MICROBIAL TRANSFORMATIONS OF ORGANIC COMPOUNDS: EUGENOL AND ISOEUGENOL

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The usefulness of microorganisms in organic synthesis has now been widely recognised. By the judicious choice of microorganisms or enzymes many desired transformations of compounds can be achieved. One of the outstanding features of microbial transformations is that various specific reactions which had been presumed not feasible by means of conventional organic synthesis under normal temperature and pressure can be brought about with a good yield of high purity products. Microbial transformations have also been utilised more extensively in the introduction of functional groups for obtaining biologically useful substances, in reactions showing stereochemical specificity and also in identification of unknown organic compounds in recent years. It can be used for microbial decomposition treatment of natural and synthetic wastes for protecting the environment as well.

A microbial transformation is the conversion of one substrate to another by a microorganism. It is a chemical reaction catalysed by a particular cellular enzyme or by an enzyme originally produced within cells. Most of

such enzymes are necessary for the normal functioning of the life processes of cellular metabolism and reproduction of the microbes. In microbial transformations these enzymes simply act as catalysts for chemical reactions. In addition to their natural substrates, many of these enzymes can utilise other structurally related compounds as substrates and therefore occasionally catalyse unnatural reactions upon addition of foreign substrates to the reaction medium.

Enzymes involved in microbial transformations increase the reaction rate by lowering the activation energy as normal catalysts do. The most striking difference between enzymes and chemical catalysts lies in their substrate specificities. They catalyse specific reactions involving one or only a few structurally related compounds, distinguishing almost absolutely between stereo isomers or regio isomers. Only a small amount of enzyme is needