction process[82].

A strain of the fungus Marasmius alliaceus could transform the sesquiterpene, alliacide (111) to alliacolide (112) and 12-hydroxy alliacolide (113)[83].



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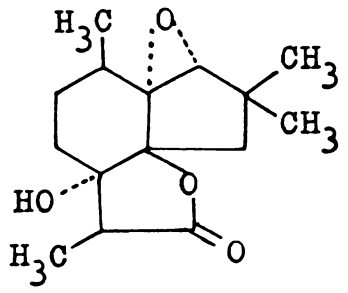

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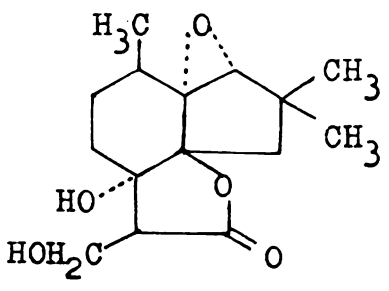
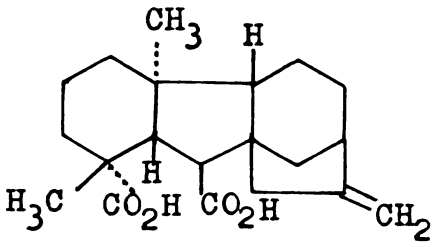


113

Cross and his colleagues, during their studies on the biosynthesis of the gibberellins were able to demonstrate the conversion of gibberellin A_{12}(114) to gibberellic acid (gibberellin A_3,115) and gibberellin A_{13} (116) by the gibberellin producing fungus Gibberella fujikuroi[84]. Gibberella fujikuroi converted (-)-16-kaurene (117) to gibberellic acid (115)[85]. Calonectria



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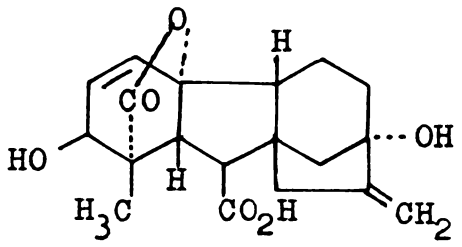

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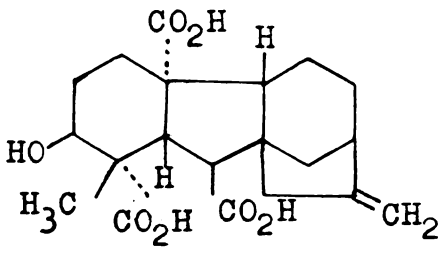

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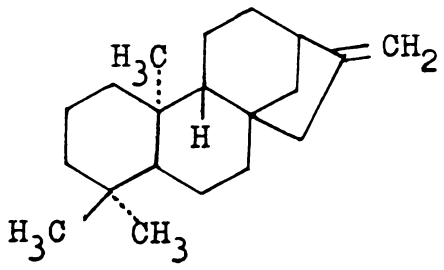
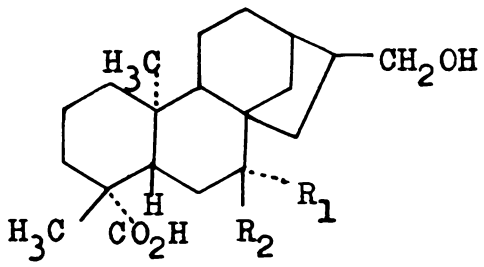


117

decora transformed ent-17-hydroxykauran-19-oic acid (118) to the 7α and 7β hydroxy derivatives, 119 and 120[86]. Transformation of ent-18-acetoxykaur-16-en-3,7-dione (121) was carried out with a strain of Aspergillus niger to give, 122, as a metabolite, after 48 hours of incubation. Compound 121, on prolonged incubation gave many hydroxylated derivatives of 122[87].



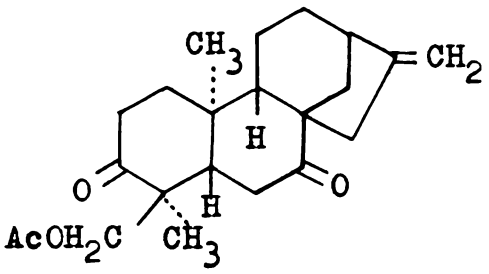
118, $R_1 = R_2 = H$

119, $R_1 = OH$; $R_2 = H$

120, $R_2 = OH$; $R_1 = H$

The microbial degradation of diterpenic acids has been investigated by Delaporte and Daste[88]. Biellmann et al. found

that dehydroabietic acid (123) could be metabolised by Flavobacterium resinovorum, to yield traces of a number of products, one of which, 124, might have been formed by the oxidation of a probable hydroxylated intermediate followed by decarboxylation[89].



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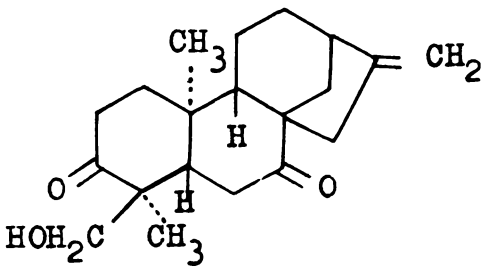

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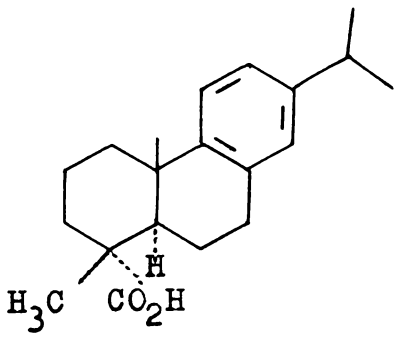

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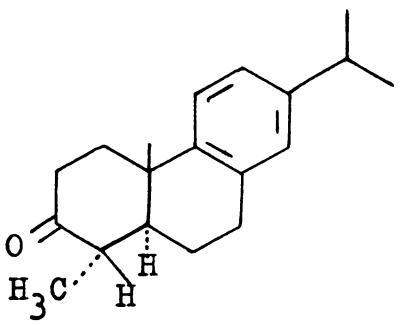
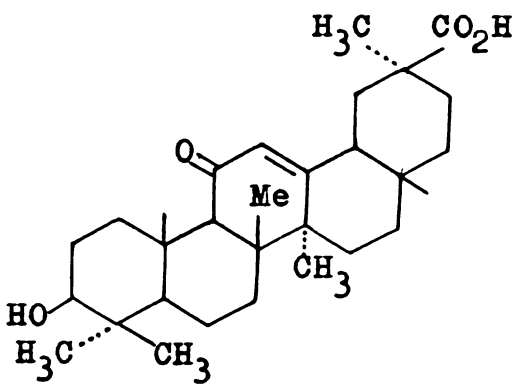


124

Canonica et al. reported that Curvularia lunata (ATCC 13432) oxidised glycyrrhetic acid (125), a triterpene, to 3-keto glycyrrhetic acid (126) and 7β-hydroxy glycyrrhetic acid (127)[90].



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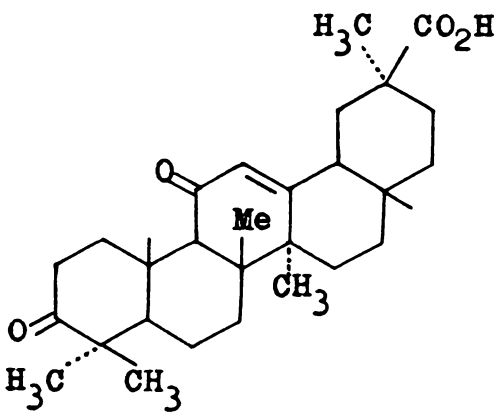

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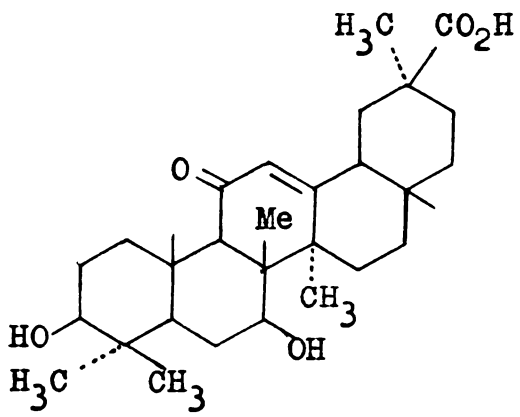

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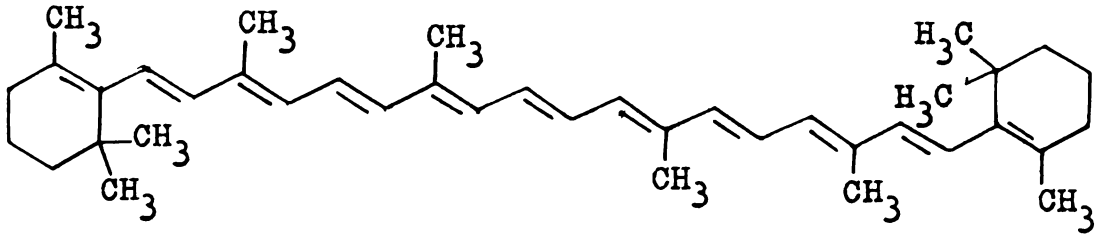
Dmitrovskii and Starikova reported the cleavage of β-carotene (128) to vitamin A (129) by a strain of Pseudomonas aeruginosa[91].



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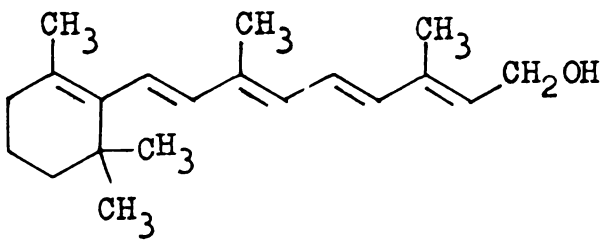


129

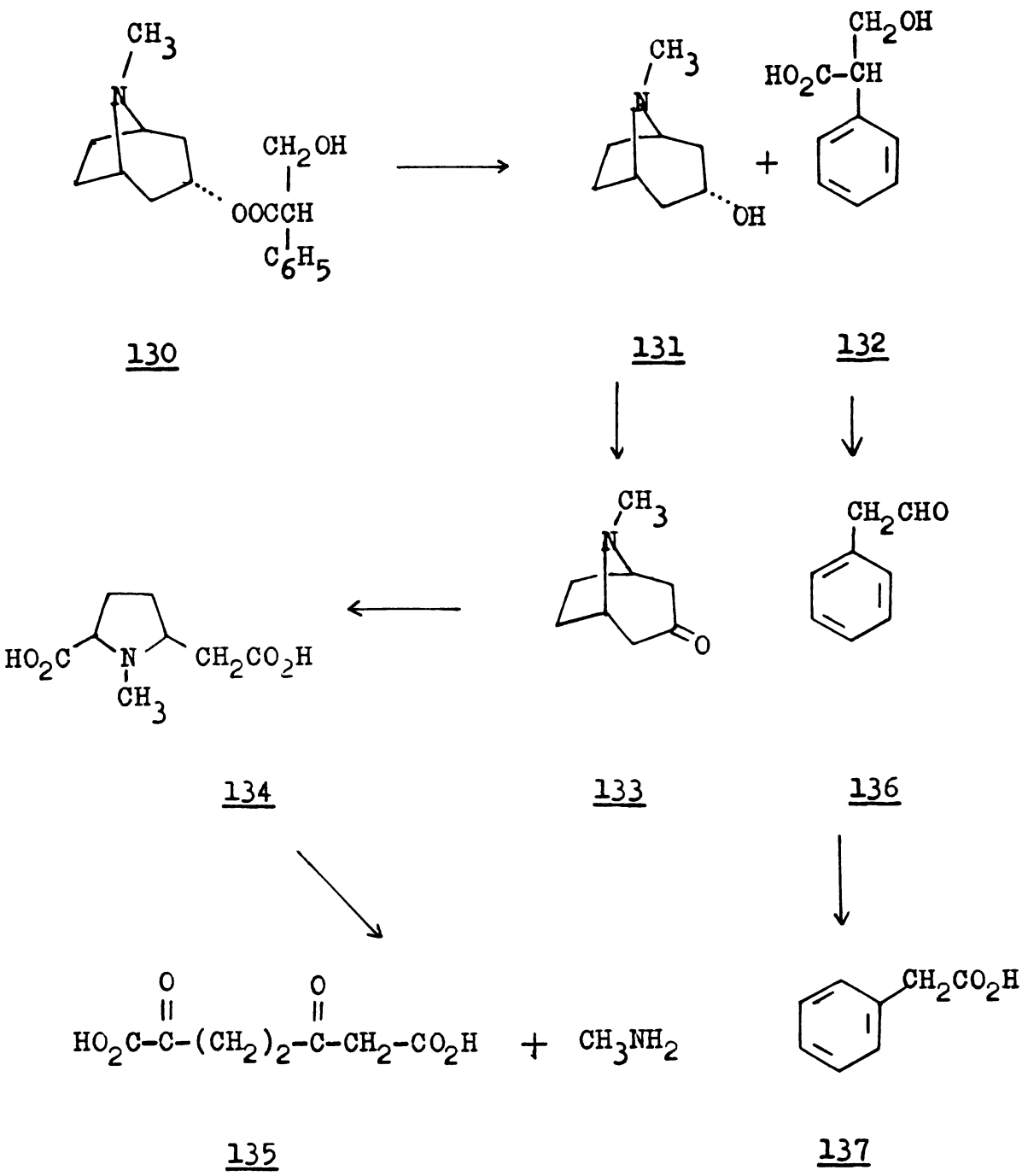
2.5 Microbial transformations of alkaloids

Compared to alicyclic compounds, alkaloids with heterocyclic structures are generally more difficult to be transformed. A heterocyclic N-atom is frequently a target of attack for undesired degradations. This is shown by low yields and usually by unfavourable material balances. On the other hand, ring linkages via such heteroatoms may yield sterically unfavourable substrate structures, which are resistant to attack or are only attacked non-specifically[4]. The work on the microbial transformation of alkaloids have been reviewed by Tamm[1], Vining[92] and recently by Iizuka and Naito[18] and Davis[93].

2.5.1 Tropa alkaloids

Niemer et al. isolated a bacterium, Arthrobacter oxydans, from soil which was overgrown with 'deadly night shade' bushes (Atropa belladonna) capable of utilising atropine (dl-hyoscyamine, 130) as the sole C and N source[94,95]. The sulphate of 130 was hydrolysed by this organism to tropine (131) and tropic acid (132). Compound 131, was oxidised via tropinone (133) to tropinic acid (134) and probably, split by oxidation to yield 2,5-dioxoheptanedioicacid (135) and methyl amine. Tropic acid (132) was subsequently decarboxylated and oxidized via phenyl acetaldehyde (136) to phenyl acetic acid (137) which inhibited further bacterial

growth[94,96]. Hyoscyamine and scopolamine (138) were broken down in the same way by this organism but nortropine (139) was not transformed[96-98]. Tamm reported that tropinone (133)

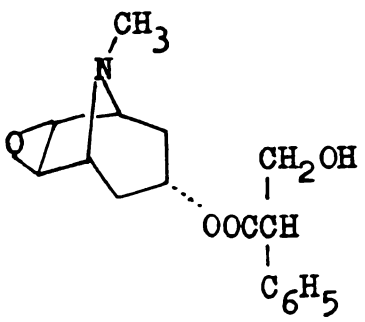


130 131 132

134 133 136

135 137

was reduced by Fusarium lini to ψ-tropine (140) and tropine (131)[99]. The transformation of 131 to 140 was effected by the synergistic action of two bacterial strains, an aerobic spore-producing Bacillus alvei and an enterococcal strain - Diplococcus[100]. The reaction did not occur via the intermediate keto compound, 133. It proceeded via the dehydration product 2-tropene (141), to which addition of water took place, as shown by the incorporation of tritiated water in the final product, 140[101].



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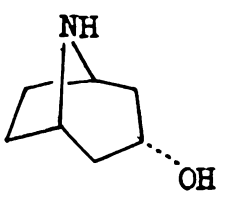

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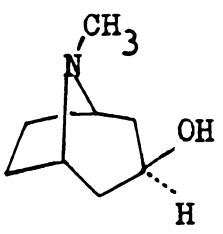

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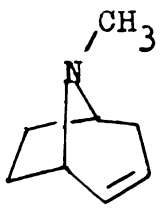
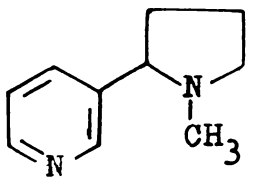


141

2.5.2 Pyridine alkaloids

The microbial degradation of nicotine (142) has been reviewed by Kuffner et al. in 1963[102]. A strain of Pseudomonas species degraded nicotine (142) to give 3-(3-carboxypropanoyl)-pyridine (143)[103]. Compound 143, was produced via the intermediate, pseudooxynicotine (144), subsequently isolated from the same fermentation reaction[104]. Nicotine (142) on incubation with Pseudomonas nicotinophaga gave 143, 144 and 3-(3-carboxypropanoyl)-6-hydroxy pyridine (145) and methylamine[105]. Micro-



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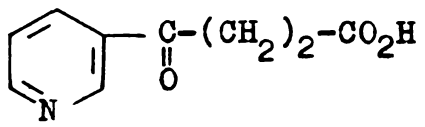

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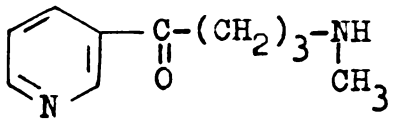

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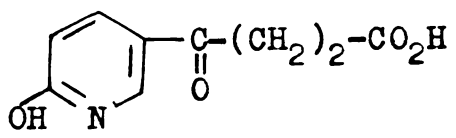
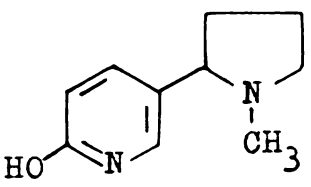


145

organisms belonging to the genera Pseudomonas, Alcaligenes, Achromobacter, Bacterium, Bacillus and Arthrobacter, utilised nicotine as the sole C and N source[106,107]. After a series of investigations, Rittenberg et al.[108-112] and Decker et al.[113-116] concluded that the initial step in the degradation of nicotine (142) by Arthrobacter oxydans was the 6-hydroxylation of the pyridine ring to give 6-hydroxynicotine (146). Further transformation of 146 was investigated with cell-free enzyme preparations. 6-Hydroxynicotine (146) was dehydrogenated to give 6-hydroxy-N-methylmyosmine (147), which on hydration gave 6-hydroxypseudooxynicotine (148)[109] and on further degradation yielded 2,6-dihydroxypyridine (149) and 4-methylaminobutyric



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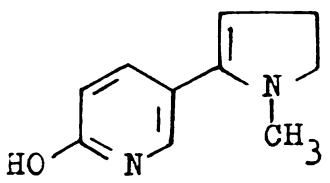

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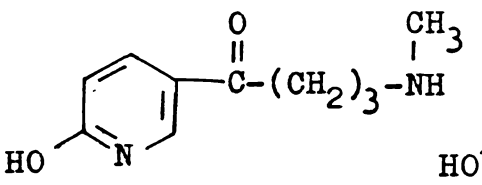

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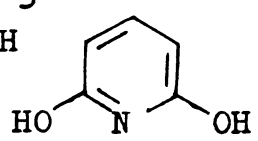
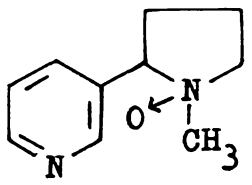


149

acid[111,112]. Arthrobacter globiformis was also reported to degrade nicotine (142) to give 146 and 147[117]. Nicotine N'-oxide (150) was degraded by this organism to give N-methylmyosmine (151) and 3-(3-carboxypropanoyl)-pyridine (143). Thus it is seen that nicotine and its N'oxide are metabolised in different pathways. Pellicularia filamentosa (JTS 208), a pathogen of tobacco, transformed nicotine (142) to nornicotine (152) and Cunninghamella echinulata (IFO 4444) converted nicotine (142) to give 151 and 152[118].



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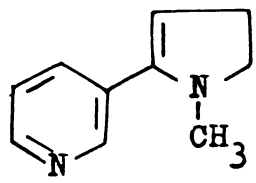

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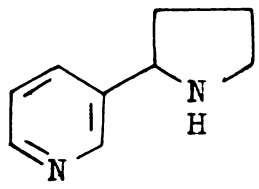
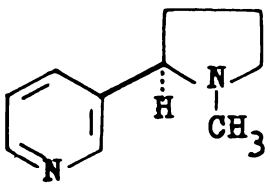


152

Yamashita et al. reported the isolation of R (+) nicotine (153) from R,S nicotine by selective degradation of S (-) nicotine (154) using cell-free enzymes from Arthrobacter oxydans[119]. Some strains of Pseudomonas putida were also able to effect this resolution[120-122]. It was reported that 153 was also degraded by P. putida at a slower rate in this resolution[121].



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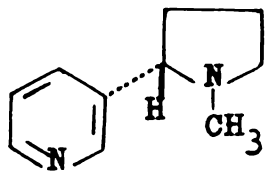
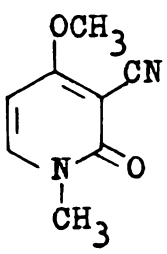


154

Hydrolysis of ricinine (155) (which is difficult to hydrolyse chemically) was carried out by a Pseudomonas strain to give the acid, 156[123].



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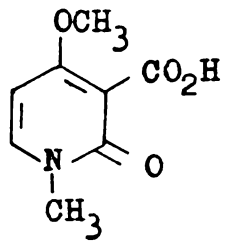
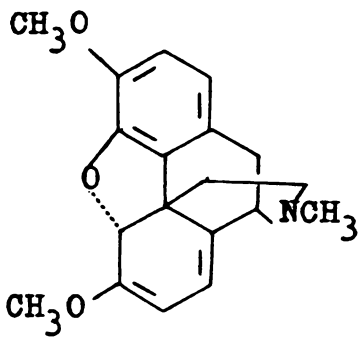


156

2.5.3 Isoquinoline alkaloids

The microbial transformation of morphine alkaloids was investigated by Iizuka and coworkers[124]. Thebaine (157) was converted to 14 β -hydroxycodeinone (158) and 14 β -hydroxycodeine (159) by the fungus Trametes sanguinea. The yield of the products varied depending upon the composition of the medium[124]. This fungus converted codeinone (160) to 14β -hydroxycodeinone (158), 14β-hydroxycodeine (159) and codeine (161)[125]. The same organism also produced



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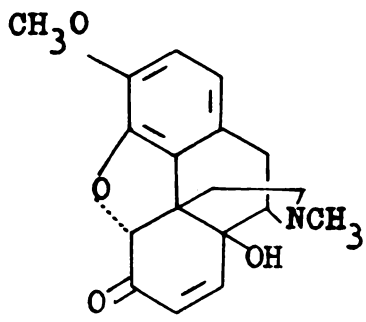

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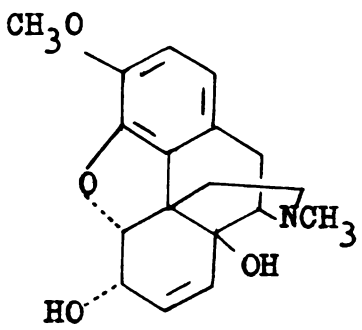

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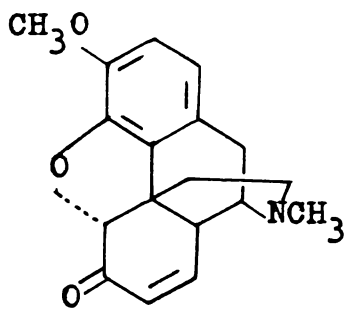
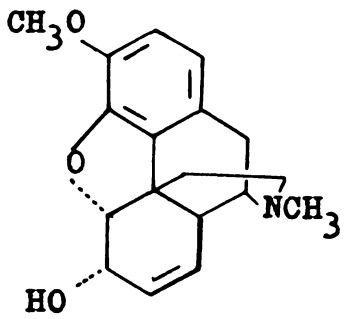


160

158, 159 and 161 from neopinone (162), depending upon the composition of the medium[125].



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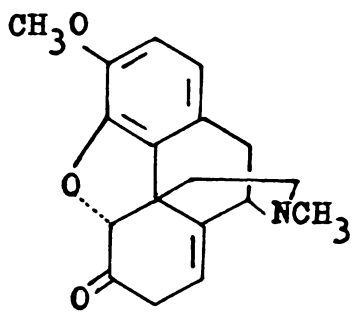
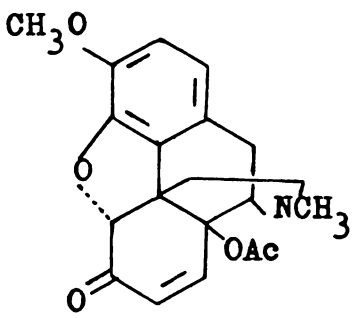


162

Several 14-substituted morphine alkaloids with α , β unsaturated C_6 carbonyl groups were found to be stereospecifically reduced[126]. Thus 14 β -acetoxycodeinone (163) was converted into 14 β -hydroxycodeine (159) by Trametes sanguinea in 70% yield[127]. On the other hand, 14 β -bromocodeinone (164) underwent only an enzymatic reduction to give 14 β -bromocodeine (165)[127].

The microbial transformationof 6,14-endo-ethenotetrahydrothebaine derivatives, which are very powerful analgesics, were

studied using Cunninghamella and Xylaria strains[128]. Thus, 7α-



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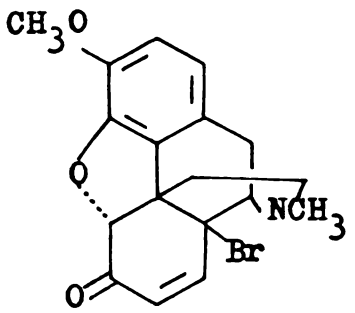

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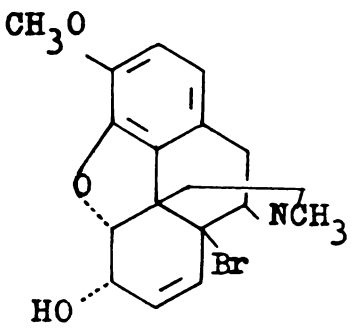
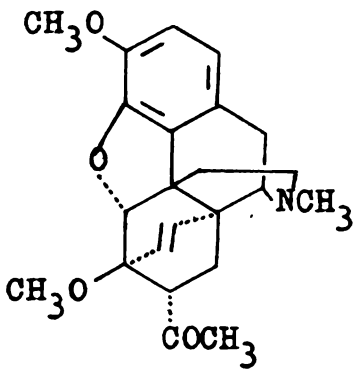


165

acetyl-6,14-endo-ethenotetrahydrothebaine (166) was transformed

to 167 and 168. Microbial dealkylation of these compounds was found to be superior to chemical methods[128].



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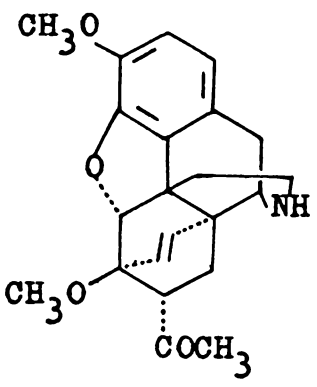

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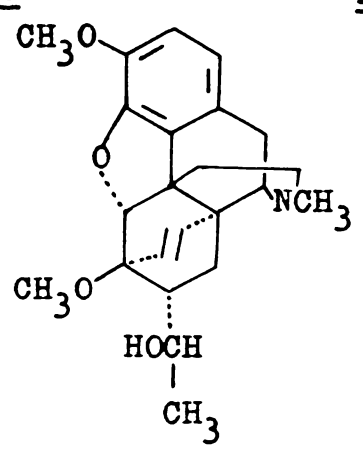
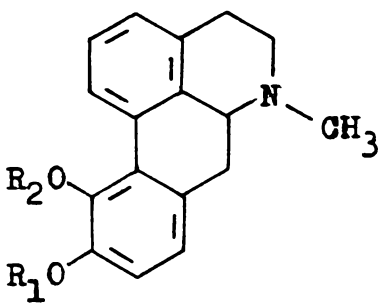


168

Microbial transformations of 10,11-dimethoxy-aporphine (169) were studied to determine the potential of micro-

organisms to produce monomethoxyaporphines. Thus, Cunninghamella elegans (ATCC 9245) converted 169 quantitatively into isoapocodeine (170). A Streptomyces species (SP-WISC 1158) gave a mixture of isoapocodeine (170) and apocodeine (171) in 20 and 24% yields respectively. It was found that the 10-methoxyl group was more susceptible to metabolic cleavage than the sterically hindered 11-methoxyl group[129]. A preparative-scale regiospecific conversion of 169 to 170 was conducted using C. elegans (ATCC 9245) by Smith and Davis[130].



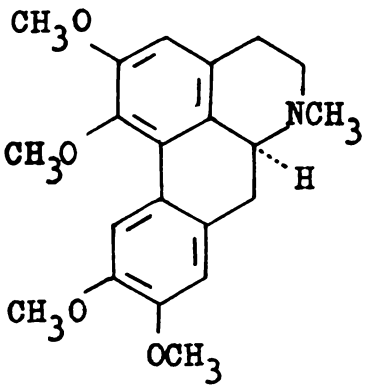
169, $R_1 = R_2 = CH_3$

170, $R_2 = CH_3$; $R_1 = H$

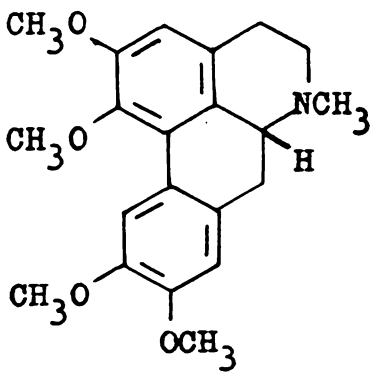
171 $R_1 = CH_3$; $R_2 = H$

Fusarium solani stereospecifically and quantitatively oxidised S-(+)-glaucine (172a) to dehydroglaucine (173), whereas R-(-)-glaucine (172b) was not metabolised[131].

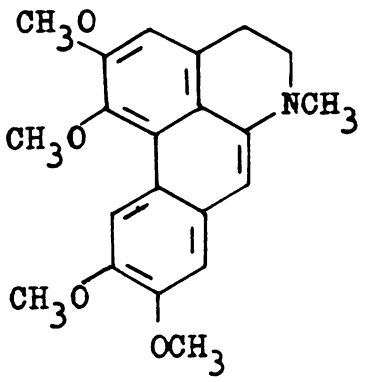
A strain of Nocardia species, isolated from soil by enrichment technique, was found to use papaverine (174) as the sole



172a



172b



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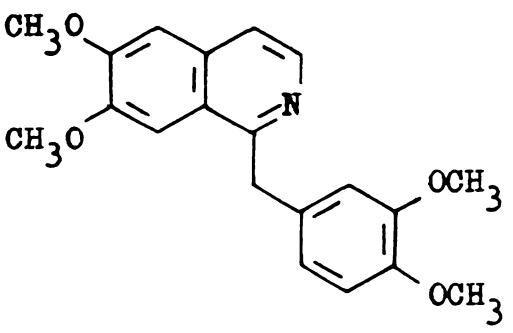
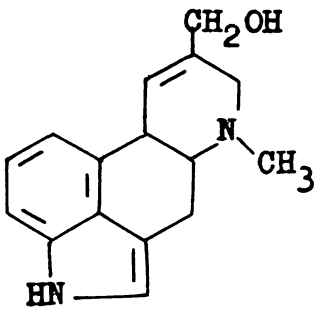


174

C and N source, producing a number of metabolites and a probable pathway of degradation was proposed[132].

2.5.4 Ergot alkaloids

Brack, Brunner and Kobel first described the microbial transformation of ergot alkaloids with the fungus Psilocybe semperviva[133]. This microorganism hydroxylated elymoclavine (175) at the 8-position to give penniclavine (176) and isopenniclavine (177). Agroclavine (178) was converted by the same fungus



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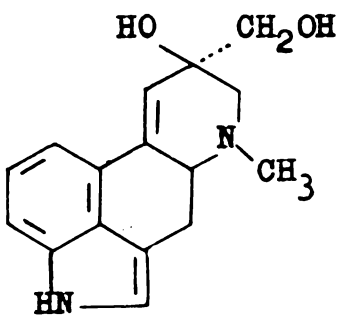

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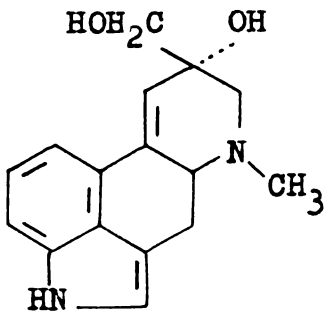
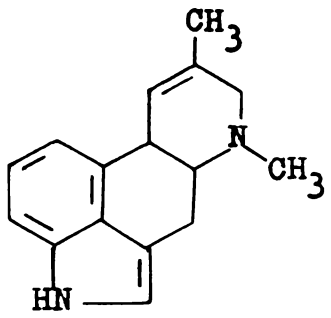


177

to setoclavine (179) and isosetoclavine (180) in 35% and 1% yields

respectively[133]. Studies by Beliveau and Ramstad showed that many fungi and streptomycetes were also capable of this enzymatic hydroxylation reaction[134]. The Takeda research group disco-



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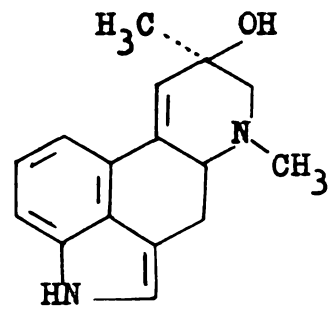

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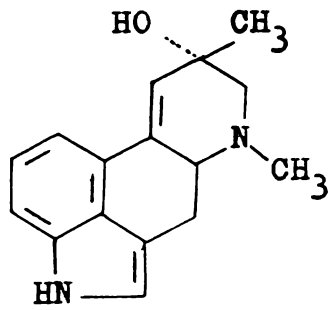
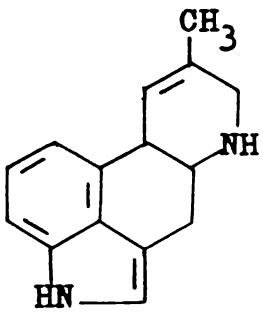


180

vered that Streptomyces roseochromogenes, S. rimosus and S. purpurescens demethylated agroclavine (178) to give noragroclavine (181)[135]. Also 178 was hydroxylated by Corticium sasakii to

give the 2-hydroxy derivative, 182[135]. Tyler et al. found that 178 was hydroxylated to elymoclavine (175) with mycelial homogenates of Claviceps paspali, which normally produces large



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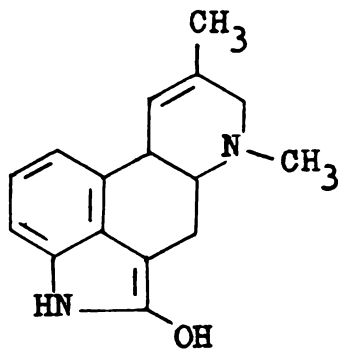

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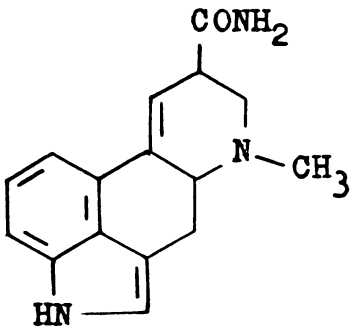
amounts of elymoclavine[136]. The same reaction was also carried out using cultures of Aspergillus and Penicillium[137]. C. paspali (Li 189) transformed elymoclavine (175) to lysergic acid amide (183)[138]. The hydrolysis of 183 and isolysergic acid amide (184) with C. purpurea gave the acids, 185 and 186 respectively[139]. The yields of these microbial hydrolysis (85 to 95%) were far better than those fromchemical means (30 to 50%)[139].

The conversion of the ergoline alkaloid, pergolide (187)

by a strain of Helminthosporium species gave the



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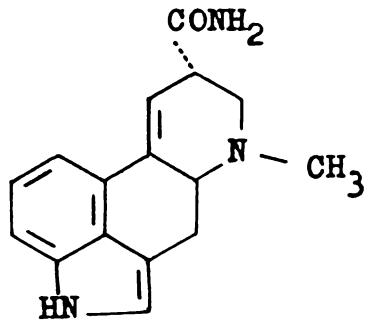

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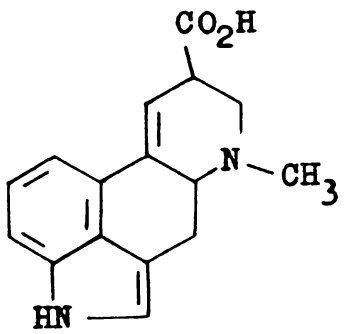

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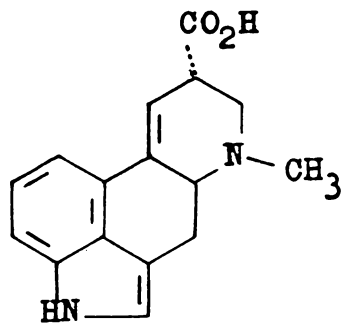

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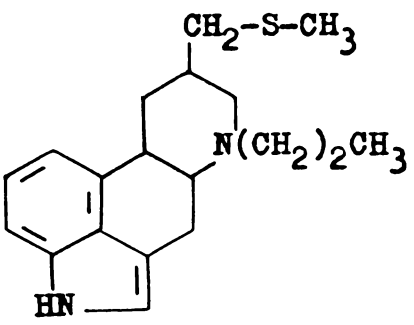
sulphoxide, 188, in significant amounts. Several organisms

including a strain of Aspergillus alliaceus formed another metabolite pergolide sulphone (189)[140].



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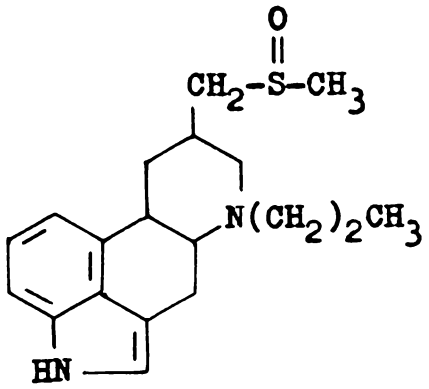

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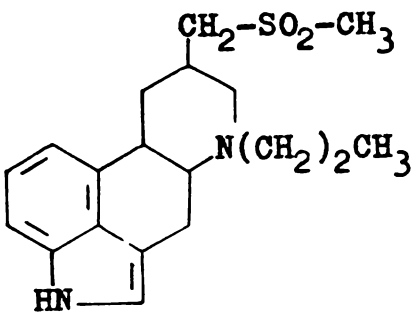
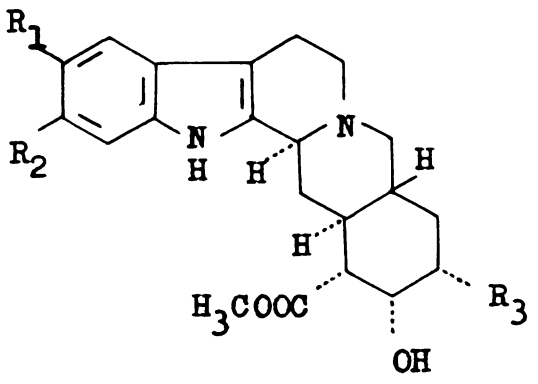


189

2.5.5 Rauwolfia alkaloids

The hydroxylation at C_{10} is more common among the yohimbine type-alkaloids. Meyers and Pan showed that yohimbine (190a) was hydroxylated at C_{10} by a wide range of microorganisms

including Actinomycetes, Phycomycetes, Ascomycetes and Fungi imperfecti[141]. This property, was strain specific and not found in any of the bacteria or yeast tested. Hartman et al. found that Cunninghamella bainieri (ATCC 924), C. echinulata (NRRL A-11498) and Streptomyces platensis (NRRL 2364) hydroxylated yohimbine (190a), α -yohimbine (191a), β -yohimbine (192a) and corynanthine (193a) in the 10 position to give 190b, 191b, 192b and 193b respectively[142]. C. bainieri (Campbell x 48), C. bertholletiae (NRRL A 11497) and C. elegans (NRRL A 11499) transformed 190a and 191a to the 11 hydroxy derivatives, 190c and 191c respectively[142]. Calonectria decora (CBS) converted 190a to give 18 α -hydroxy derivative, 190d[142]. Alakloids 190a and 191a were metabolised by Streptomyces rimosus (NRRL 2234) or S. aureofaciens (ATCC 11834) to give the 18 α -hydroxy derivatives, 190d and 191d respectively[143].



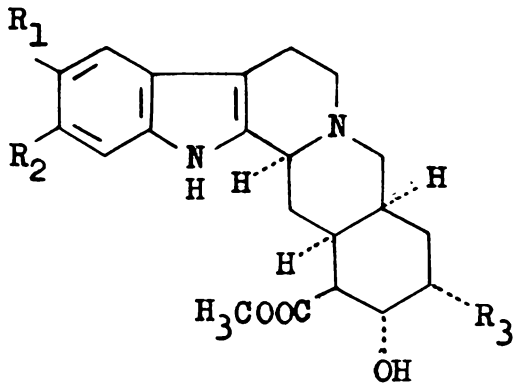
190a, $R_1=R_2=R_3=H$

190b, $R_2=R_3=H$; $R_1=OH$

190c, $R_1=R_3=H$; $R_2=OH$

190d, $R_1=R_2=H$; $R_3=OH$

Ajmalicine (194) was hydroxylated in the 10-position by

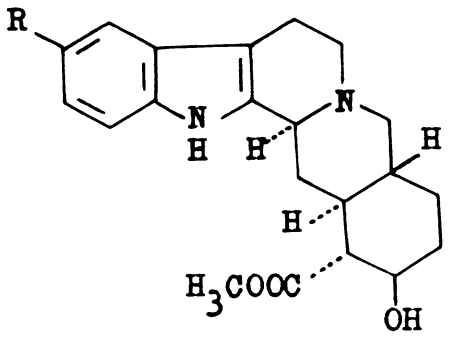


191a, $R_1=R_2=R_3=H$

191b, $R_2=R_3=H$; $R_1=OH$

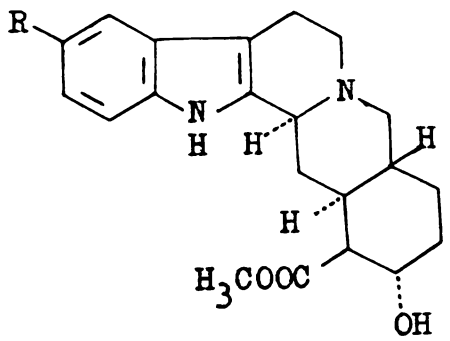
191c, $R_1=R_3=H$; $R_2=OH$

191d, $R_1=R_2=H$; $R_3=OH$



192a, R = H

192b R = OH

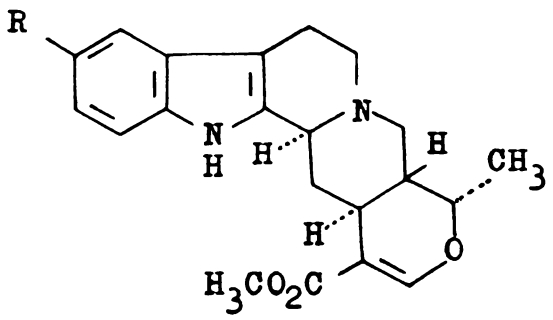


193a, R = H

193b, R = OH

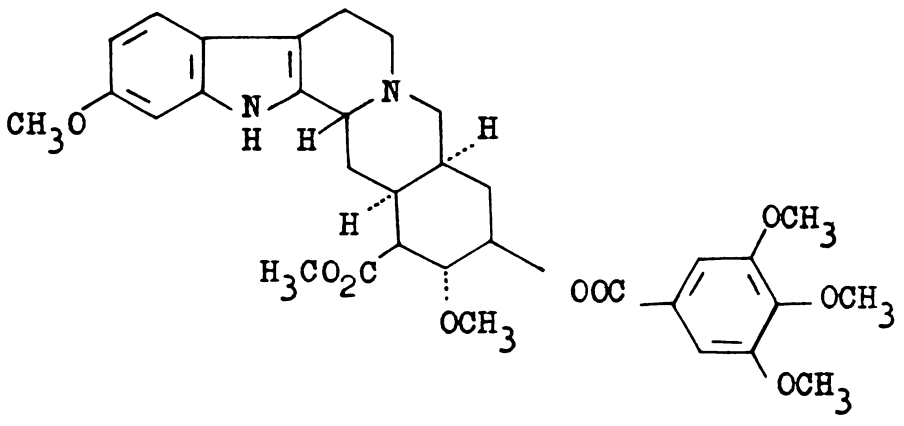
Gongronella urceolifera to give 195 in 40% yield[144].

Probably because reserpine (196) and its derivatives already have hydroxy functions at 11 and 18 positions, they cannot



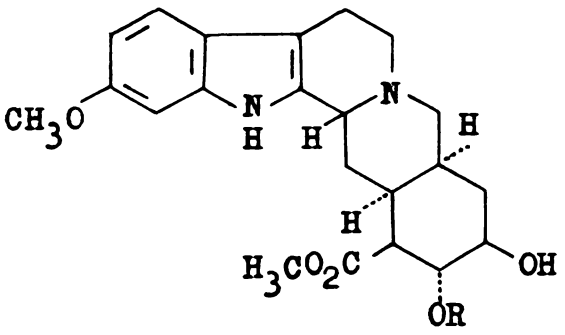
194, R = H

195, R = OH



196

undergo the same hydroxylation reactions as yohimbine[4]. Methyl reserpate (197) on incubation with Streptomyces aureofaciens (ATCC 13132) or S. rimosus (NRRL 2234) gave methyl pseudo-reserpate (198)[145].



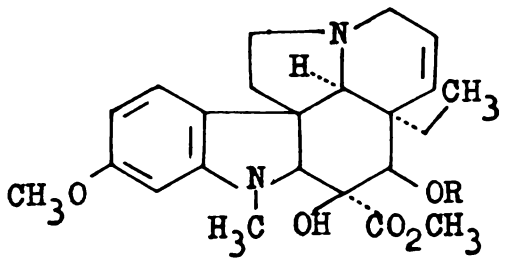
197, R = CH_3

198, R = H

2.5.6 Vinca alkaloids

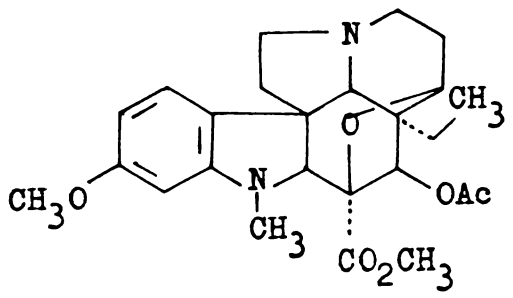
Mallett et al. initiated the studies of biotransformations of this group of alkaloids. A strain of Streptomyces cinnamonensis (A 15167) was found to transform vindoline (199) to deacetylvindoline (200) and deacetyldihydrovindoline ether[146]. Alkaloid 199 on incubation with Streptomyces griseus (UI 1158) gave good yields of dihydrovindoline ether (201) and a novel dimeric vindoline

derivative, 202. This metabolite 202 consisted of two dihydrovindoline ether moieties joined by a C-C bond, which was most



199, R = $COCH_3$

200, R = H



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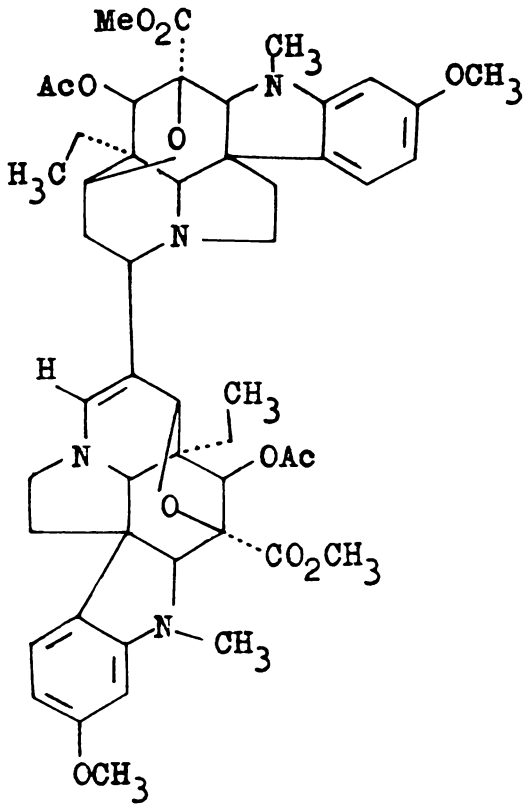
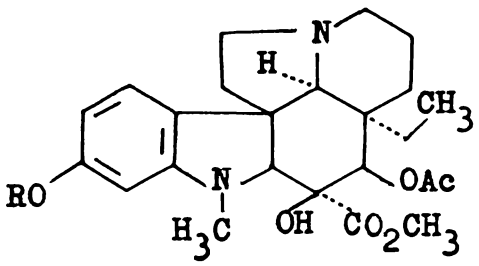


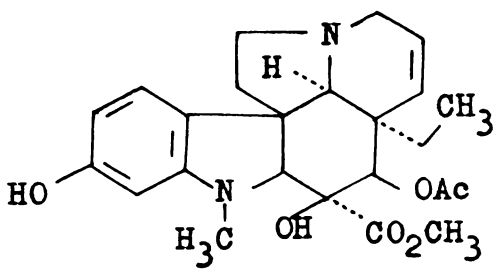
202

probably formed by an enamine condensation[147]. But, dihydrovindoline (203) when incubated with resting cells of Streptomyces griseus produced 11-O-demethyldihydrovindoline (204) in 10% yield[148]. A strain of the fungus Sepedonium chrysospermum (ATCC 13378) was found to transform vindoline (199) to O-demethylvindoline, 205, in 33% yield[149]. A detailed review on the biotransformation of vinca alkaloids has appeared elsewhere[150].



203, R = CH_3

204, R = H



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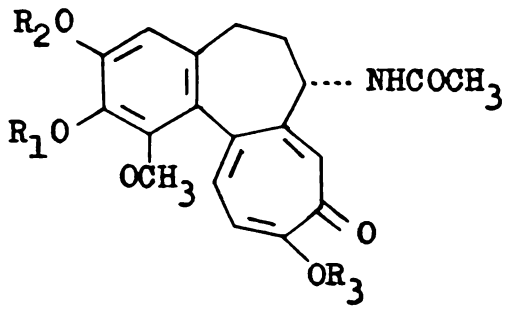
2.5.7 Colchicine

Colchicine (206) was converted to the monodemethyl derivatives by various Streptomyces species. Thus, S. griseus (ATCC 10137) demethylated 206 at the C_{10} position to give 207[151]. Alkaloid, 206 on incubation with S. spectabilis or S. griseus (a different strain) gave the 2-O-demethyl derivative, 208, and 3-O-demethyl derivative, 209[152].



206, $R_1 = R_2 = R_3 = CH_3$

207, $R_1 = R_2 = CH_3$; $R_3 = H$

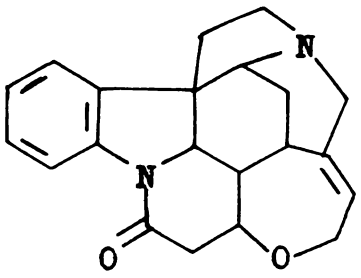
208, $R_2 = R_3 = CH_3$; $R_1 = H$

209, $R_1 = R_3 = CH_3$; $R_2 = H$

2.5.8 Strychnos alkaloids

Practically very little work has been reported on strychnos alkaloids. Bellet and Gerard reported the conversion of strychnine (210) and brucine (211) to the corresponding N-oxides, 212 and 213 respectively[153-155]. The N-oxidation was found to be a very

general reaction of various microorganisms such as bacteria (Bacillus thuringiensis, B. subtilis, Propionibacterium freudenreichii), actinomycetes (Streptomyces species U.C. 57, Nocardia asteroides, N. coralina) and fungi imperfecti (Rhizopus arrhizus, Cunninghamella blakesleeana). However, Bacillus thuringiensis (ATCC 10792) is the only organism that provided good yields (50%) of the N-oxide[153].



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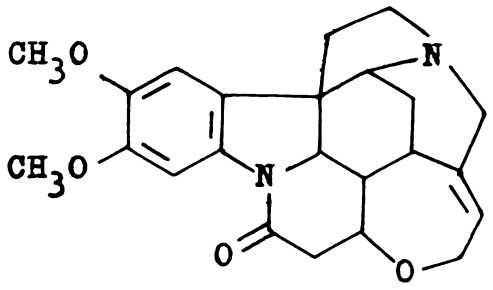

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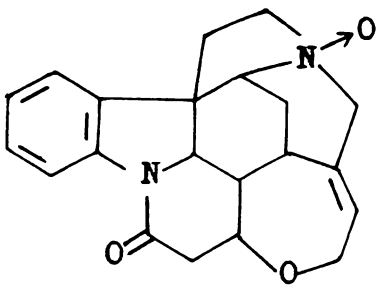

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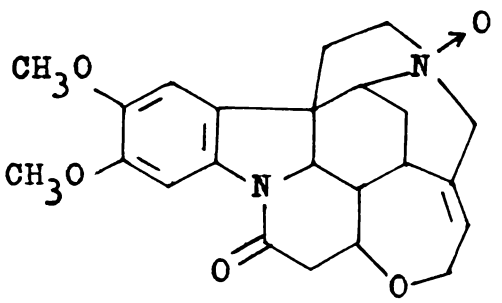
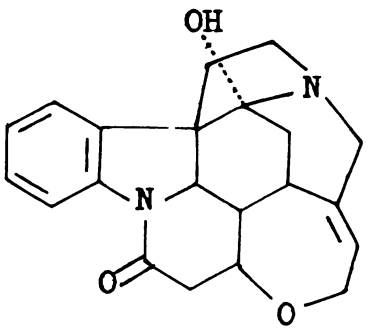


213

Helicostylum piriforme transformed strychnine (210) to the N-oxide, 212, and to the 16α-hydroxy derivative, 214[156].

Bucherer isolated different strains of Arthrobacter species capable of decomposing strychnine[157]. One of these strains, A. strychnovorum utilised strychnine only as a source of carbon since it could grow in the mineral strychnine solution only in the presence of an inorganic nitrogen source. C_{16}-Hanssen acid (215) was isolated as a degradation product in this fermentation[158].

The same culture was not able to transform the closely related alkaloids, brucine (211) or vomicine (216). A. belladonnae, which



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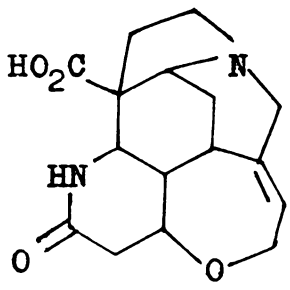

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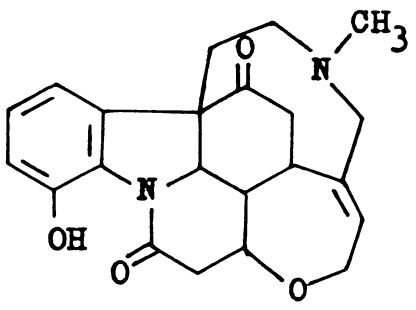
was capable of decomposing atropine, also decomposed strychnine in the presence of an inorganic nitrogen source forming a violet dye. Brucine and vomicine were not transformed by this organism[157]. A. strychnophagum utilised strychnine both as C and N source producing low molecular weight fragments which were not completely characterised. Brucine and vomicine were also degraded by this strain[157].



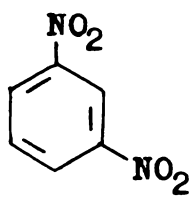
216

2.6 Microbial transformations of aromatic nitro and amino compounds

2.6.1 Reactions involving nitro groups

Neuberg, as early as 1914 reported the reduction of nitrobenzene to aniline with top yeast[159]. 1,3-Dinitrobenzene (217) gave 3-nitroaniline (218) and bis-(3-nitrophenyl)-diazene N-oxide (219) in about equal proportions[160]. The enzyme responsible for the reductions of aromatic nitro groups, nitroaryl reductase, was

later isolated from Aspergillus niger (Mulder strain)[161]. It reduced o-nitrobenzoic acid, m-nitrobenzoic acid and p-nitrobenzoic acid to the corresponding amino acids. The nitro group of p-nitrobenzenesulphonamide (220) was also reduced by this enzyme system to give 221.



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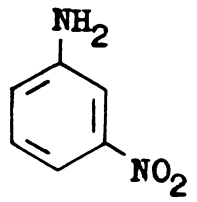

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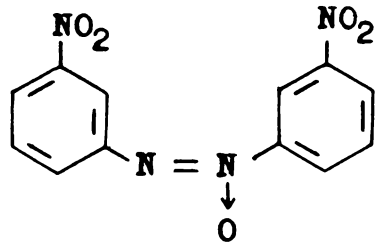

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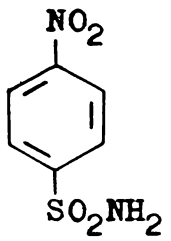

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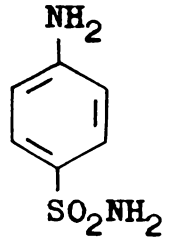

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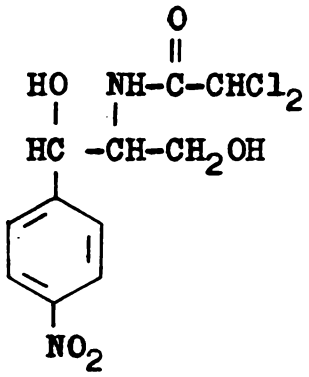
Chloramphenicol (222), a broad spectrum antibiotic, was inactivated by strains of Escherichia coli, Bacillus subtilis and Pseudomonas vulgaris with the reduction of the nitro group and the simultaneous hydrolysis of the N-acetyl group and oxidation of the secondary hydroxyl group to give the product, 223[162,163]. Various mycobacteria have been found to reduce picric acid (224)

to 6-amino-2,4-dinitrophenol (225)[164]. The herbicide 3,5-dinitro-o-cresol (DNOC, 226) was broken down by strains of Pseudomonas and Rhizobium via 3-amino-5-nitro-o-cresol (227)[165,166]. Gundersen and Jensen isolated a strain of Arthrobacter simplex, from soil, actively metabolising DNOC (226) which eliminated the nitro groups as nitrite ions, producing the metabolite, 2,3,5-trihydroxy-toluene (228)[167].



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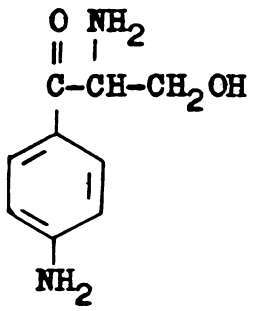

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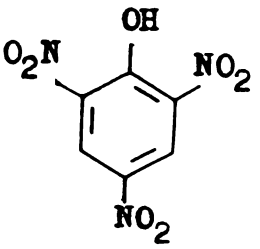

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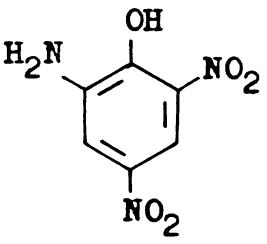

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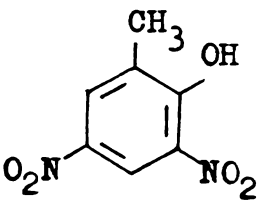

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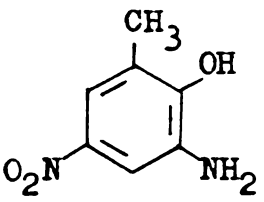

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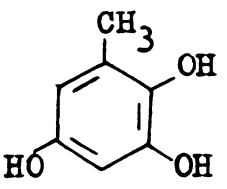
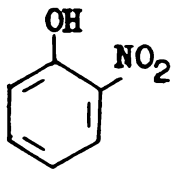


228

A strain of Pseudomonas putida, isolated from soil, was found to degrade o-nitrophenol (229), initially to catechol, by eliminating the nitro group as nitrite ions. m-Nitrophenol (230) was also degraded by the same bacteria, releasing ammonium ions into the culture medium. But, this organism failed to metabolise p-nitrophenol (231)[168]. A mixed culture of Bacillus species was reported to completely degrade p-nitrophenol (231) producing p-nitrophenetol (232) and p-phenetidine (233) as the intermediate products[169].



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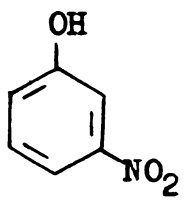

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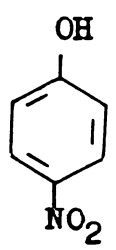

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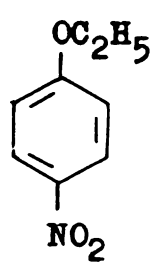

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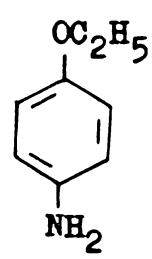
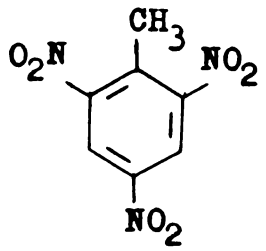


233

Some strains of Pseudomonas denitrificans and Escherichia coli preferentially reduced the 2-nitro group of TNT (2,4,6-trinitrotoluene, 234) to give 2-amino-4,6-dinitrotoluene (235)[170]. TNT was reduced by a large number of yeasts and fungi to yield mainly 235. In this transformation, a small amount of 4-amino-2,6-dinitrotoluene (236) was also formed[171].

The pesticide pentachloronitrobenzene (237) was primarily

reduced to the amine, 238, by Streptomyces aureofaciens[172].



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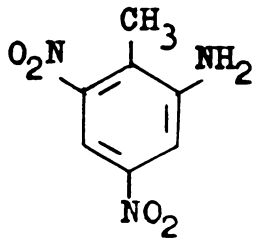

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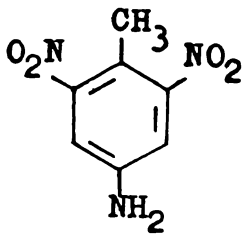

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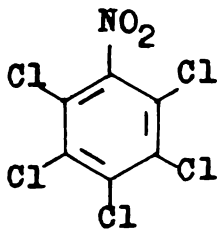

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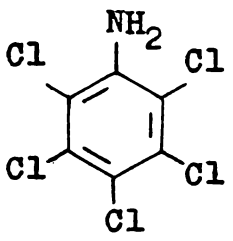
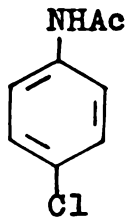


238

A basidiomycetous yeast of genus Rhodosporidium metabolised 4-chloronitrobenzene (239) by a reductive pathway to give 4-chloroacetanilide (240) and 4-chloro-2-hydroxyacetanilide (241) as the major final metabolites[173]. The metabolism of 2,4-dichloro-1-nitrobenzene (242) by a strain of the fungus Mucor javanicus gave the amino derivative, 243 and two other metabolites, 244 and

245[174]. 3,4-Dichloronitrobenzene (246) on incubation with a strain

239



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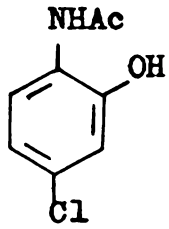

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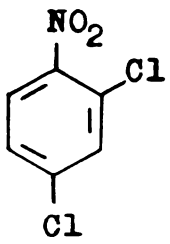

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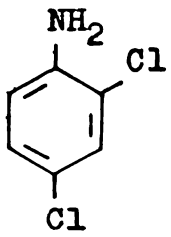

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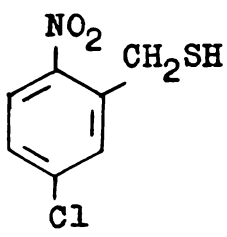

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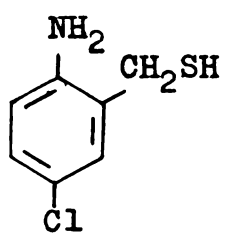
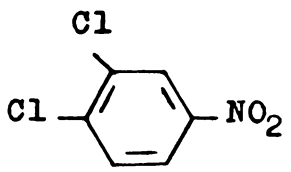


245

of Escherichia coli was converted to tetrachloroazobenzene, 247 and tetrachloroazoxybenzene, 248[175]. 1-Nitropyrene (249) was slowly reduced by Salmonella typhimurium to yield 1-aminopyrene (250) and N-acetyl-1-aminopyrene (251)[176]. 5-Nitro-2-furaldehyde

semicarbazone (252) was reduced by a strain of Aerobacter aerogenes to give the amino compound, 253[177].



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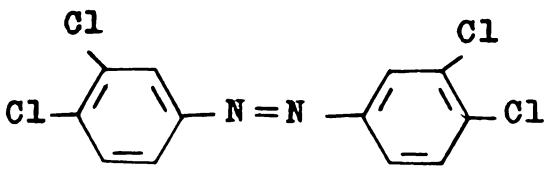

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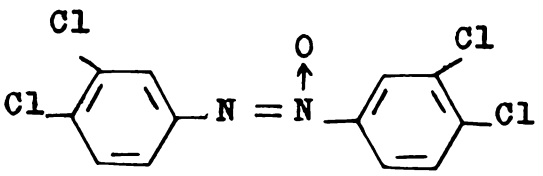

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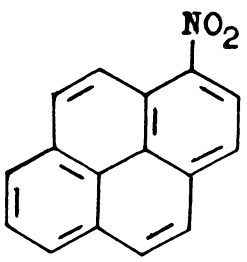

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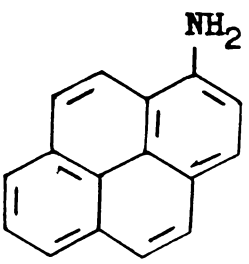

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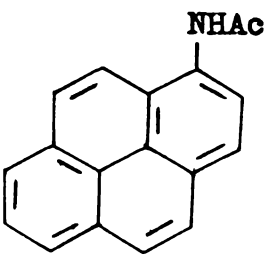

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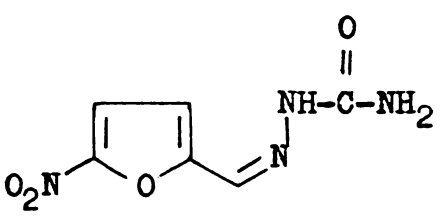

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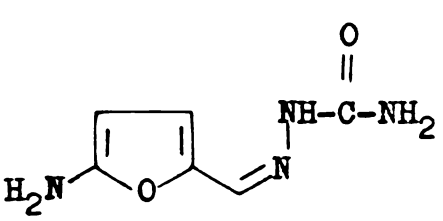
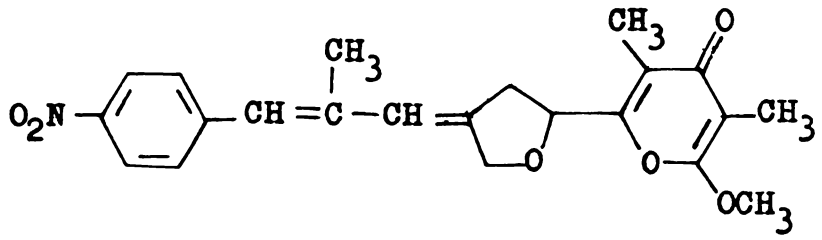


253

2.6.2 Reactions involving amino groups

Several microorganisms produce the metabolites containing the nitro derivatives by oxidising the corresponding amino compounds. For example, Streptomyces thioluteus which produces the nitro compound aureothin (254), oxidised p-aminobenzoic acid to p-nitrobenzoic acid[178,179]. A strain of Streptomyces species (LE 3342) producing the antibiotic, azomycin (2-nitroimidazole, 255), was able to oxidize several aminoimidazoles to nitroimidazoles[180,181]. 2-Aminoimidazole(256) itself was converted to 255 by this organism in about 50% yield.



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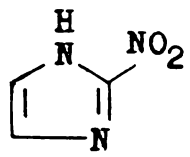

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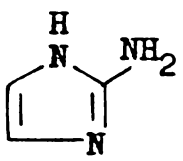

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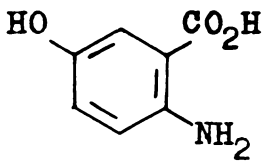
The oxidation of amino group to hydroxyl group can result in a phenol, an alcohol, an aldehyde or acid, depending on the substrate[182]. Nocardia opaca converted 5-hydroxy-anthranilic acid (257) to 2,5-dihydroxy benzoic acid (258)[183]. The transformation of 3,4-dichloroaniline (259) in partly anaerobic cultures of Escherichia coli in the presence of nitrate ions yielded 3,4-dichlorophenol (260) as one of the products[175]. Amino groups are also



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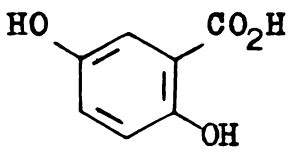

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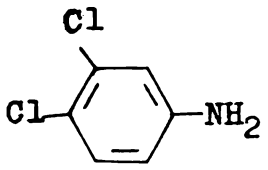

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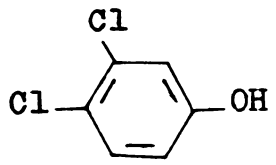


260

oxidised to N-oxides. Since chemical oxidation to N-oxides can be accomplished in excellent yields, only few microbial conversions have been studied for this purpose. Some examples are found in the alkaloid field as described earlier[153].

CHAPTER III

MICROBIAL DEGRADATION OF STRYCHNINE

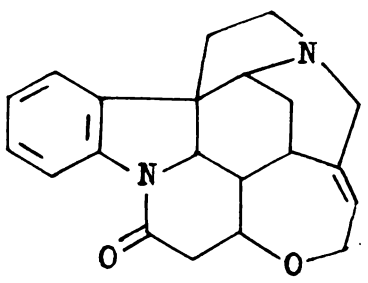
3.1 Materials and methods

3.1.1 Chemicals

Strychnine (1), brucine (2) and C_{16}-Hanssen acid (3) were used as the substrates.

3.1.1.1 Isolation of strychnine (1) from Strychnos nuxvomica seeds

Strychnine (1) was isolated from seeds of Strychnos nuxvomica by a known method[184]. The ground nuts (200 g) were thoroughly mixed with 200 ml of a suspension of 10% calcium hydroxide in water and left overnight at room temperature. After air drying, the slurry was extracted with chloroform in a soxhlet extractor for 3 hours. The chloroform solution was extracted several times with 5% sulphuric acid solution and the combined acid extract was basified with 10% aqueous sodium hydroxide. After cooling, the crystals were separated, 1.5 volumes of 50% ethyl alcohol was added and the mixture was refluxed until most of the solid had dissolved.



1

The solution was decolourised with activated charcoal and filtered hot and left overnight. The crystals of strychnine formed were filtered and washed with a little 50% ethyl alcohol to give 3.0 g of crude strychnine. The mother liquor and washings were kept for the isolation of brucine.

The crude strychnine was dissolved in 30 ml of boiling water by the addition of 15% hydrochloric acid until the mixture was slightly acidic to congo red. Activated charcoal was added and the solution was refluxed for 1 hour and filtered hot. The hydrochloride which crystallised on cooling was filtered and washed with cold water to give 3.0 g of strychnine hydrochloride, darkened at 220^{o}, but not melting upto 290^{o}C.

This hydrochloride was dissolved in 40 ml of water at 80^{o}C and neutralised with 10% aqueous sodium carbonate. After decolourisation with charcoal, the solution was filtered hot. Strychnine precipitated on addition of aqueous sodium carbonate and cooling. The precipitate was filtered, washed with cold water, and recrystallised from ethanol to yield 2.0 g of strychnine, m.p. 286 - 288^{o}C (lit[185] m.p. 286 - 288^{o}C)

3.1.1.2 Isolation of strychnine (1) from commercially available strychnine hydrochloride

Strychnine hydrochloride[186] was purified as follows:

A saturated solution of the hydrochloride in water at 80°C was prepared, filtered hot and left overnight for crystallisation. The crystallised hydrochloride was filtered and washed with cold water. It charred at 215° but not melted upto 290°C. Strychnine (free base) was prepared by dissolving this hydrochloride in water, followed by treatment with ammonium hydroxide to pH 8. The precipitated free base was filtered, washed with cold water and dried. Recrystallisation from ethanol yielded pure strychnine, m.p. 278-280°C.

$[\alpha]_D^{20}$ = -138.2°, c 0.13 ($CHCl_3$)

UV : λ_{max} 211 nm ($\epsilon = 5.7 \times 10^4$), 254 nm ($\epsilon = 2.8 \times 10^4$)

IR (KBr) : 1670 cm^{-1} (C=O)

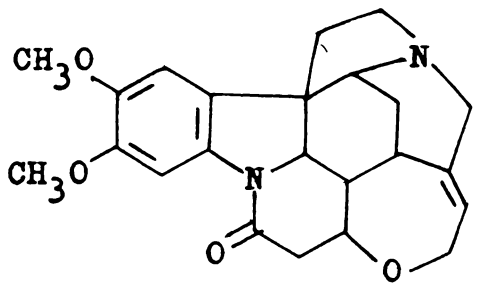
(Lit[185]) m.p. = 286 - 288°, $[\alpha]_D^{18}$ = -139.9° ($CHCl_3$);

UV : λ_{max} 254 nm ($\epsilon = 1.5 \times 10^4$), 278 nm ($\epsilon = 4.3 \times 10^3$), 288 nm ($\epsilon = 3.4 \times 10^3$).

3.1.1.3 Isolation of brucine (2) from *Strychnos nuxvomica* seeds[184]

The mother liquor remaining after the separation of strychnine (Section 3.1.1.1) was concentrated under reduced pressure on a water bath until most of the alcohol was removed. The residue was acidified to pH 6 with dilute sulphuric acid and concentrated to a volume of 3.4 ml. After standing in

a refrigerator overnight, the product was filtered and washed with cold water. Brucine sulphate was purified by dissolving in 4.5 volumes of hot distilled water and boiling with a little charcoal for 1 hour. It was filtered hot and cooled in a



2

refrigerator. The crystals formed were filtered to give 1 g of brucine sulphate. This was dissolved in 20 ml of water at 80°C and the solution was basified with 10% aqueous sodium carbonate. The separated brucine was washed with cold water and recrystallised from acetone-water to give pure brucine, m.p. 176°C (lit[185] m.p. 178°C).

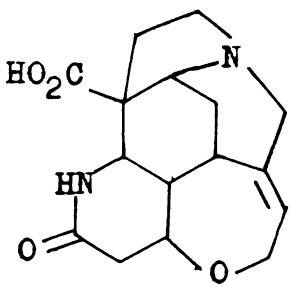
3.1.1.4 Isolation of brucine (2) from commercially available brucine sulphate[186]

Brucine sulphate was recrystallised by dissolving in

water at 80°C followed by filtration of the solution to remove impurities. The solution, left overnight in refrigerator, yielded pure crystals of brucine sulphate. This material was redissolved in water and treated with ammonium hydroxide to pH 8. The precipitated brucine was filtered, washed with cold water and recrystallised from acetone-water to yield pure brucine of melting point 177°C. $[\alpha]_D^{20}$ = -126.3°, c 0.13 ($CHCl_3$); UV : λ_{max} 303 nm (ϵ = 9.4 x 10^3)
(lit[185]) m.p. 178°C, $[\alpha]_D^{20}$ = -127° ($CHCl_3$) UV : λ_{max} 263 nm (ϵ = 1.2 x 10^4), 301 nm (ϵ = 8.5 x 10^3)

3.1.1.5 C_{16}-Hanssen acid (3)[187]

Brucine (10.0 g, 0.0254 mol) was added in portions to 50 ml concentrated sulphuric acid and the solution was diluted to 500 ml with water. A solution of 30 g of chromic anhydride in 50 ml water was added to the cold solution of



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brucine in sulphuric acid. The temperature was maintained at 60°C for 3 hours, at 70°C for three more hours, and then the solution was heated over a boiling water bath for 12 hours. To this green solution was added a saturated solution of barium hydroxide until no more precipitate was formed. The precipitate was filtered and washed thrice with boiling water and the filtrate concentrated to 300 cc under reduced pressure. The solution was just neutralised with dilute sulphuric acid and the precipitate was filtered off. The filtrate was concentrated to 15 ml and cooled to obtain a powdery colourless precipitate which was filtered, washed with water to give 0.25 g (2.5%) of crude acid, $C_{17}H_{22}N_2O_6$. The compound was recrystallised from water. It became brown at 278°C and did not melt above 310°C. (lit[187] not melting below 345°C)

The mother liquor on further concentration and addition of alcohol gave 0.35 g (3.5%) of crude C_{16}-Hanssen acid ($C_{16}H_{20}N_2O_4$). The product was recrystallised from water-acetone mixture. The product became brown at 254°C and melted with decomposition at 304°C, $[\alpha]_D^{24} = -114.5^\circ$ (water),
IR (KBr) : See figure 3.1. 1670 cm^{-1} (C = O),
3500 - 3180 cm^{-1} (broad, OH)
(lit[187]) m.p. 311°C (decomp) $[\alpha]_D^{24} = -116.3^\circ$ (water)

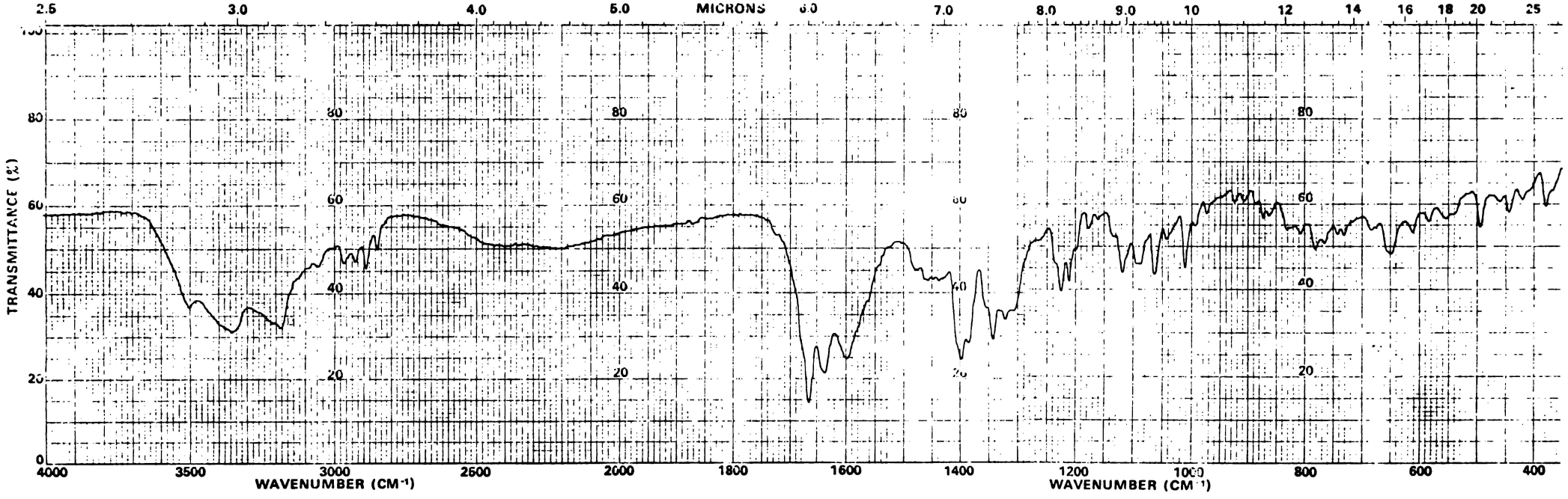


Fig. 3.1 IR spectrum of C_{16}-Hanssen acid - Synthetic sample

3.1.2 Source of microorganisms

Microorganisms used in the present investigations were obtained from two different sources.

i. National Collection of Industrial Microorganisms, (NCIM), National Chemical Laboratory, Poona, India.

ii. Soil samples collected from the natural habitat of Strychnos nuxvomica L.

3.1.2.1 NCIM cultures

The cultures obtained from the National Collection of Industrial Microorganisms (NCIM), of National Chemical Laboratory, Poona are listed below with their reference numbers.

a) Bacteria

i. Arthrobacter simplex 2449

ii. Arthrobacter albidus 2447

iii. Bacillus thuringiensis 2130, 2159

iv. Micrococcus glutamicus 2168

v. Micrococcus luteus 2169

vi. Micrococcus flavus 2376

vii. Pseudomonas aeruginosa 2074

viii. Pseudomonas desmolyticum 2112

ix. Pseudomonas putida 2152

b) Streptomyces

i. Streptomyces rimosus 2213

ii. Streptomyces griseus 2622

c) Fungi

i. Aspergillus niger 589, 595

ii. Cunninghamella blakesleeana 687, 688

iii. Cunninghamella echinulata 691, 693

iv. Cunninghamella elegans 689, 690

v. Curvularia lunata 716

vi. Rhizopus arrhizus 997

vii. Rhizopus nigricans 880

3.1.2.2 Maintenance and preservation of NCIM cultures

All the cultures were maintained on appropriate agar slants. The slants were incubated at room temperature (28^{o}-30^{o}C) for 24 to 48 hours. They were stored in a refrigerator and transferred every month to keep them viable. The maintenance media for the cultures are described below. They were sterilised by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes.

a) Bacteria

The bacterial cultures were maintained in the nutrient

agar medium of the following composition [188].

Beef extract	:	3 g
Peptone	:	5 g
Sodium chloride	:	8 g
Agar	:	20 g
Distilled water	:	1000 ml
pH	:	7.0 - 7.2

b) Streptomyces

The streptomyces cultures were maintained in the following medium[188].

Dextrose	:	10 g
Sodium chloride	:	5 g
Peptone	:	5 g
Yeast extract	:	5 g
$CoCl_2.6H_2O$	:	0.002 g
Agar	:	20 g
Distilled water	:	1000 ml
pH	:	7.2

c) Fungi

The fungal cultures were maintained in the potato dextrose agar medium of the following composition[188].

Potatoes, Infusion from	:	200 g
Dextrose	:	20 g
Agar	:	20 g
Distilled water	:	1000 ml

The peeled, sliced potatoes (200 g) was boiled in 500 ml water for 30 minutes. The extract was decanted and made upto 1000 ml, after the addition of agar (20 g) and dextrose (20 g). Heated to dissolve and then autoclaved.

3.1.2.3 Screening of NCIM cultures

The NCIM cultures were screened for their ability to transform strychnine using the following different media, containing 0.05 g of strychnine hydrochloride, sterilised by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes.

a) Bacteria

The screening of the bacteria was carried out in a medium with the following composition[189].

Glucose	:	5 g
Tryptone	:	2 g
Peptone	:	3 g
Yeast extract	:	5 g
Water	:	1000 ml
pH	:	7.0

b) Streptomyces

The streptomyces cultures were screened in the following medium[190].

Glucose	:	10 g
Yeast extract	:	5 g
Beef extract	:	5 g
Water	:	1000 ml
pH	:	7.0

c) Fungi

The fungal cultures were screened in the following medium[190].

Glucose	:	30 g
$NaNO_3$	:	3 g
KH_2PO_4	:	1 g
KCl	:	0.5 g
$MgSO_4 7H_2O$	:	0.5 g
$FeSO_4 7H_2O$	:	0.01 g
Water	:	1000 ml
pH	:	6.0

The three different media described above, were prepared and distributed in 50 ml aliquots into 250 ml Erlenmeyer flasks. The media were autoclaved and they were inoculated with the cultures and incubated at room temperature (28^{o} - $30^{o}C$) on a rotary shaker (220 rpm) for 4 - 8 days. The fermented broths were analysed regularly by extracting with chloroform-methanol (8:2) after bringing the pH to 9.0

by the addition of ammonium hydroxide. The extracts were dried with anhydrous sodium sulphate, the solvent evaporated and concentrated under reduced pressure. The concentrated extracts were analysed by TLC (Thin Layer Chromatography) to detect the metabolites as described in Section 3.1.5.3.

3.1.2.4 Screening of soil samples

The screening of the soil samples was done in the following mineral salts medium (MSM), which is a modification of the medium reported by Neimer and Bucherer[158].

KH_2PO_4	:	0.1 g
$(NH_4)_2SO_4$	:	0.1 g
$NaNO_3$	:	0.1 g
$MgSO_4 7H_2O$	:	0.01 g
Strychnine hydrochloride	:	0.1 g
Tap water	:	100 ml
pH	:	7.0

Addition of 2% agar resulted in a solid medium.

About 100 g of the soil sample was suspended in 900 ml of sterile tap water and thoroughly shaken. The supernatant solution served as the inoculum. About 10 ml of this inoculum was mixed with 90 ml of the MSM broth.

The inoculated media were incubated at 28°-30°C for 8-10 days. The turbidity in the broth was continuously observed. After 48 hours of incubation, 1 ml of the broth culture was mixed with 20 ml of the melted MSM agar medium and poured into petri dishes at regular intervals of 24 hours. The inoculated plates were incubated at 28°-30°C. They were also subjected to daily observation for colony growth. The colonies developed on the agar plates were isolated and purified to auxenic strain by repeatedly streaking over nutrient agar plates and pure strains which showed growth over strychnine agar medium (MSM agar) were propagated and preserved in nutrient agar under oil[191].

The efficiency of the strains to metabolise strychnine was confirmed by growing them in the MSM broth and analysing the broth during the course of the incubation period (8 days). The broth was exhaustively extracted with a mixture of chloroform:methanol (8:2), after bringing the pH to 9.0. The pooled extracts were dried and concentrated under reduced pressure and subjected to TLC studies (Section 3.1.5.3). The strain which registered comparatively good growth, designated as ACM 1, was only selected for further characterisation studies.

3.1.3 Identification of the isolated microorganisms

The isolated strains were identified to the generic

level based on their morphological and biochemical characters[192].

3.1.3.1 Morphological characters

These tests included the cell morphology, gram staining, spore staining, motility, chromogenesis and pleomorphism of cells during growth. Hucker's modification of gram staining was adopted[193]. Chromogenesis was tested by growing the cultures on nutrient agar. Motility was tested by hanging drop method.

3.1.3.2 Biochemical characters

These included tests for production of indole, utilisation of citrate, reduction of nitrate, hydrogen sulphide production, gas from glucose, oxidation-fermentation test, cytochrome oxidase activity, catalase activity and sensitivity to penicillin G sodium[194]. The strains were tested for their ability to elaborate the hydrolytic enzymes namely gelatinase, caseinase, amylase and lipases[194].

3.1.4 Growth studies of the isolated organism

The effect of physicochemical factors on the growth of bacteria was studied employing the selected strain ACM 1 as detailed below.

3.1.4.1 Medium

Prepared the MSM basal broth with the following composition.

KH_2PO_4	:	0.1 g
$(NH_4)_2SO_4$	:	0.1 g
$NaNO_3$	:	0.1 g
$MgSO_4 7H_2O$	:	0.01 g
Tap water	:	100 ml

The pH was adjusted to 7.0 after the addition of appropriate concentration of strychnine hydrochloride.

3.1.4.2 Preparation of inoculum

A loopful of the growth from the maintenance culture in nutrient agar slants was transferred to 100 ml nutrient broth and incubated at room temperature, 28^{o}-30^{o}C, for 16-18 hours and the turbidity of the culture was observed at 600 nm and the absorbance was adjusted, with MSM basal broth to give a final absorbance of 1.0 at 600 nm (A_{600}). This cell suspension was used as inoculum (2ml/100ml).

3.1.4.3 Incubation procedure

To the dispensed medium of 10 ml quantity in each test tube, 0.2 ml of the inoculum was added. Inoculated tubes were incubated at 28^{o}-30^{o}C for a period of 48 hours

on a rotary shaker (220 rpm) unless otherwise specified. Controls were maintained for all the experiments. All the experiments were done in duplicate.

3.1.4.4 Measurement of growth

The turbidity formed in the inoculated tubes due to growth of bacteria was measured at 600 nm in a UV-visible spectrophotometer (Hitachi Model 200-20) at the end of 48 hours. Growth was expressed as optical density values (OD).

3.1.4.5 Effect of concentration of strychnine on growth

The effect of concentration of strychnine on growth of bacteria was tested by inoculating the test strain of bacteria in MSM basal broth adjusted to various concentrations of strychnine hydrochloride viz 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 mg per 100 ml (0.01 to 0.07%). The dispensed medium was inoculated, incubated and the growth was measured as outlined in Section 3.1.4.4.

3.1.4.6 Effect of pH on growth

The effect of pH on the growth of bacteria was tested in MSM basal broth prepared after incorporating the substrate at its optimal concentration and adjusting it to various levels of pH viz 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0,

10.5 & 11.0. The dispensed medium was inoculated, incubated for 48 hours and the growth was measured as outlined in Section 3.1.4.4.

3.1.4.7 Effect of concentration of sodium chloride on growth

The effect of sodium chloride concentrations on the growth of bacteria was tested in MSM basal broth containing the optimal concentration of strychnine hydrochloride, adjusted to various concentrations of sodium chloride (1.0, 3.0, 5.0, 7.5, 10.0 and 15.0%). The dispensed medium was inoculated, incubated for 48 hours and the growth was measured as outlined in section 3.1.4.4.

3.1.4.8 Effect of temperature on growth

The effect of temperature on the growth of bacteria was tested in the MSM basal broth adjusted to optimal conditions by incubating the inoculated tubes at various temperatures viz $4^{o}C$, $15^{o}C$, $30^{o}C$, $37^{o}C$, $45^{o}C$ and $56^{o}C$. The dispensed medium was inoculated, incubated at the above temperatures and the growth was estimated after 48 hours as mentioned in Section 3.1.4.4.

3.1.5 Effect of physicochemical factors on the degradation of strychnine

The effect of physicochemical factors namely pH,

sodium chloride and glucose on the rate of degradation of strychnine by the selected strain of bacteria was studied.

3.1.5.1 Medium

MSM broth was prepared with the composition mentioned in Section 3.1.4.1 with the addition of 0.04% strychnine hydrochloride. The prepared medium was dispensed in 50 ml aliquots in 250 ml Erlenmeyer flasks and autoclaved at 15 lbs pressure for 15 minutes.

3.1.5.2 Inoculation and incubation procedures

Preparation of inoculum and inoculation procedures were the same as mentioned under Section 3.1.4.2. The inoculated flasks were incubated at $30^{o}C$ for specified periods of 12, 24, 36, 48 and 60 hours on a rotary shaker (220 rpm). All the experiments were done in duplicate.

3.1.5.3 Analytical procedures

a) Extraction procedure

The fermented broth in each flask was analysed at every 12 hours interval. The broth was made alkaline (pH 9) by the addition of ammonium hydroxide solution and then exhaustively extracted by a mixture of chloroform-methanol(8:2). The pooled extracts were dried using anhydrous

sodium sulphate and concentrated under reduced pressure. The residue was redissolved in 50 ml of methyl alcohol and subjected to TLC studies and quantitative studies.

b) Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on 0.25 mm thick layers of silica gel G prepared on glass plates with a spreader. The following solvent systems were used.

i) Chloroform : Methanol - (9:1)

ii) Chloroform : Acetone : Diethylamine - (5:4:1)

iii) Butanol : Conc. Hydrochloric acid : Water - (10:2:1)

The spots were detected either by exposing the plates to iodine vapours or by spraying them with Dragendorf's reagent[195].

c) Estimation of strychnine

Standard solutions of pure strychnine in methanol were prepared and the absorbance at 254 nm, was measured in a UV-vis spectrophotometer (Hitachi Model 200-20). A standard curve was drawn from which concentration of strychnine was estimated.

3.1.5.4 Effect of concentration of sodium chloride on degradation of strychnine

The effect of sodium chloride on the degradation

of strychnine was tested in MSM broth having various sodium chloride concentrations viz 0.0, 0.5 and 1.0%. The dispensed medium, in 50 ml aliquots, in 250 ml Erlenmeyer flasks were sterilised, inoculated and incubated for the five desired time intervals for each concentration of sodium chloride. After the required incubation period, the contents of the flasks were analysed as mentioned under Sec 3.1.5.3 for residual strychnine.

3.1.5.5 Effect of concentration of glucose on degradation of strychnine

The effect of glucose on the degradation of strychnine was tested in MSM broth by incorporating glucose at concentrations of 0.1, 0.5 and 1.0%. The dispensed medium was autoclaved, inoculated and incubated for the five desired periods, for each concentration of glucose. After the required incubation period, the contents of the flasks were analysed as mentioned under Section 3.1.5.3 for residual strychnine.

3.1.5.6 Effect of pH on the degradation of strychnine

The effect of pH on the degradation of strychnine was tested in MSM broth, adjusted to pH values of 6.5, 7.0 and 8.0. The pH values above 8 were not studied because the substrate started precipitating above the neutral pH. The dispensed medium was autoclaved, inoculated and incubated

for the five desired time intervals for each of the pH value. After the specified incubation period, the broth was analysed for the residual strychnine content as mentioned under Section 3.1.5.3.

3.1.6 Isolation of C_{16}-Hanssen acid (3)

The mineral salts medium (MSM) described under Section 3.1.5.1 was prepared. Each batch of fermentation broth having a volume of 3 litres, distributed in 10 one litre Erlenmeyer flasks each containing 300 ml aliquots was autoclaved. The flasks were inoculated and incubated on a rotary shaker (220 rpm) at room temperature (28^{o}-30^{o}C) for 14 to 18 hours. At the end of the incubation period the pH of the broth was raised to 9.0 by the addition of ammonium hydroxide solution. The pooled fermented broth was evaporated under reduced pressure removing the last traces of water by azeotropic evaporation with benzene. The dried residue was subjected to exhaustive extraction with chloroform to remove the unconverted alkaloid and then with absolute methanol. Both the extracts were evaporated to dryness under reduced pressure. The chloroform extract when subjected to TLC examination showed a spot at the same Rf value as strychnine. The residue (200 mg) from the methanolic extracts were further dissolved in methanol and chromatographed over a column of silica gel using methanol as eluant.

The fractions yielding the metabolite were pooled and concentrated under reduced pressure. Recrystallisation from water-acetone mixture gave 120 mg (10%) of the product. The product became brown at 250°C and melted with decomposition at 302°C. $[\alpha]_D^{24}$ = -114.5° (water), IR (KBr) : See figure 3.2, 1670 cm^{-1} (C = O), 3500 - 3180 cm^{-1} (broad, OH)
(lit[187]) m.p. 311°C (decomp), $[\alpha]_D^{24}$ = -116.3° (water)

3.1.6.1 Methiodide of C_{16}-Hanssen acid

A suspension of 50 mg (0.00016 mol) of C_{16}-Hanssen acid in a mixture of 5 ml of methanol and 1 ml of methyl iodide at room temperature was left for 2 to 3 hours for completion of reaction. The solvent was evaporated and the product recrystallised from ethanol to give 40 mg (80%) of methiodide, m.p. 234 °C (lit[158] m.p. 237 °C).

3.1.6.2 Perchlorate of C_{16}-Hanssen acid

A solution of 50 mg (0.00016 mol) of C_{16}-Hanssen acid in a little water was mixed with a 50% ethanolic solution of perchloric acid. The mixture was warmed on a water bath for 1 hour and concentrated under reduced pressure. On cooling, crystals were formed. Recrystallisation from ethanol-ether gave the perchlorate melting at 215°C (lit[158] m.p. 220 °C).

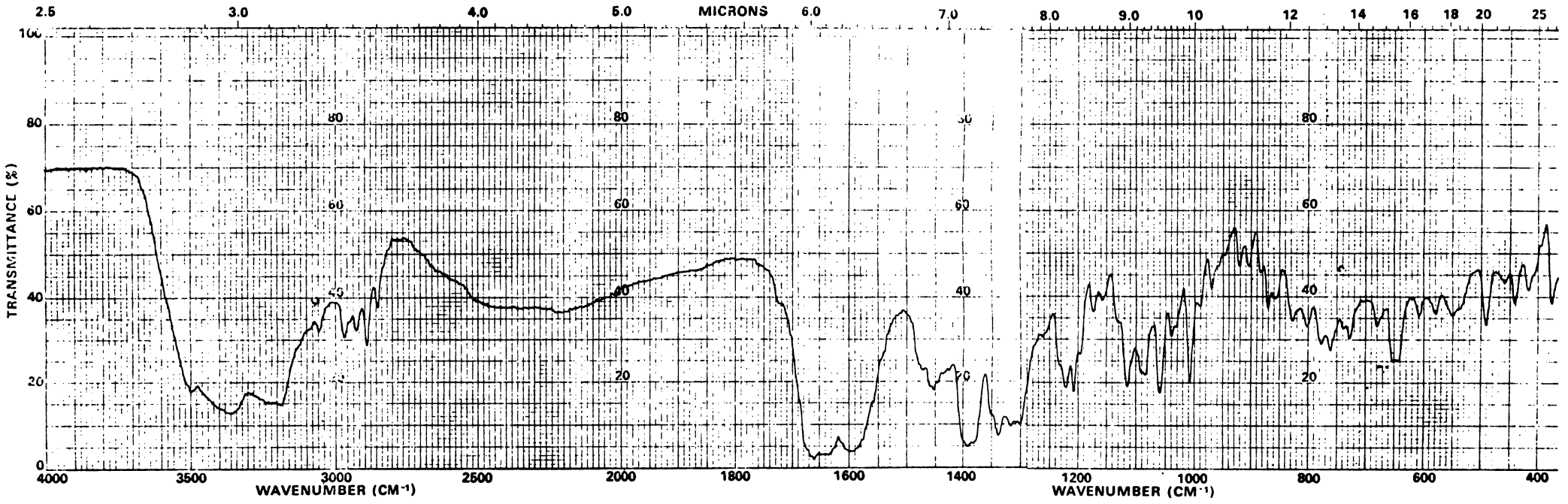


Fig. 3.2 IR spectrum of C_{16}-Hanssen acid - Microbial degradation product

3.1.7 Utilisation of strychnine both as a C and N source

The efficiency of the organism to utilise strychnine both as C and N source was tested in the following broth devoid of inorganic nitrogen sources.

KH_2PO_4	:	0.1 g
$MgSO_4 7H_2O$	:	0.01 g
Strychnine hydrochloride	:	0.04 g
Distilled water	:	100 ml
pH	:	7.0

This broth was inoculated with the test strain and incubated at room temperature, (28^{o} - 30^{o}C) on a rotary shaker (220 rpm) for 10 days. The growth of the organism was visually observed and the broth was extracted with a mixture of chloroform:methanol (8:2) in alkaline conditions (pH 9.0) and the concentrated extract was examined by TLC to detect the completeness of degradation.

3.1.8 Growth on brucine (2)

The growth of the test strain on brucine was studied in the MSM basal broth (Section 3.1.4.1) supplemented with brucine (0.02%). The medium was inoculated with the test strain and incubated at room temperature (28^{o}-30^{o}C) on a rotary

shaker (220 rpm) for a period of 10 days. During the period of incubation the broth was daily subjected to visual observation. The contents in the flasks were exhaustively extracted with a mixture of chloroform:methanol (8:2) after bringing the pH to 9.0 and the concentrated extracts were analysed by TLC.

3.1.9 Selective utilisation of strychnine in a mixture of strychnine and brucine

To the MSM basal broth, described under Section 3.1.4.1, strychnine hydrochloride (0.04%) and brucine (0.02%) were added. The pH of the medium was adjusted to 7.0 and was distributed in 200 ml aliquots each and autoclaved. Then the medium was inoculated with the test strain and incubated at room temperature (28^{o}-30^{o}C) on a rotary shaker (220 rpm) for 10 days. During the period of incubation the growth of the organism was visually observed and the broth was extracted with chloroform:methanol (8:2) in alkaline conditions. The concentrated extracts were examined by TLC with authentic samples of strychnine and brucine.

3.1.10 Growth on C_{16}-Hanssen acid (3)

The growth of the test strain on C_{16}-Hanssen acid was studied by growing it in the MSM basal medium

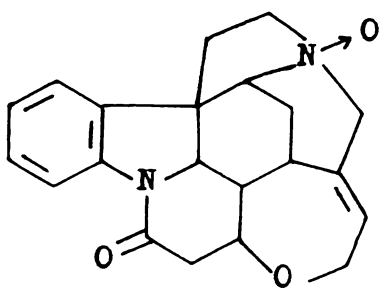
described under Section 3.1.4.1, supplemented with C_{16}-Hanssen acid (0.03%). The inoculated broth was incubated at room temperature (28^{o}-30^{o}C) on a rotary shaker (220 rpm) for 10 days. The growth was visually observed to find out the increase in turbidity. The broth was evaporated to dryness, and the residue dissolved in methanol was subjected to TLC studies to detect the metabolites formed.

3.2 Results

3.2.1 Introduction

Strychnine (1) and brucine (2), the alkaloids present in the seeds, barks and leaves of Strychnos nuxvomica, were extracted from the seeds and purified to get 1.5% of strychnine and about 0.4% of brucine according to the procedure reported by Ikan[184]. As this was a tedious method and the yield was poor, strychnine was purchased as its hydrochloride and brucine as its sulphate for the present studies[186]. The microorganisms obtained from NCIM, National Chemical Laboratory, Poona and isolated from soil were tested for their ability to convert or degrade strychnine. The NCIM cultures, Bacillus thuringiensis 2159, Cunninghamella blakesleeana 687 and C. echinulata 691, transformed strychnine in very small amounts to its N-oxide, 4, as detected by TLC studies. The other cultures, however, failed to transform strychnine into any other material.

Since N-oxidation was a known reaction with strychnine[153], this type of transformation was not further studied. Hence, the soil samples were screened to obtain strychnine degrading organisms. The enrichment culture technique yielded a few microorganisms metabolising strychnine and the three strains isolated were identified as mentioned in Section 3.1.3. Interestingly all the three isolates were of the same species as indicated by their morphological, biochemical and physiological characters (Table 3.1). The strains were confirmed to belong to



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Arthrobacter species as they also showed pleomorphism during their growth (Fig 3.3a - d) which is a characteristic feature of this species[192]. The 10 hours old culture (Fig 3.3a) showed the elongation of the coccoid cells to give rods of varying size and shape. The 15 hours (Fig 3.3b) and 24 hours old cultures (Fig 3.3c) showed the gradual shortening of the rods as the

incubation proceeded and the 72 hours old culture (Fig 3.3d) showed the characteristic coccoid cells.

3.2.2 Growth studies

The strains were grown in the MSM broth and the turbidity resulting from the growth was visually observed. The strain ACM 1, which showed comparatively good turbidity was selected to find out the optimal requirements of physico-chemical factors influencing the growth. The results are given below.

3.2.2.1 Effect of concentration of strychnine on growth

The effect of different concentrations of strychnine on the growth of the bacteria was studied in detail in MSM broth as outlined earlier. Results presented in Table 3.2 indicate that lower and higher concentrations of the substrate failed to elicit appreciable response in growth (Fig 3.4). The maximal growth was observed at a concentration of 0.04% of strychnine. Concentrations above 0.055% resulted in poor growth. However, a gradual increase in growth proportional to increase in the concentration of strychnine was observed from 0.01 to 0.04% and the growth declined on further increase in the substrate concentration.

3.2.2.2 Effect of pH on growth

The effect of pH on the growth of bacteria was studied by growing the microorganism in MSM broth adjusted to various levels of pH. The results presented in Table 3.3 indicate that pH 7.0 was the optimum pH for maximum growth (Fig 3.5). However, a relatively wider range of pH (6.5 to 9.5) favoured good growth. But, the substrate got precipitated above the neutral pH 7.0.

3.2.2.3 Effect of concentration of sodium chloride on growth

The effect of various concentrations of sodium chloride on the growth of the bacteria was studied in MSM broth. The results presented in Table 3.4 suggest that the optimum sodium chloride requirement of this strain was 1.0% since it could record maximum growth only at this concentration of sodium chloride when compared to other sodium chloride concentrations (Fig 3.6). However, the bacteria recorded fairly good growth at 0% and above 1% upto 7.5% indicating an euryhaline nature. The growth started decreasing slowly as the concentration of sodium chloride was increased from 1.0% upto 7.5% and further increase of NaCl concentration to 10% caused a sudden and sharp decrease in growth.

3.2.2.4 Effect of temperature on growth

The effect of temperature on the growth of the

bacterial strain was studied by growing the microorganism in MSM broth at different temperatures and the results are presented in Table 3.5. Of the various temperatures tested, incubation at 30°C and 37°C alone favoured good growth of the organism (Fig 3.7). Both low and high temperatures had a detrimental effect on the growth and cell division. Results suggest that 30°C was the optimum growth temperature for this organism.

3.2.3 Effect of physicochemical factors on degradation of strychnine

The effect of sodium chloride, glucose and pH on the rate of degradation of strychnine was studied in MSM broth at 30°C. Results obtained for the various experiments are presented below.

3.2.3.1 Effect of concentration of sodium chloride on degradation of strychnine

The effect of sodium chloride concentrations on the rate of degradation of strychnine was studied in MSM broth. The test was performed with sodium chloride concentration of 0, 0.5 and 1% only. Results are presented in Table 3.6. Irrespective of the concentration of sodium chloride in the medium, the strain showed activity on strychnine (Fig 3.8). At all the three concentrations of sodium chloride, a rapid

decline in the concentration of strychnine was recorded throughout the incubation period. The amount of strychnine remaining at the end of 12 hours was 36.5, 41.0 and 42.1% for 0, 0.5 and 1.0% sodium chloride concentrations respectively. More than 98% of strychnine was degraded by the organism at the end of 36 hours in all the three sets.

3.2.3.2 Effect of concentration of glucose on degradation of strychnine

The effect of glucose concentrations on the rate of degradation of strychnine was tested by incorporating glucose at different concentrations (0.1, 0.5 and 1.0%) in the MSM broth. Results presented in Table 3.7 suggest that increase in the glucose concentration affected the strychnine degradation considerably at the early hours (Fig 3.9). Major quantity of strychnine was degraded by 12 hours itself at 0.1% glucose compared to the other concentrations of glucose. Further, almost all strychnine was degraded at 36 hours at 0.1% glucose concentration, whereas, the strain took 48 hours and 60 hours to achieve the same, at 0.5% and 1.0% glucose concentrations respectively.

3.2.3.3. Effect of pH on the degradation of strychnine

The effect of pH on the degradation of strychnine

was tested in MSM broth adjusted to different pH values (6.5, 7.0 and 8.0). Results presented in Table 3.8 point out that at pH 7.0 the organism degraded strychnine very fast (Fig 3.10). At the end of 12 hours the residual content of strychnine at pH 7.0 was 19% whereas the value recorded for both pH 6.5 and 8.0 was 40%. Within 24 hours of incubation at neutral pH, the organism degraded about 98.5% of strychnine. But when the initial pH of the medium was 6.5 and 8.0, the strain degraded only 95% and 76% of strychnine respectively, within 24 hours. However, the amount of strychnine remaining at the close of 48 hours was more or less same (less than 1.5%) in all the three cases. The initial lag recorded in the utilisation of strychnine at both the pH values of 6.5 and 8.0 was overcome as the fermentation proceeded.

3.2.4 Utilisation of strychnine both as C and N source

The organism registered good growth in the media devoid of inorganic N sources and the TLC examination of the chloroform-methanol (8:2) extract showed that strychnine was completely degraded by the organism. But no metabolites were detected in this extract.

3.2.5 Growth on brucine (2)

The organism could not show any growth in this medium and the TLC of the recovered material showed only a spot corresponding to brucine. About 16 mg (80%) of brucine was recovered.

3.2.6 Selective utilisation of strychnine in a mixture of strychnine and brucine

There was progressive increase in turbidity during the course of incubation. The TLC examination of the chloroform-methanol (8:2) extract showed that strychnine was totally absent in the extract and only a spot at the same Rf value of brucine was detected in the TLC. About 15 mg (75%) of brucine was reisolated.

3.2.7 Growth on C_{16}-Hanssen acid (3)

The inoculated broth registered an increase in turbidity indicative of growth and the TLC (Butanol : Conc.HCl : H_2O, 10:2:1) of the methanolic extract of the broth showed the formation of a metabolite moving just below C_{16}-Hanssen acid which was not accumulated in amounts enough to be characterised.

3.3 Discussion

The organism was identified to belong to

Arthrobacter species, based on the morphological and biochemical characters. The strains exhibited pleomorphism during cell growth, clearly evident from the photomicrographs of the culture taken at 10, 15, 24 and 72 hours. The pleomorphism exhibited during cell growth is characteristic of Arthrobacter species[192]. The growth studies of the selected strain, ACM 1, had revealed many interesting facts. The strain was able to use strychnine both as a C and N source, which was conclusively established by growing the strain in a medium devoid of inorganic nitrogen sources. However, the strain failed to convert brucine into any metabolite. But brucine had no inhibitory effect on either the growth of the organism or on the ability of the strain to degrade selectively strychnine from a mixture of strychnine and brucine. This finding has an important practical application for the organism can be used to remove traces of strychnine present in commercially available brucine.

The present strain was found to be quite different from the three strains, Arthrobacter strychnovorum, A. belladonnae and A. strychnophagum reported earlier[157]. The strain A. strychnovorum degraded strychnine to C_{16}-Hanssen acid, only in the presence of an inorganic nitrogen source and did not metabolise brucine[157,158]. But A. belladonnae was reported to degrade strychnine, only in the presence of an inorganic

nitrogen source and brucine was not metabolised[157]. However, the other strain A. strychnophagum utilised strychnine both as a C and N source yielding only low molecular weight fragments which were not characterised. Further A. strychnophagum metabolised brucine also yielding low molecular weight compounds. But the strain, ACM 1, isolated in the present studies is different from A. strychnovorum and A. belladonnae as it utilised strychnine both as a C and N source. It is also different from A. strychnophagum as it produced C_{16}-Hanssen acid as a product in 10% yield and also because it did not metabolise brucine. The identity of the C_{16}-Hanssen acid was established by direct comparison with an authentic sample prepared by the chemical oxidation of brucine[187]. The two samples had identical infrared spectra (See Figs 3.1 and 3.2) and melting points. Further the derivatives, methiodide and perchlorates also had identical physical constants as were reported[158]. Thus it was established that the bacterium ACM 1 converts strychnine into C_{16}-Hanssen acid in 10% yield. The strain ACM 1 also metabolised C_{16}-Hanssen acid producing further breakdown products which was not accumulated in amounts enough to be characterised. Thus, the strain, ACM 1, isolated here is clearly a new strain different from the strains of Arthrobacter species reported earlier[157].

The strain, ACM 1, showed good growth at neutral pH and at room temperature typical of the mesophilic nature of the organism. The poor growth at higher concentrations of strychnine might be probably due to the toxic nature of the substrate at higher concentrations. The organism grew well upto a salt concentration of 7.5% indicating its euryhaline nature. The growth studies thus revealed the optimal parameters for the growth of the bacteria.

Neutral pH favoured a speedier degradation of strychnine than acidic or alkaline pH. Sodium chloride did not influence the rate of degradation of strychnine. Hence, sodium chloride was not incorporated in the fermentation medium for product isolation. The inclusion of an easily available carbon source like glucose made strychnine less preferable for the organism. The organism seemed to attack the alkaloid only after the easily available carbon source was exhausted. The results obtained from these studies gave the optimum conditions effecting a quick transformation of strychnine. These parameters were taken into consideration, in selecting the conditions for the fermentation of strychnine to obtain C_{16}-Hanssen acid.

The formation of C_{16}-Hanssen acid might have resulted from the hydroxylation of the aromatic ring of strychnine and the subsequent ring opening and further breakdown of the molecule as known in other systems[196].

Table 3.1 Identification tests

Tests	Results
Gram reaction	Positive
Spore formation	Negative
Form and arrangement	Cocci to coccoids Arranged in bunches
Motility	Non motile
Chromogenesis in nutrient agar	Rose pink colour
Production of indole	Negative
Cytochrome oxidase	Negative
Catalase	Positive
H_2S production	Negative
Ox-ferm test	Oxidative
Citrate utilisation	Negative
Nitrate reduction	Positive
Gelatinase	Negative
Caseinase	Negative
Amylase	Positive
Lipases	
Tween 20	Positive
Tween 40	Negative
Tween 60	Negative
Tween 80	Negative
Sensitivity to Penicillin (2.5 I.U. of penicillin-G sodium)	Insensitive

Table 3.2 Effect of concentration of strychnine on growth

Strychnine hydrochloride (%)	0.01	0.015	0.02	0.025	0.03	0.035	0.04	0.045	0.05	0.055	0.06	0.065	0.07
Optical density (600 nm)	0.099	0.107	0.113	0.165	0.196	0.210	0.272	0.247	0.235	0.212	0.074	0.045	0.035

Table 3.3 Effect of pH on growth

pH	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0
Optical density (600 nm)	0.018	0.033	0.051	0.159	0.249	0.236	0.224	0.220	0.204	0.185	0.131	0.110	0.105

Table 3.4 Effect of concentration of sodium chloride on growth

NaCl (%)	0	1.0	3.0	5.0	7.5	10.0	15.0
Optical density (600 nm)	0.249	0.264	0.258	0.250	0.240	0.105	0.055

Table 3.5 Effect of incubation temperature on growth

Temp. ^{o}C	4	15	30	37	45	56
Optical density (600 nm)	0.003	0.015	0.311	0.275	0.082	0.005

Table 3.6 Effect of concentration of sodium chloride on degradation of strychnine

NaCl (%)	Residual Strychnine (%)					
	0hr	12hr	24 hr	36 hr	48hr	60hr
0.0	100	36.5	21.0	1.39	0.99	0.98
0.5	100	41.0	24.41	1.56	1.22	1.11
1.0	100	42.16	22.19	1.61	1.44	0.99

Table 3.7 Effect of concentration of glucose on degradation of strychnine

Glucose (%)	Residual Strychnine (%)					
	0hr	12 hr	24hr	36hr	48 hr	60 hr
0.1	100	27.18	16.1	1.39	1.28	1.28
0.5	100	81.0	45.48	21.08	1.33	0.94
1.0	100	92.08	57.69	31.07	2.39	1.33

Table 3.8 Effect of pH on degradation of strychnine

pH	Residual Strychnine (%)					
	0 hr	12 hr	24 hr	36 hr	48 hr	60 hr
6.5	100	39.94	4.77	2.22	1.44	1.05
7.0	100	19.4	1.55	1.39	1.33	0.99
8.0	100	39.94	24.41	9.89	1.11	0.99

Fig 3.3 a - Photomicrograph of Arthrobacter sp. - 10 hours old

Fig 3.3 b - Photomicrograph of Arthrobacter sp.- 15 hours old

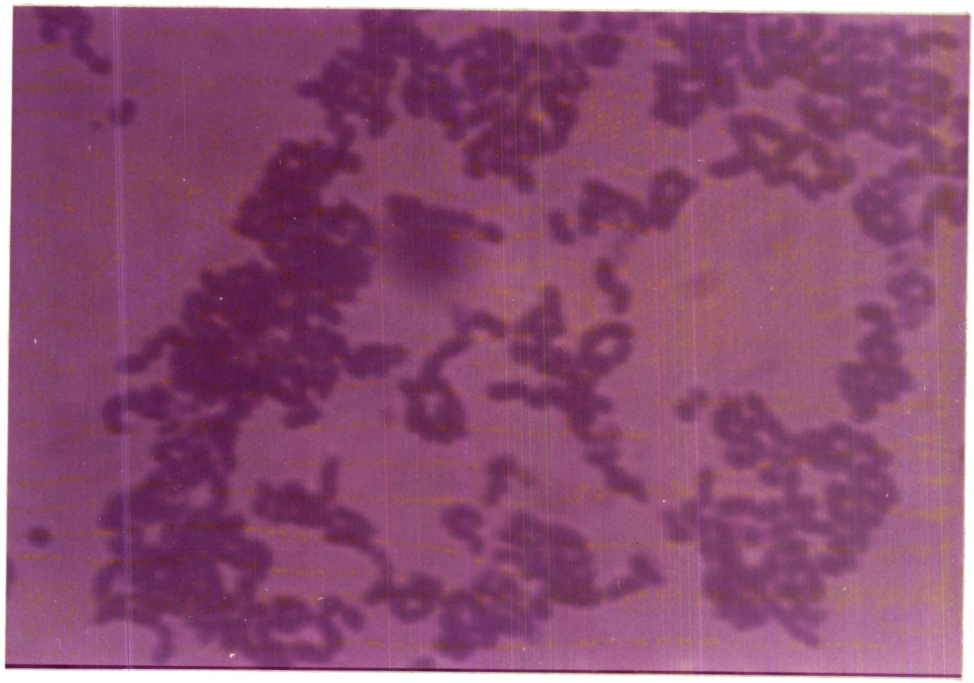


Fig. 3.3 c - Photomicrograph of Arthrobacter sp.- 24 hours old

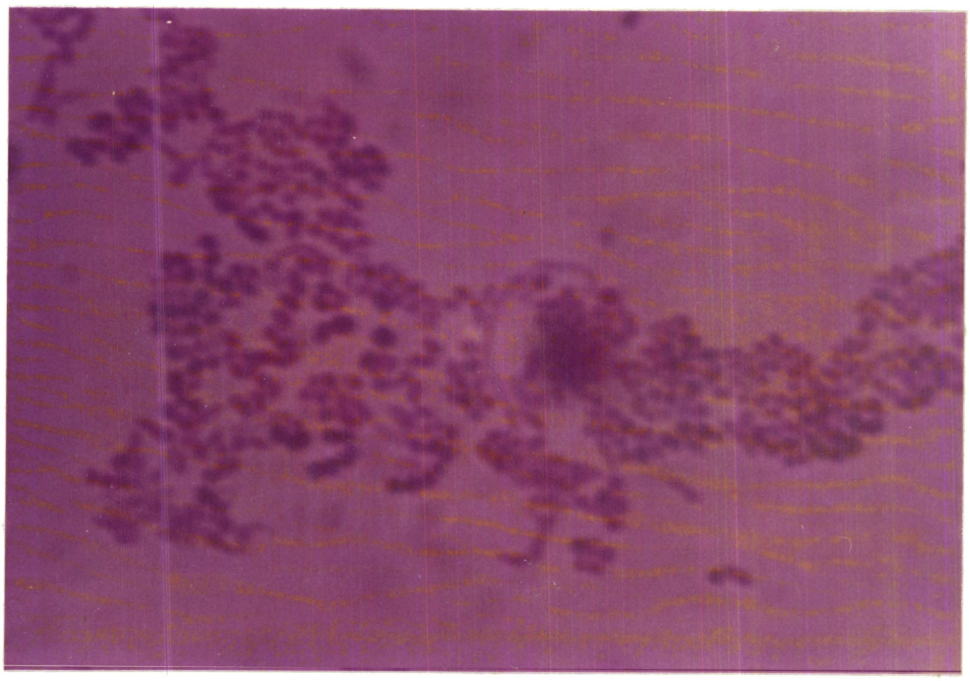


Fig. 3.3 d - Photomicrograph of Arthrobacter sp.- 72 hours old

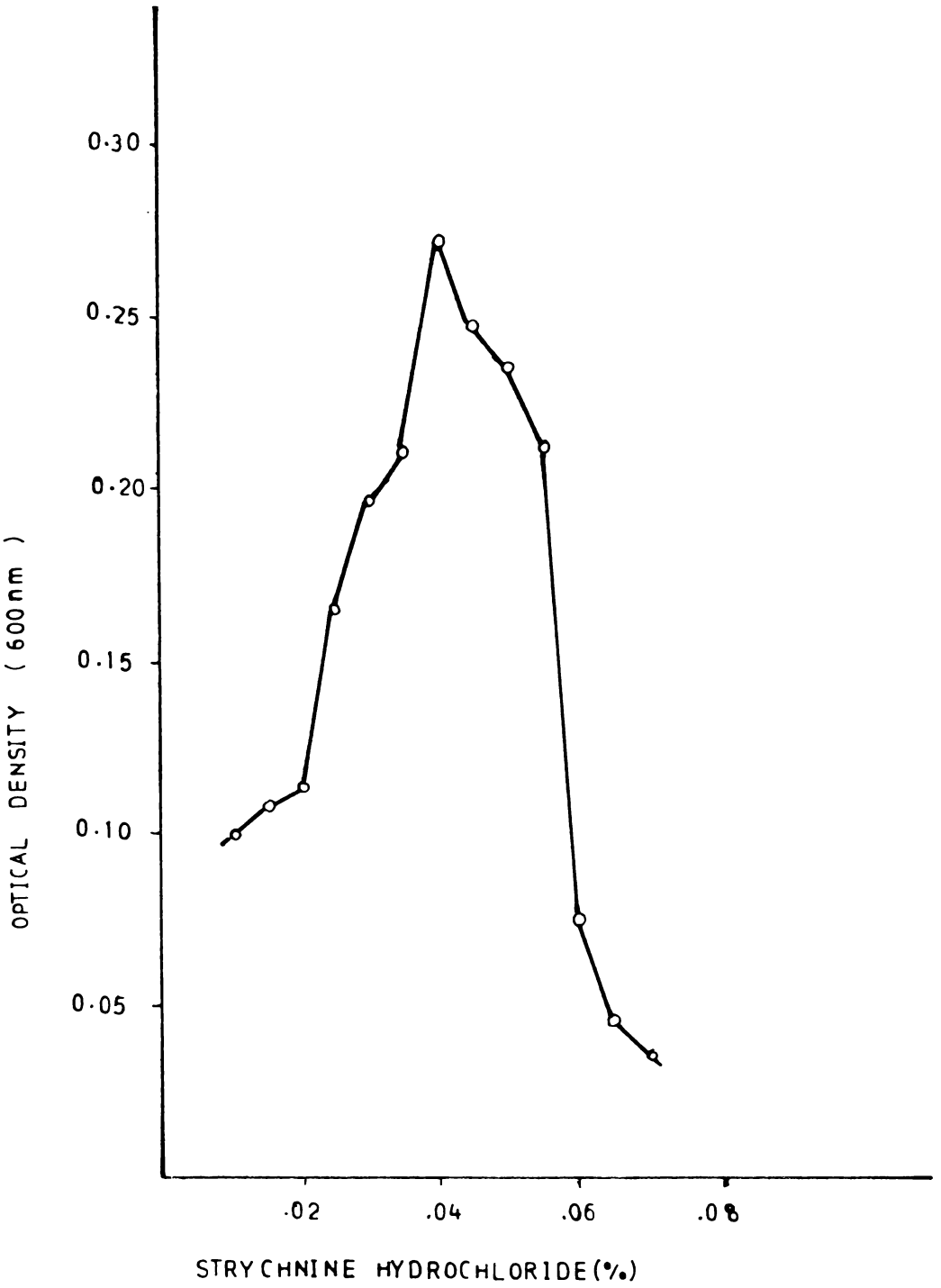


Fig 3.4 Effect of concentration of strychnine on growth

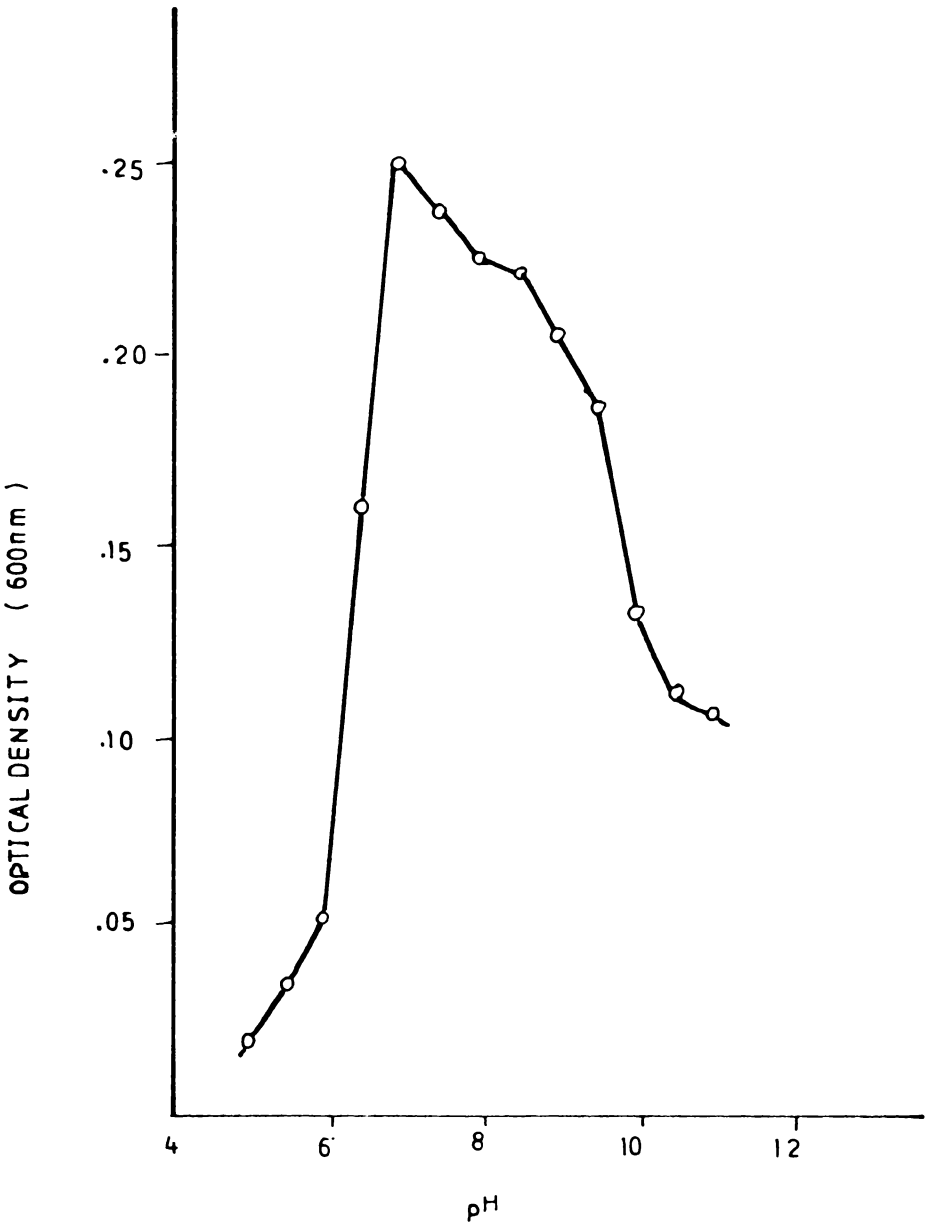


Fig. 3.5 Effect of pH on growth

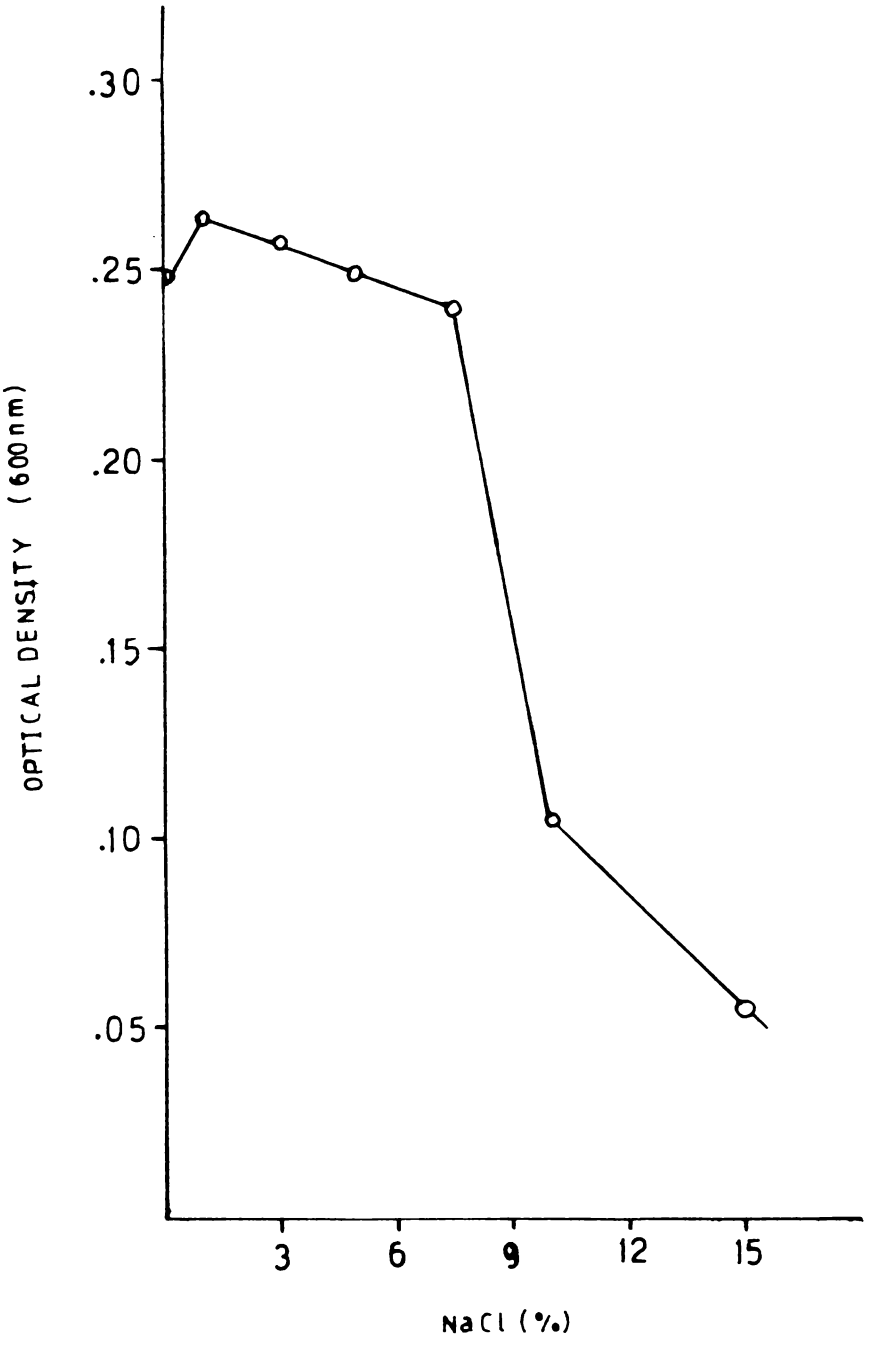


Fig. 3.6 Effect of concentration of sodium chloride on growth

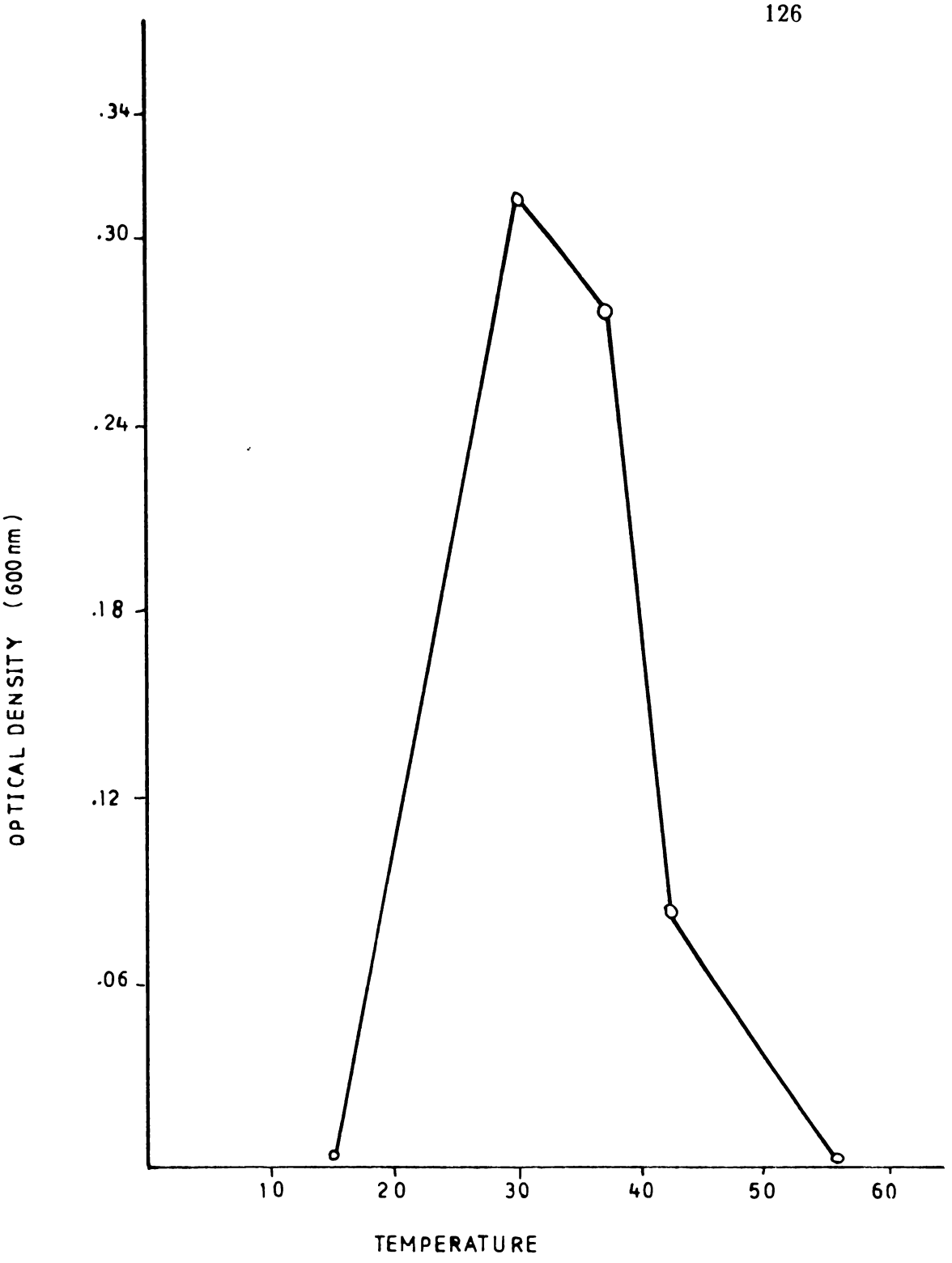


Fig. 3.7 Effect of incubation temperature on growth

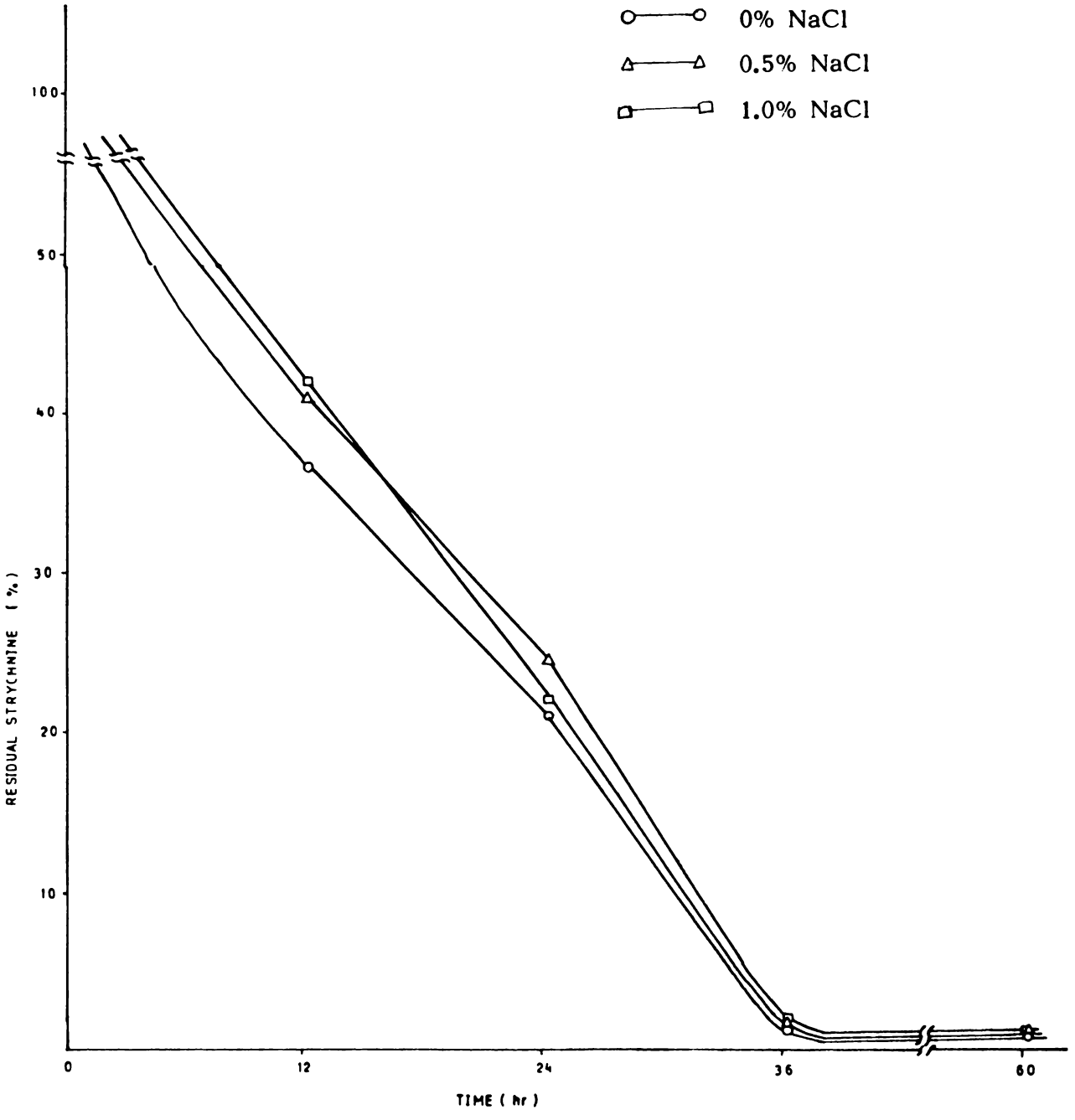


Fig. 3.8 Effect of concentration of sodium chloride on degradation of strychnine

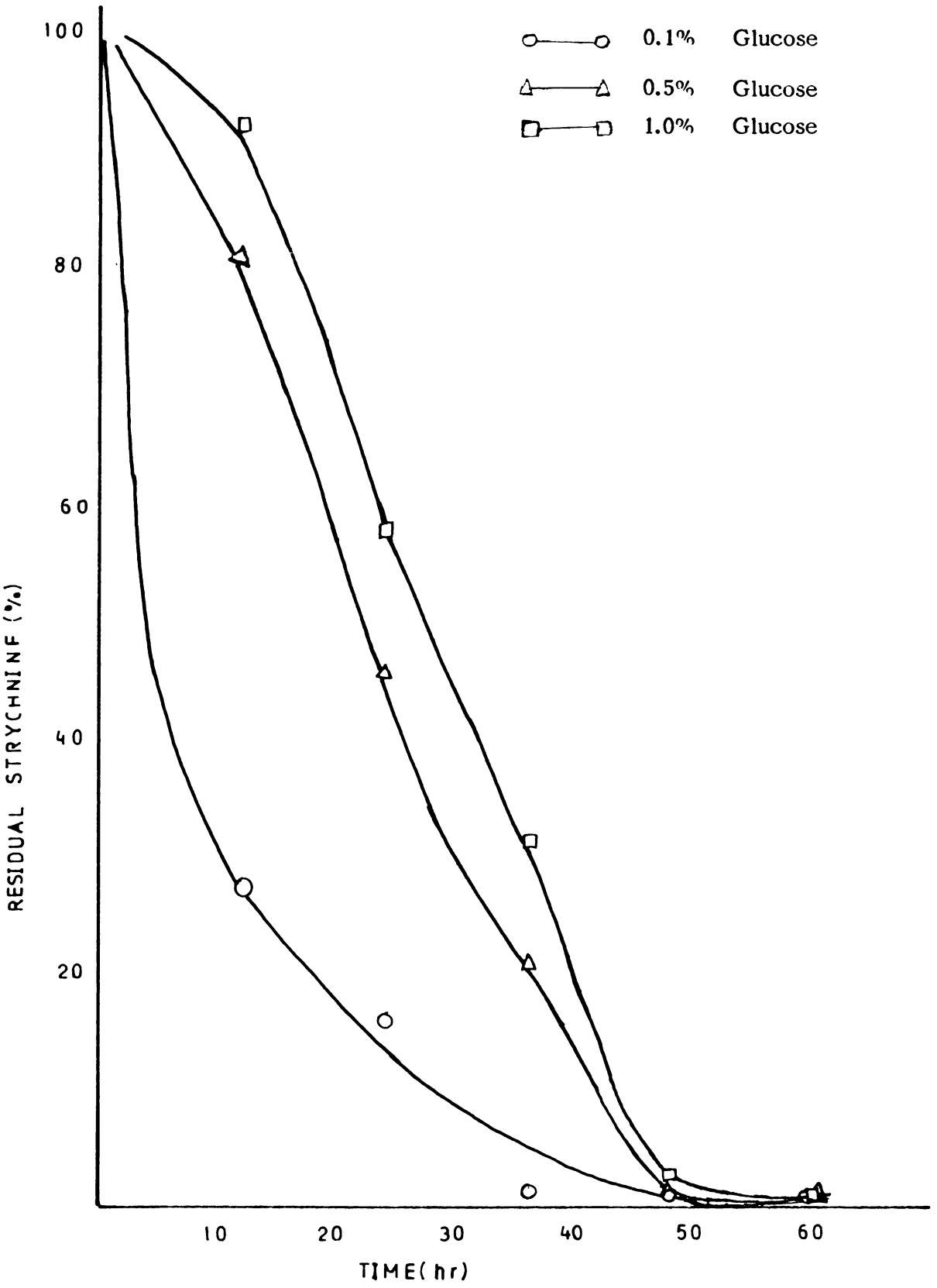


Fig. 3.9 Effect of concentration of glucose on degradation of strychnine

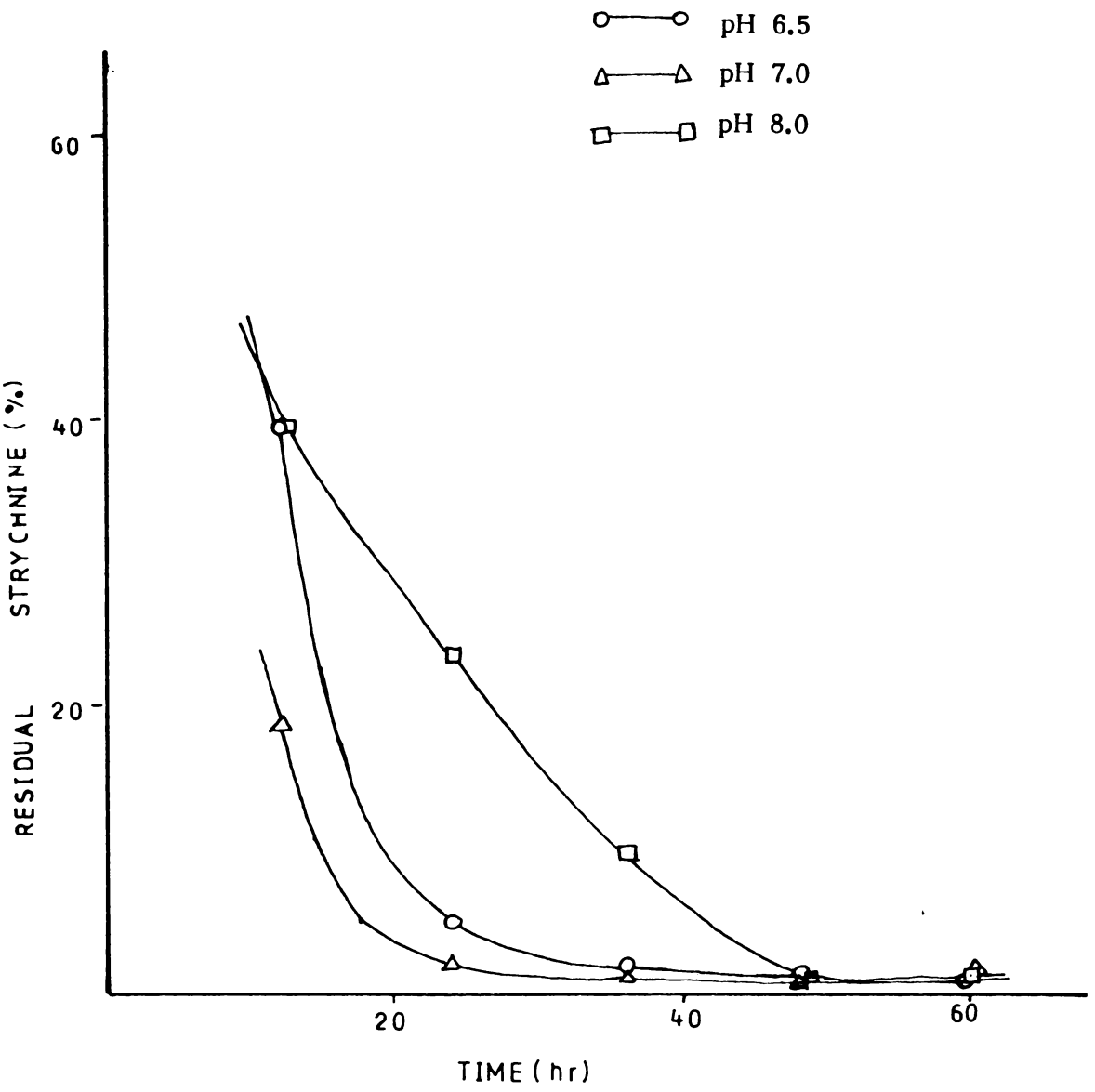


Fig 3.10 Effect of pH on degradation of strychnine

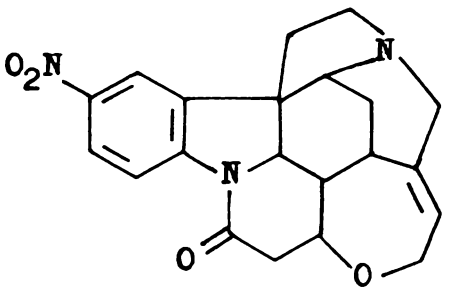
CHAPTER IV

MICROBIAL REDUCTION OF 2-NITROSTRYCHNINE TO 2-AMINOSTRYCHNINE

4.1 Materials and methods

4.1.1 Chemicals

2-Nitrostrychnine (1), 2-aminostrychnine (2), nitrobenzene (3), 2-nitrophenol (4) and 3-chloronitrobenzene (5) were used as the substrates.



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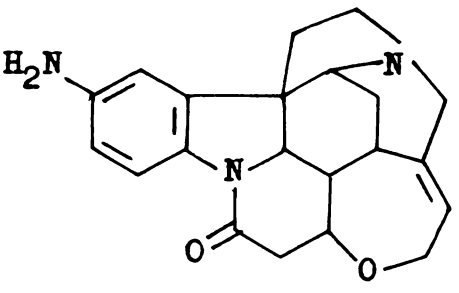

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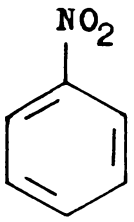
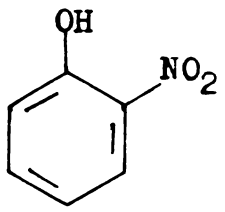


3



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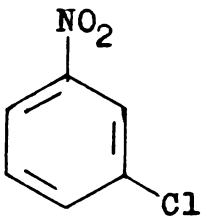


5

4.1.1.1 Preparation of 2-nitrostrychnine (1)[197]

Strychnine (2.5 g, 0.0075 mol) was added in portions to 10 ml of a nitrating mixture (H_2SO_4-HNO_3, 1:2) at 0°C with stirring. The addition required 3 to 4 hours. Towards the end of the reaction the temperature was allowed to rise to 20°C. The viscous product was poured into 75 ml of warm water (50°C) and then cooled. The crystals of nitrostrychnine nitrate formed were collected by filtration, washed with chilled water and recrystallised from boiling water containing a small amount of nitric acid. On cooling 2.0 g (80%) of 2-nitrostrychnine nitrate was obtained as fine light yellow crystals. 2-Nitrostrychnine (free base) was prepared by dissolving the nitrate in water followed by treatment with ammonium hydroxide to pH 8. The precipitated free base was filtered, washed with cold water and dried. It was recrystallised from ethanol-chloroform to get pure 2-nitrostrychnine. It melted at 239 - 240°C. (lit[197] m.p. 240 - 243°C)

$[\alpha]_D^{20}$ = -65.8°, c 0.15 (DMF)

UV : λ_{max} 339 nm (ϵ = 2.7 x 10^4), 231 nm (ϵ = 2.8 x 10^4)
207 nm (ϵ = 4.1 x 10^4)

IR (KBr) : 1680 cm^{-1} (C = O), 1530 cm^{-1} (NO_2)

4.1.1.2 Preparation of 2-aminostrychnine (2)

(a) By reduction of 2-nitrostrychnine with sodium dithionite[198]

A suspension of 1.5 g (0.004 mol) of 2-nitrostrychnine in 10 ml of hot water was dissolved by adding few drops of 10% hydrochloric acid adjusting the pH between 3 and 4. Sodium dithionite (3 g) was added in small portions while stirring. The addition was continued till the yellow colour disappeared. Ten ml of 50% sulphuric acid was added and the reaction mixture was heated on a boiling water bath for 3 hours. A tlc examination ($CHCl_3$ - MeOH, 6:4) of the reaction mixture indicated complete conversion of 2-nitrostrychnine to aminostrychnine. The solution was filtered while hot and ammonium hydroxide solution was added to pH 8. The crystalline precipitate obtained was recrystallised from chloroform-ether containing traces of methanol to give 1.2 g (80%) of the product, melting at 274 - 277°C. (lit[198] m.p. 276 - 278°)

$[\alpha]_D^{20}$ = -159.5°, c 0.09 (DMF)

UV : λ_{max} 272 nm (ϵ =2.8 x 10^4), 263 nm (ϵ =1.4 x 10^4)
209 nm (ϵ =1.0 x 10^4)

IR (Nujol) : 3430 and 3310 cm^{-1} (NH_2), 1650 cm^{-1} (C=O)

(b) By reduction of 2-nitrostrychnine with Raney nickel[199]

Nitrostrychnine base (0.3 g, 0.0008 mol) was added to 5 g of freshly prepared Raney nickel[200] in 2-propanol under stirring. The temperature was maintained at 60°C and the reaction was allowed to proceed for 3 hours. The Raney nickel residue was repeatedly washed with 2-propanol and then with methanol. The washings were collected and concentrated. TLC ($CHCl_3$ - MeOH, 6:4) showed a major spot corresponding to aminostrychnine with traces of nitrostrychnine. This material was purified by crystallisation from methanol-chloroform mixture to give 0.12 g (40%) of the product, m.p. 275 - 277°. Mixed melting point with the previously prepared sample was undepressed.

$[\alpha]_D^{20}$ = -159.5°, c 0.09 (DMF)

UV : λ_{max} 272 nm (ϵ =2.8 x 10^4), 263 nm (ϵ = 1.4 x 10^4)
209 nm (ϵ = 1.0 x 10^4)

IR (Nujol) : 3430 and 3310 cm^{-1} (NH_2), 1650 cm^{-1} (C = O)

4.1.2 Source of microorganisms

Microorganisms used in the present studies were obtained from two different sources.

a. National Collection of Industrial Microorganisms, (NCIM), National Chemical Laboratory, Poona, India.

b. Soil samples collected from the natural habitat of Strychnos nuxvomica L.

The details of NCIM cultures with their maintenance media are listed out in Chapter III under Section 3.1.2.

4.1.3 Screening of cultures

Both the NCIM cultures and soil samples were screened to select microorganisms capable of effecting transformation of 2-nitrostrychnine.

4.1.3.1 Screening of NCIM cultures

The following media were used in these studies. The media were sterilised by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes.

a) Medium for bacteria[189]

Glucose	:	5 g
Tryptone	:	2 g
Peptone	:	3 g
Yeast extract	:	5 g
2-nitrostrychnine	:	0.05 g
Water	:	1000 ml
pH	:	7.0

b) Medium for streptomyces[190]

Glucose	:	10 g
Yeast extract	:	5 g
Beef extract	:	5 g
2-Nitrostrychnine	:	0.05 g
Water	:	1000 ml
pH	:	7.0

c) Medium for fungal cultures[190]

Glucose	:	30 g
$NaNO_3$	:	3 g
KH_2PO_4	:	1 g
KCl	:	0.5 g
$MgSO_4 7H_2O$	:	0.5 g
$FeSO_4 7H_2O$	:	0.01 g
2-Nitrostrychnine	:	0.05 g
Water	:	1000 ml
pH	:	6.0

The three different media described above were prepared, distributed in 50 ml aliquots in 250 ml Erlenmeyer flasks and autoclaved. The bacterial medium was inoculated with a loopful of 18-24 hours old agar slant cultures. Similarly, the media for streptomyces and fungi were inoculated with loopfuls of

18-24 hours old cultures. The inoculated media were incubated at room temperature (28^{o}-30^{o}C) on a rotary shaker (220 rpm) for 4-8 days. These fermented broths were regularly analysed by extraction with chloroform-methanol (8:2) after the broth was made alkaline (pH 9) by the addition of ammonium hydroxide. The extracts were dried with anhydrous sodium sulphate and the solvent removed under reduced pressure. The concentrated extracts were analysed by TLC to detect any metabolites formed, as described in Section 4.1.6.1.

4.1.3.2 Screening of soil samples

About 100 g of the soil sample, collected from the natural habitats of Strychnos nuxvomica trees, was suspended in 900 ml of sterile tap water and thoroughly shaken. The supernatant solution was used as the primary inoculum. Ten ml of this inoculum was added to 90 ml of the medium, which had the following composition.

Peptone	:	3 g
Yeast extract	:	5 g
Glucose	:	5 g
2-Nitrostrychnine	:	0.05 g
Tap water	:	1000 ml
pH	:	7.0

The medium was sterilised by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes.

The inoculated medium was incubated at room temperature (28^{o}-30^{o}C) on a rotary shaker (220 rpm) for 4-8 days. About 1 ml of this broth culture was spread over nutrient agar plates and incubated for 24 hours at room temperature. The colonies developed on the plates were isolated and purified to auxenic strain by repeatedly streaking over nutrient agar plates and the pure strains were propagated and maintained in nutrient agar under oil[191]. The remaining broth was made alkaline (pH 9.0) with ammonium hydroxide solution and extracted with a mixture of chloroform-methanol (8:2). The pooled extracts were dried with anhydrous sodium sulphate and the solvent removed under reduced pressure. The concentrated extracts were examined by TLC as described in Section 4.1.6.1 to detect transformed products. The efficiency of the isolated pure strains to transform 2-nitrostrychnine was further tested by growing them in the above mentioned medium and confirmed by analysing the fermented broth as described above. By this method two strains ACM 11 and ACM 12 were isolated.

4.1.4 Identification of the microorganisms

The isolated strains were identified to the generic level based on their morphological and biochemical characters[201].

4.1.4.1 Morphological characters

These tests include mainly cell morphology, gram reaction, spore staining, motility and chromogenesis. Hucker's modification of gram staining was adopted[193]. Motility was tested by hanging drop method. Chromogenesis was tested by growing the cultures on nutrient agar.

4.1.4.2 Biochemical characters

The cultures were characterised for the production of indole, utilisation of citrate, reduction of nitrate, hydrogen sulphide production, ox-ferm test, cytochrome oxidase activity, catalase activity and sensitivity to penicillin-G-sodium[194]. The strains were also tested for their ability to produce the hydrolytic enzymes namely gelatinase, caseinase, amylase and lipases[194].

4.1.5 Growth studies

The influence of physicochemical factors namely concentration of substrate, pH, concentration of sodium chloride and temperature on the growth of bacteria was investigated as outlined below.

4.1.5.1 Medium

The medium was prepared as described in Section 4.1.3.2 with appropriate concentration of 2-nitrostrychnine. The medium was sterilised in an autoclave at 15 lbs pressure (121^{o}C) for 15 minutes.

4.1.5.2 Preparation of inoculum

A loopful of the growth from nutrient agar slants was aseptically transferred to 50 ml of nutrient broth and incubated at room temperature (28^{o}-30^{o}C) for 16-18 hours. The turbidity of the culture was observed at 600 nm and the absorbance was adjusted to give a value of 0.8. This prepared cell suspension was used as the inoculum (1 ml/100 ml).

4.1.5.3 Incubation methods

The medium was dispensed in 10 ml aliquots in each test tube and was inoculated with 0.1 ml of the prepared cell suspension, and incubated at room temperature (28^{o}-30^{o}C) for a period of 24 hours on a rotary shaker (220 rpm), unless otherwise stated. Controls were maintained for all the experiments. The experiments were carried out in duplicate.

4.1.5.4 Measurement of growth

The growth of the organism was measured by observing

the turbidity at 600 nm in a UV visible spectrophotometer (Hitachi Model 200-20) after 24 hours of incubation. The growth was expressed as optical density values (OD).

4.1.5.5 Effect of concentration of 2-nitrostrychnine on growth

The influence of concentration of 2-nitrostrychnine on the growth of bacteria was tested by inoculating the test strains, ACM 11 and ACM 12 in the medium (Section 4.1.5.1) supplemented with various concentrations of 2-nitrostrychnine (0, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06%). The dispensed medium was sterilised, inoculated and incubated on a rotary shaker at room temperature (28^{o}-30^{o}C) for 24 hours. Then, the growth was measured as outlined in Section 4.1.5.4.

4.1.5.6 Effect of pH on growth

The influence of pH of the medium on the growth of bacteria was tested by inoculating the selected test strain, ACM 11 in the medium (Section 4.1.5.1) adjusted to various pH levels (6.0, 6.5, 7.0, 7.5 and 8.0) after incorporating the substrate at its optimal concentration. Controls (basal medium) were maintained to cover these pH ranges. The dispensed medium was autoclaved, inoculated and incubated on a rotary shaker at room temperature (28^{o}-30^{o}C). The growth was measured after 24 hours as mentioned in Section 4.1.5.4.

4.1.5.7 Effect of concentration of sodium chloride on growth

The effect of sodium chloride concentrations on the growth of bacteria was observed by inoculating the selected test strain, ACM 11 in the medium containing various concentrations of sodium chloride (0, 0.5, 1.0, 1.5 and 3.0%) supplemented with optimal level of substrate. Controls (basal medium) were maintained for the above sodium chloride ranges. The dispensed broth was autoclaved, inoculated and incubated at room temperature (28^{o}-30^{o}C). After 24 hours of incubation the growth was measured as mentioned in Section 4.1.5.4.

4.1.5.8 Effect of temperature on growth

The growth of the strain ACM 11 was tested by incubating the inoculated medium at various temperatures :- 25^{o}C, 30^{o}C and 37^{o}C. Controls (basal broth) were also incubated at these temperatures. After 24 hours of incubation the growth was observed as mentioned in Section 4.1.5.4.

4.1.6 Transformation of 2-nitrostrychnine

4.1.6.1 Analytical methods

a) Thin layer chromatography (TLC)

Thin layer chromatography was performed on

0.25 mm thick layers of silica gel G prepared on glass plates with a spreader. The plates were developed in the following solvent systems.

i) Chloroform : Methanol - (9:1)

ii) Chloroform : Acetone : Diethylamine - (5:4:1)

The spots were detected either by spraying the plates with Dragendorff's reagent[195] or by exposing them to iodine vapours.

b) High pressure liquid chromatography (HPLC)

High pressure liquid chromatography was used both for the detection and estimation of the substrate and metabolites. The instrument used was a Waters Associates Model 440. The column used was an analytical reverse phase C_{18}-μ-Bondapack. The eluant was methanol. The UV detector (254 nm) and refractive index detector were used.

c) Estimation of 2-nitrostrychnine by HPLC

Standard solutions of 2-nitrostrychnine in methanol were prepared and 10μl each was injected into HPLC system and eluted with methanol. The progress of elution was monitored with the refractive index detector. From the peak heights observed in the chromatogram a caliberation curve was drawn, from which concentration of 2-nitrostrychnine was computed.

d) Estimation of 2-aminostrychnine by HPLC

Standard solutions of 2-aminostrychnine in methanol were prepared and 10 µl each was injected into the HPLC system and the eluant used was methanol. The progress of elution was monitored by the UV detector at the fixed wavelength 254 nm. From the peak heights observed, a standard caliberation curve was drawn, from which concentration of 2-aminostrychnine was obtained.

4.1.6.2 Rate of transformation of 2-nitrostrychnine and rate of formation of 2-aminostrychnine

The rate of transformation of 2-nitrostrychnine was studied employing only the culture ACM 11 as the other culture ACM 12 showed lesser tolerance in a medium with 2-nitrostrychnine. The fermentation was monitored by analysing the fermented broth at every 6 hours interval upto 48 hours.

The medium was prepared (Section 4.1.3.2) after incorporating 2-nitrostrychnine at its optimal concentration (0.03%) and the pH adjusted to 7.0. The medium dispensed in 50 ml aliquots in eight 250 ml Erlenmeyer flasks was autoclaved. The flasks were inoculated with 0.5 ml of the prepared inoculum (Section 4.1.5.2) and incubated at room temperature (28^{o}-30^{o}C) on a rotary shaker (220 rpm). At every six hours interval

the broth was made alkaline with ammonium hydroxide to pH 9.0 and exhaustively extracted with a mixture of chloroform-methanol (8:2). The extracts were pooled and dried using anhydrous sodium sulphate and the solvent distilled under reduced pressure to dryness. The dried extract was further dissolved in methanol and made upto known volume and analysed by TLC and HPLC as mentioned in 4.1.6.1.

4.1.7 Isolation of 2-aminostrychnine (2)

The fermentation was conducted in three litres of the medium (Section 4.1.3.2) containing 0.03% of 2-nitrostrychnine distributed in 300 ml aliquots in 1 litre Erlenmeyer flasks. The flasks were autoclaved, inoculated with the inoculum (Section 4.1.5.2). They were incubated over a rotary shaker (220 rpm) at room temperature (28^{o}-30^{o}C) for 40-50 hours. The progress of fermentation was closely monitored by conducting TLC analysis of the broth (Section 4.1.6.1). When the TLC registered maximum conversion the fermented broth in all the flasks were pooled, made alkaline with ammonium hydroxide to pH 9 and exhaustively extracted with a mixture of chloroform-methanol (8:2). The extract was dried over anhydrous sodium sulphate and the solvent evaporated under reduced pressure, to dryness. The residue was further dissolved in minimum amount of methanol and chromatographed over

a column of silica gel. The elution was carried out with chloroform and different combinations of chloroform and methanol. The fractions containing the metabolites as shown by TLC was concentrated under reduced pressure, and left overnight in the refrigerator. The crude crystalline precipitate obtained was recrystallised from chloroform-methanol mixture to give 0.1 g (11.1%) of 2-aminostrychnine, m.p. 274-275^{o} (lit[198] m.p. 276-278^{o}), mixed m.p. with an authentic sample (Section 4.1.1.2) was undepressed.

4.1.8 Fermentation of 2-aminostrychnine (2)

Fermentation of 2-aminostrychnine was attempted in the medium mentioned under Section 4.1.3.2 using 0.03% of 2-aminostrychnine instead of 2-nitrostrychnine. The medium was dispensed in 100 ml aliquots in 500 ml Erlenmeyer flasks and autoclaved. They were inoculated and incubated over rotary shaker at room temperature (28^{o}-30^{o}C) for 7 days. After the incubation period the broth was made alkaline (pH 9.0) with ammonium hydroxide solution and exhaustively extracted with a mixture of chloroform-methanol (8:2). The extract was evaporated under reduced pressure and the residue was analysed by TLC and HPLC.

4.1.9 Fermentation of other aromatic nitro compounds

Attempts were made to reduce simple aromatic nitro compounds by fermentation using the strain ACM 11. Fermentation medium with the composition described under Section 4.1.3.2 was prepared substituting 2-nitrostrychnine with nitrobenzene (3), 2-nitrophenol (4) and 3-chloronitrobenzene (5) at concentrations of 0.02%. The medium was dispensed in 100 ml aliquots in 500 ml Erlenmeyer flasks and sterilised by autoclaving. They were inoculated and incubated for 7 days on a rotary shaker at room temperature (28^{o}-30^{o}C). After the incubation period the broth was extracted with chloroform and, the extracts concentrated after drying with anhydrous sodium sulphate and analysed by TLC.

4.2 Results

4.2.1 Introduction

In the present investigation, microorganisms obtained from NCIM (National Chemical Laboratory, Poona) and isolated from soil were tested for their ability to transform 2-nitrostrychnine. However, the NCIM cultures were unsuccessful in transforming 2-nitrostrychnine. But, the two cultures ACM 11 and ACM 12 isolated from the local soil were able to convert 2-nitrostrychnine to 2-aminostrychnine as shown

initially by TLC. These two cultures were identified to belong to Pseudomonas species based on their morphological and biochemical characters (Table 4.1).

4.2.2 Growth studies

The results of the growth studies of the selected strains of Pseudomonas species, ACM 11 and ACM12 carried out in order to optimise physicochemical environmental factors are presented below.

4.2.2.1 Effect of concentration of 2-nitrostrychnine on growth

The effect of substrate concentration on the growth of bacteria was tested at various concentrations of 2-nitrostrychnine (1). Results presented in Table 4.2 indicate that of the two strains, ACM 11 showed preference to 2-nitrostrychnine (1) as it recorded an increase in growth when the concentration of 1 was increased from 0% to 0.03% (Fig 4.1). On further increase in the concentration of 2-nitrostrychnine the growth declined. But, ACM 12, interestingly, recorded a decrease in growth with increase in concentration of 2-nitrostrychnine from 0% to 0.06%. This indicated that the strain ACM 11 exhibited more tolerance and preference to 2-nitrostrychnine than the strain ACM 12 which exhibited greater

sensitivity to the substrate. Hence, the strain ACM 11 alone was included in further investigations.

4.2.2.2 Effect of pH on growth

The effect of pH on the growth of bacteria ACM 11 was tested at various pH levels and the results are presented in Table 4.3. The strain exhibited an increase in growth with increase in pH in the basal medium as well as the medium supplemented with 2-nitrostrychnine (Fig 4.2). Comparatively, the growth of the bacteria was more in the medium containing 2-nitrostrychnine than in the basal medium without the substrate.

4.2.2.3 Effect of concentration of sodium chloride on growth

The effect of concentration of sodium chloride on growth of bacteria ACM 11 was tested at various concentrations of NaCl in both media (basal as well as medium containing 2-nitrostrychnine). The results are presented in Table 4.4. The organism recorded maximum growth only in the absence of sodium chloride in both the media (Fig 4.3). Increase in sodium chloride concentration affected the growth adversely in both the media. Comparatively, the strain exhibited more growth in the medium containing 2-nitrostrychnine than in the basal medium. Thus, it is seen that the strain did not exhibit any preference to sodium chloride.

4.2.2.4 Effect of temperature on growth

The effect of temperature on the growth of bacteria was tested by incubating the inoculated medium at different temperatures. The results presented in Table 4.5 show that there was proportionately more growth in the medium containing the substrate than in the basal broth, at all temperatures (Fig 4.4). Of the three temperatures (25°, 30° and 37°C) tested, the organism recorded maximum growth at 30°C in both the media.

4.2.3 Rates of transformation of 2-nitrostrychnine and formation of 2-aminostrychnine

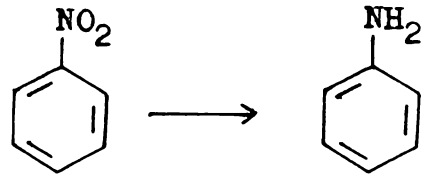
Based on the growth studies, the strain ACM 11 was selected for studying the rate of transformation of 2-nitrostrychnine to 2-aminostrychnine. The results are presented in Table 4.6. As the incubation proceeded, 2-aminostrychnine started slowly accumulating giving a value of 8.0% at the end of 12 hours (Fig 4.5). At the close of 42 hours, the concentration of 2-aminostrychnine rose to a maximum value of 16.7%. It is seen that, on further incubation, the concentration of 2-aminostrychnine started decreasing. The amount of residual 2-nitrostrychnine was also recorded (Fig 4.5). It is seen that 74% of 2-nitrostrychnine was degraded within 24 hours and more than 94% of 2-nitrostrychnine was degraded by 42 hours.

4.2.4 Fermentation of 2-aminostrychnine (2)

The chloroform-methanol extract of the fermented broth was analysed by TLC and HPLC. No transformation products were detected. Only a small amount of the material was recovered. However, the recovered 2-aminostrychnine was not quantitatively estimated.

4.2.5 Fermentation of other aromatic nitro compounds

The strain ACM 11 was able to convert nitrobenzene (3) to aniline (6) but the conversion was not complete as both nitrobenzene and aniline were detected by TLC on comparison with authentic samples. Also 2-nitrophenol (4) was partially converted to 2-aminophenol (7) as the extract showed the presence of both compounds in TLC. But in the case of



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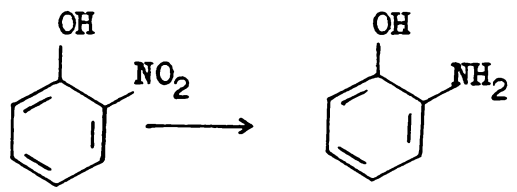

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3-chloronitrobenzene (5) no transformation took place as only 3-chloronitrobenzene was detected in the extract by TLC.

4.3 Discussion

The two organisms isolated from local soil were identified to belong to Pseudomonas species. The growth studies revealed that Pseudomonas species ACM 11 had greater growth than Pseudomonas species ACM 12 in both the basal medium and the medium supplemented with 2-nitrostrychnine. Pseudomonas species ACM 11 showed a wide tolerance range to substrate concentration. But the growth of the other strain ACM 12 was retarded by the inclusion of 2-nitrostrychnine probably because it might be toxic to the strain. However, 2-nitrostrychnine acted as a promoter of growth in the case of the strain, ACM 11. Although both the strains belonged to the same genus they demonstrated quite different patterns of preference and sensitivity to various levels of the substrate. Based on these observations, further detailed studies were carried out only with the strain ACM 11.

The strain ACM 11 registered a constant rise in growth as the pH increased from 6.0 to 8.0 in both the media. Interestingly, in both media the growth response was higher at pH 8.0 than at the neutral pH. But as the substrate precipitated

above neutral pH, pH ranges above 7.0 were not selected for further studies. The experiments with sodium chloride clearly showed the non-halophilic nature of the organism as the growth decreased in the presence of sodium chloride. The strain exhibited a preference to room temperature, than to higher temperatures which indicated their mesophilic nature and their preference to conditions which simulate their natural environment.

Based on the growth studies, the strain ACM 11 was selected for studies on kinetics of transformation. Only when the culture grew into the logarithmic phase, after 12 hours, there was appreciable formation of 2-aminostrychnine. The accumulation of the product, increased to a maximum value of only 16.7% at the close of 42 hours and thereafter concentration of 2-aminostrychnine started decreasing probably because 2-aminostrychnine was further degraded into low molecular weight products. However, the attempted fermentation of 2-aminostrychnine by the same strain did not lead to the isolation of any transformation products. This might be due to the formation of water soluble transformation products, which were not isolable under the conditions of extractions employed.

The growth studies thus revealed the optimum parameters for the growth of the organism. The results obtained from the rate studies further gave the optimum incubation period. These results were taken into consideration, in selecting the conditions for the reductive transformation of 2-nitrostrychnine (1) to 2-aminostrychnine (2). However, this yielded only 11.1% of 2-aminostrychnine.

The fermentation studies with other aromatic nitro compounds revealed an interesting feature of the possible nature of the enzyme system involved in these transformations. Of the three aromatic nitro compounds studied the culture was able to convert nitrobenzene (3) and 2-nitrophenol (4) to aniline (6) and 2-aminophenol (7) respectively, which apparently indicated the presence of a nitro-aryl reductase enzyme system involved in these processes[161]. Complete reductions of the nitro compounds were not achieved. However, detailed examination of these reductions were not carried out to pin point the optimum conditions of reduction. The failure of ACM 11 to effect any transformation of 3-chloronitro-benzene (5) may be due to the extreme insolubility of this compound in water under acidic, basic and neutral conditions.

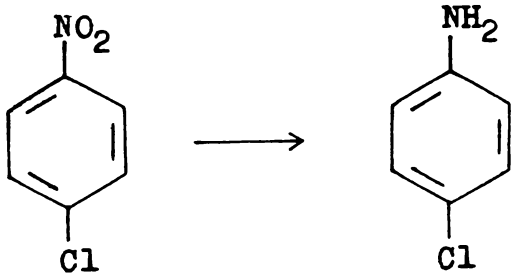
The formation of 2-aminostrychnine (2) from 2-nitro-strychnine (1) and reductions of other aromatic nitro compounds

may be visualised as taking place through the following pathway.

RNO_2 ----> RNO ----> RNHOH -----> RNH_2

8 9 10 11

The initial metabolic reactions comprising the sequential 2 e^- reductions of the nitro compound, 8 lead to the formation of nitroso and then to the hydroxylamino derivatives, 9 and 10 respectively. Further reduction of 10 gives the amino derivative, 11. A similar pathway has been proposed



12 13

for the reduction of 4-chloronitrobenzene (12) to 4-chloroaniline (13) by the yeast Rhodosporidium species[173].

Table 4.1 Identification tests

Tests	Results	
	ACM 11	ACM 12
Gram reaction	Negative	Negative
Form and arrangement	Rods No characteristic arrangement	Rods No characteristic arrangement
Motility	Motile	Motile
Chromogenesis in Nutrient Agar	Negative	Negative
Production of indole	Negative	Negative
Cytochrome oxidase	Positive	Positive
Catalase	Positive	Positive
H_2S production	Negative	Negative
Ox-ferm test	Oxidative	Oxidative
Citrate utilisation	Negative	Negative
Nitrate reduction	Positive	Positive
Gelatinase	Positive	Positive
Caseinase	Negative	Positive
Amylase	Positive	Negative
Lipases		
Tween 20	Negative	Positive
Tween 40	Negative	Positive
Tween 60	Negative	Positive
Tween 80	Negative	Positive
Sensitivity to Penicillin (2.5 I.U. of penicillin-G sodium)	Insensitive	Insensitive

Table 4.2 Effect of concentration of 2-nitrostrychnine on growth

2-Nitrostrychnine (%)		0	0.01	0.02	0.03	0.04	0.05	0.06
Optical Density (600 nm)	ACM 11	1.050	1.120	1.130	1.190	1.094	1.022	1.018
	ACM 12	0.620	0.466	0.360	0.342	0.337	0.328	0.302

Table 4.3 Effect of pH on growth of Pseudomonas sp. ACM 11

pH		6.0	6.5	7.0	7.5	8.0
Optical Density (600 nm)	Basal medium	0.730	0.842	1.060	1.306	1.342
	2-Nitrostrychnine medium	0.912	1.110	1.348	1.380*	1.500*

* This value may include 2-nitrostrychnine and 2-aminostrychnine precipitated at basic pH.

Table 4.4 Effect of concentration of sodium chloride on growth of Pseudomonas sp. ACM 11

NaCl (%)		0	0.5	1.0	1.5	3.0
Optical Density (600 nm)	Basal medium	1.250	1.030	0.858	0.684	0.464
	2-Nitro-strychnine medium	1.368	1.210	1.170	0.814	0.506

Table 4.5 Effect of temperature on growth of Pseudomonas sp. ACM 11

Temp ^{o}C		25	30	37
Optical Density (600 nm)	Basal medium	0.434	0.800	0.454
	2-Nitro strychnine medium	0.514	0.930	0.558

Table 4.6 Rates of transformation of 2-nitrostrychnine and formation of 2-aminostrychnine

Hours	0	6	12	18	24	30	36	42	48
Residual 2-Nitro-strychnine (%)	100	98.0	90.6	34.0	26.0	18.0	15.0	5.3	5.3
2-Amino-strychnine (%) formed	0	0	8.0	12.3	13.3	14.3	15.3	16.7	8.1

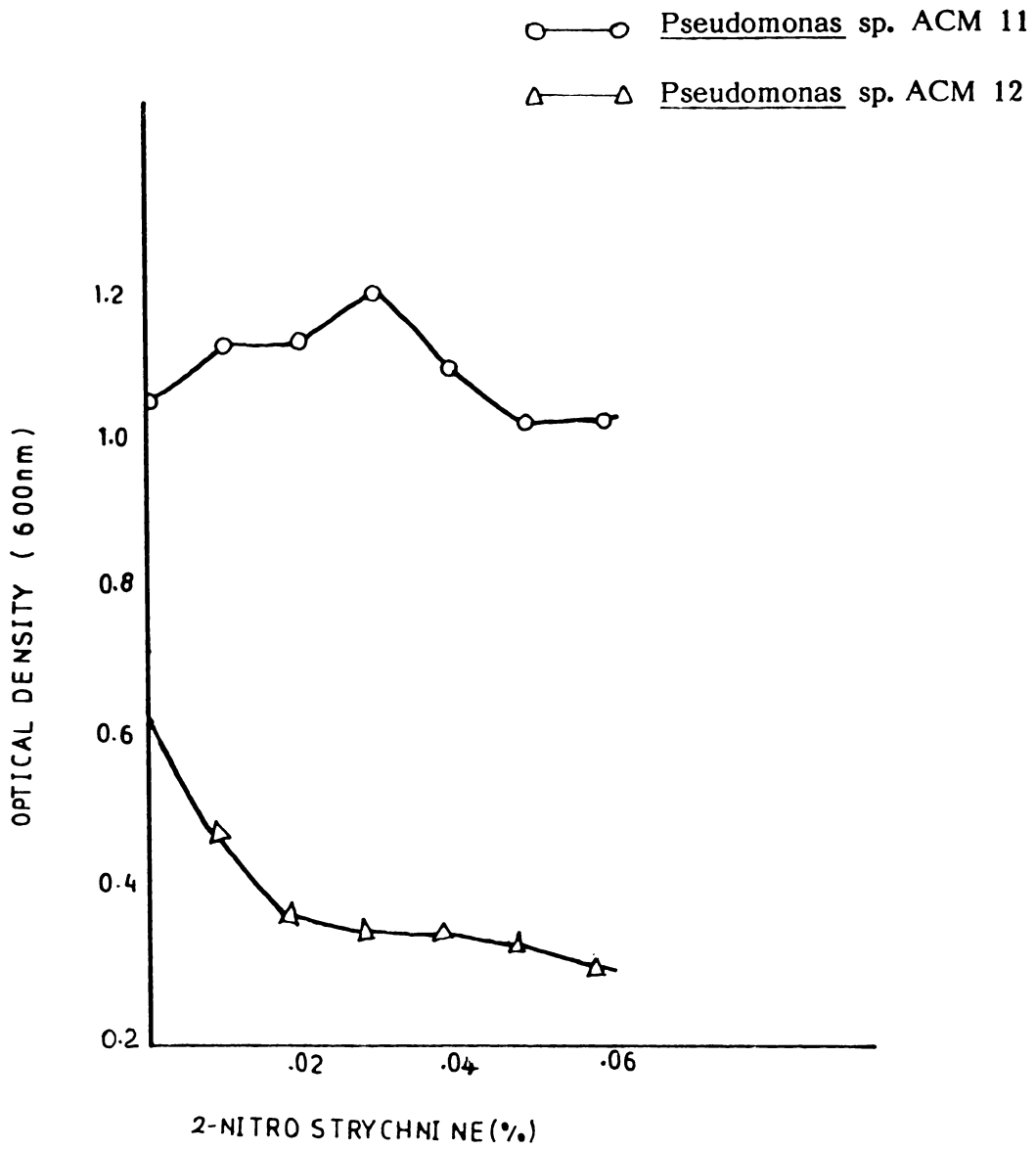


Fig. 4.1 Effect of concentration of 2-nitrostrychnine on growth

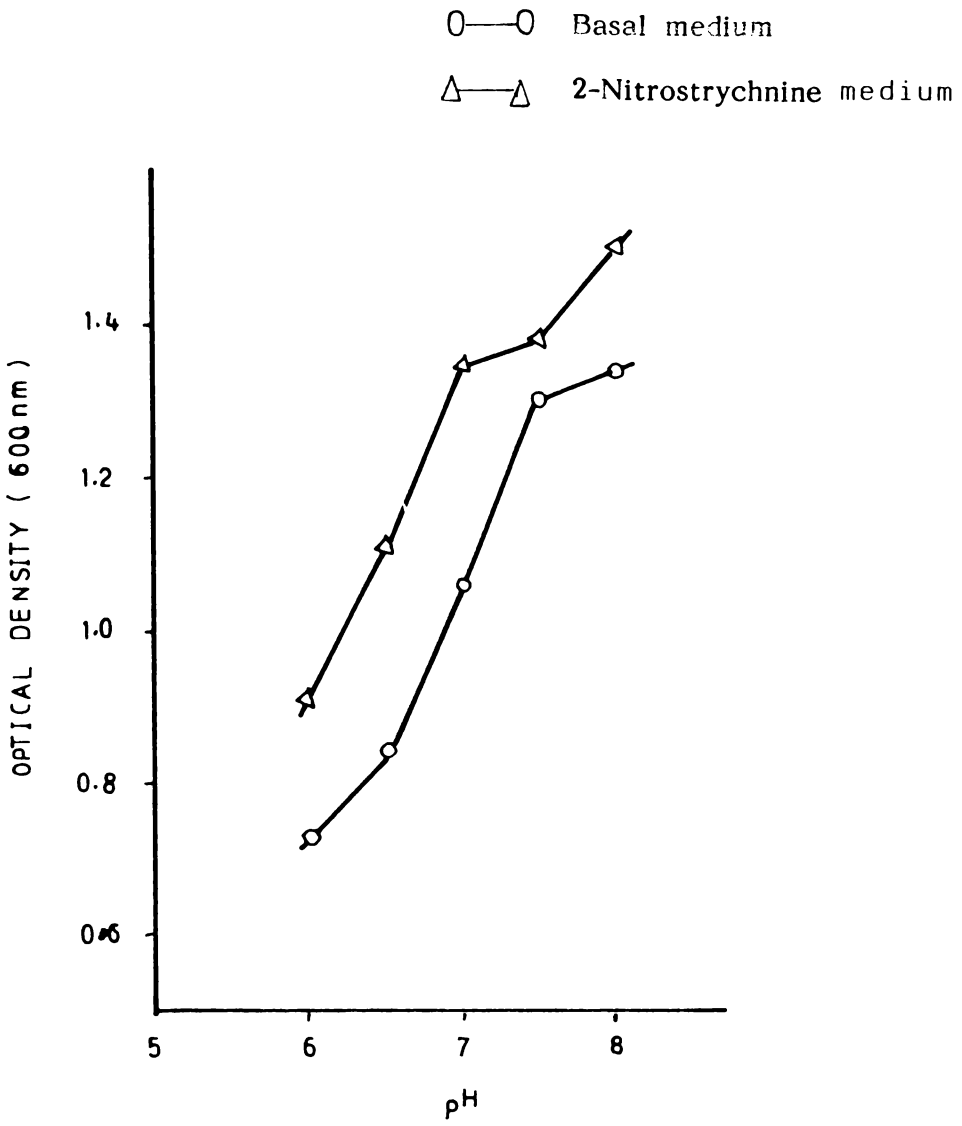


Fig. 4.2 Effect of pH on growth of Pseudomonas sp. ACM 11

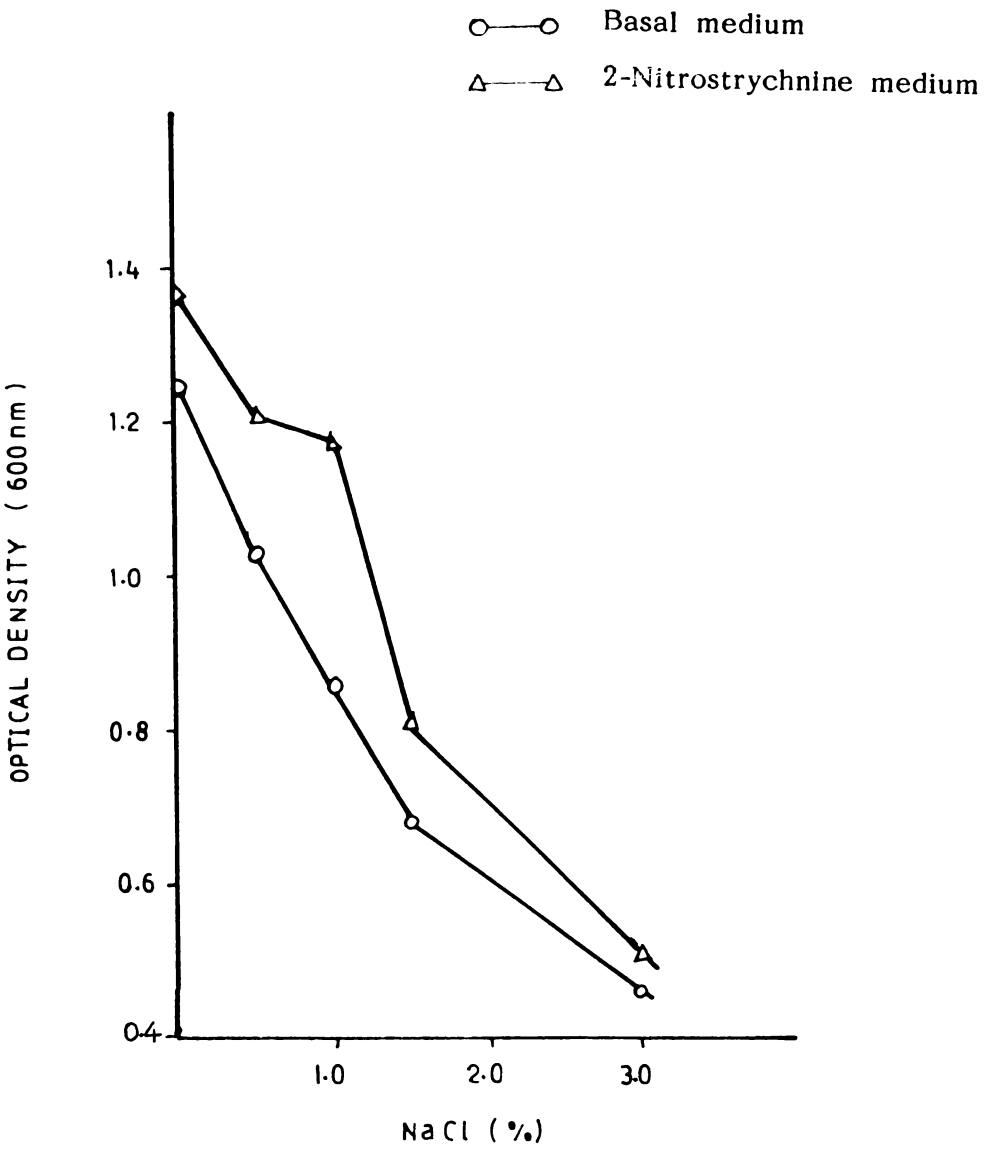


Fig. 4.3 Effect of concentration of sodium chloride on growth of Pseudomonas sp. ACM 11

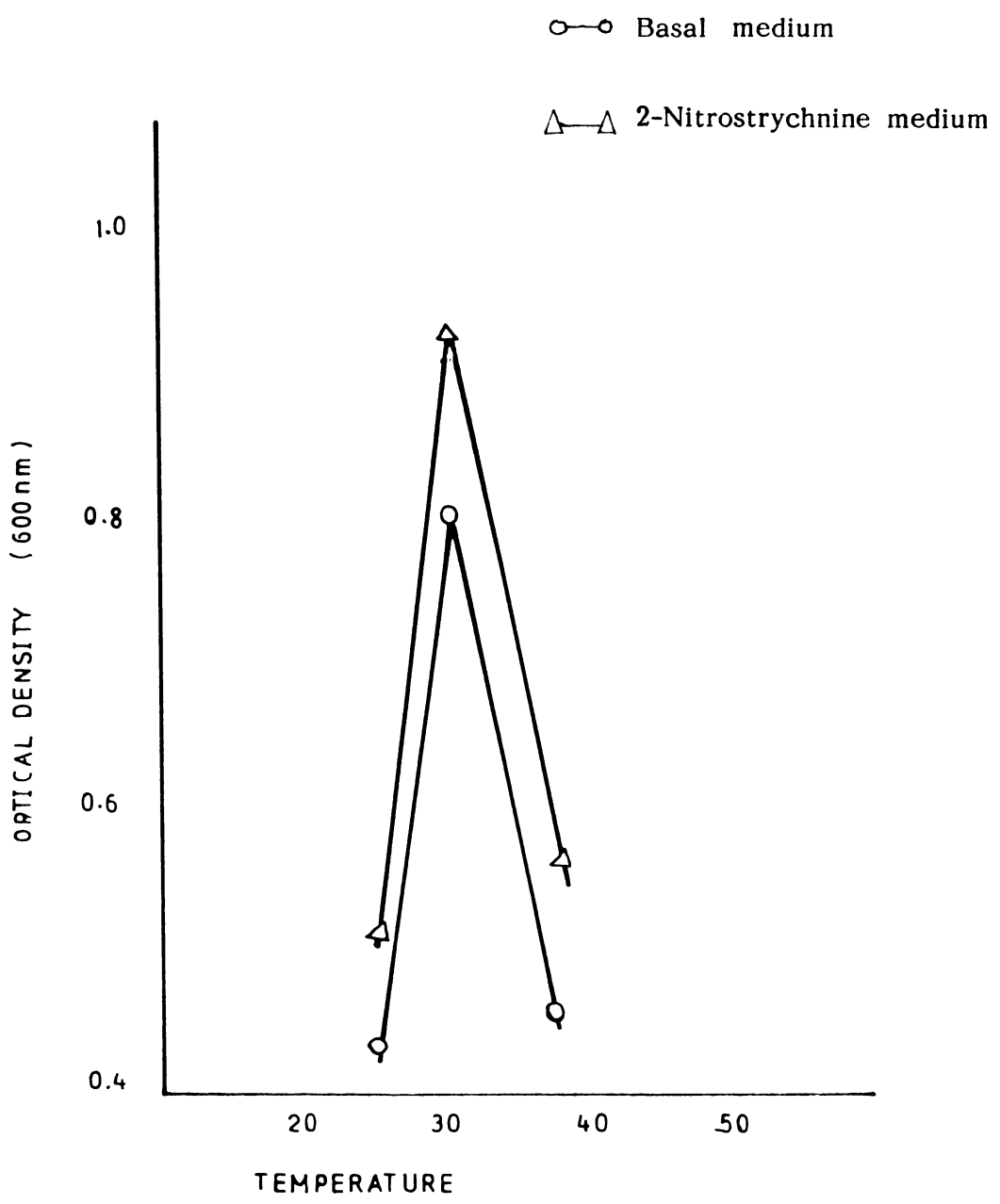


Fig. 4.4 Effect of temperature on growth of Pseudomonas sp. ACM 11

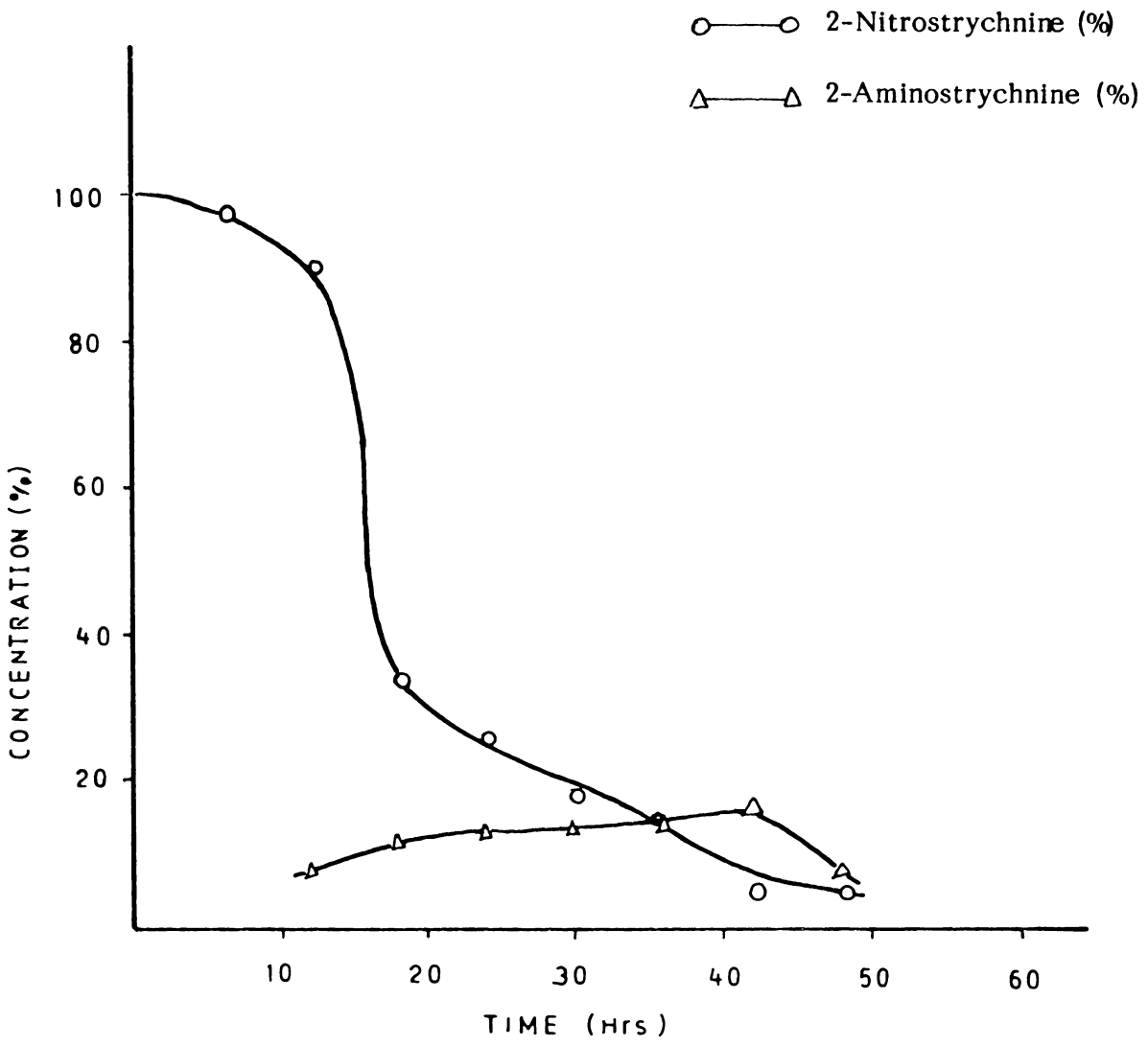


Fig. 4.5 Rates of transformation of 2-nitrostrychnine and formation of 2-aminostrychnine

CHAPTER V

SUMMARY AND CONCLUSIONS

Microbial transformations of natural products have been a useful method for the preparation of many biologically active compounds. Although microorganisms are capable of carrying out stereospecific and regiospecific chemical transformations on a wide variety of organic compounds, their utilization has not been fully exploited on substrates other than carbohydrates and steroids. Alkaloids, as a class, have received very little attention in this respect as their transformations involving heterocyclic systems are more difficult compared to alicyclic compounds. Strychnine, the major alkaloid isolated from the seeds of the Indian tree, Strychnos nuxvomica is known to stimulate all portions of the central nervous system with preference to the spinal cord. However, being a powerful convulsant it leads to death from asphyxia. As such, neither strychnine nor any of its derivatives has any therapeutic application in the western system of medicine at present.

The microbial transformations of strychnine and one of its derivatives, 2-nitrostrychnine were extensively investigated in this work. Strychnine was subjected to the actions of a variety of microorganisms with a view to convert it into a product of less toxicity and improved pharmacological activity. Many of the cultures obtained from the National

Collection of Industrial Microorganisms (NCIM) failed to convert strychnine into any useful products. Of these organisms only Bacillus thuringiensis 2159, Cunninghamella blakesleeana 687 and C. echinulata 691 produced the N-oxide in small amounts and this process itself is of no practical value as the same product can be obtained in better yields by chemical methods. Hence organisms that grow on strychnine were isolated from the local soil using the elective culture technique. The isolated organisms were purified and the auxenic strains were identified upto the generic level according to Bergey's Manual of Determinative Bacteriology, based on their morphological, cultural and biochemical characteristics. The strains were identified as belonging to Arthrobacter species. The growth of the organism under varying physicochemical environmental factors such as temperature, pH, sodium chloride concentration and substrate concentration were studied to arrive at the optimal conditions. Similarly, the influence of a second carbon source, glucose on the rate of transformation was also investigated. The kinetics of utilization of strychnine was studied by subjecting the fermented broth to exhaustive extraction using chloroform-methanol and estimating the concentration of the substrate in the extract at specified time intervals spectrophotometrically. The progress of strychnine utilization was also followed using thin layer chromatography. These

data also gave an idea about the optimum incubation period required.

By incorporating the optimal conditions of physico-chemical parameters and incubation time, experiments were designed to isolate the transformed product. The fermentation was carried out for 14-18 hours, the fermented broth was made alkaline to pH 9 using ammonium hydroxide and the solvent (water) was removed under reduced pressure. The residue was extracted with methanol and the crude product was purified by column chromatography to give about 10% of C_{16}-Hanssen acid. The identity of the product was established by comparison with an authentic sample of C_{16}-Hanssen acid obtained by the oxidation of brucine by a known procedure and also by conversion into its perchlorate and methiodide and comparison of their physical constants.

The isolated strain, Arthrobacter sp. ACM 1 could not produce a substantial yield of the product C_{16}-Hanssen acid because the organism was also found to degrade this product further. Also the same strain failed to act on brucine which is the dimethoxy derivative of strychnine and which occurrs in Strychnos nuxvomica seeds alongwith strychnine. The studies also revealed that the microorganism utilised strychnine alone in a mixture of strychnine and brucine and

this method can therefore be used to purify the commercially available brucine. That the bacterial strain is able to utilise strychnine both as a carbon and nitrogen source indicates that it differs from the earlier reported Arthrobacter strychnovorum, which could utilise strychnine only in the presence of an external inorganic nitrogen source. Also the fact that it produced C_{16}-Hanssen acid and did not metabolise brucine establishes that it is different from A. strychnophagum also reported previously.

Transformation of 2-nitrostrychnine into 2-aminostrychnine was carried out using another organism, also isolated from the local environment. This organism was identified to the generic level and was shown to belong to Pseudomonas species. Here also the growth of the organism under varying physicochemical parameters were investigated to obtain the optimal conditions. These optimal conditions were incorporated and the kinetics of transformation was followed by analysing the chloroform-methanol extract of the broth using high pressure liquid chromatography. Employing the optimum physicochemical factors and incubation time, fermentation of 2-nitrostrychnine was carried out to isolate the product, 2-aminostrychnine. Its identity was established by comparison with an authentic sample obtained by chemical reduction of 2-nitrostrychnine. This Pseudomonas strain, ACM 11 apparently contains a nitro-

aryl reductase enzyme system because the same culture was also able to reduce aromatic nitro compounds to the corresponding aryl amines. The low yield of 2-aminostrychnine indicates that the amine formed was further broken down by the same organism.

This study, thus, has led to the isolation of two new microorganisms, one belonging to Arthrobacter species that selectively degrades strychnine into C_{16}-Hanssen acid and the other, belonging to Pseudomonas species that reduces 2-nitrostrychnine into 2-aminostrychnine. The characterisation of the microorganisms upto the generic level, their growth studies under different physicochemical parameters and the proof of the structures of the transformation products have been presented in detail.

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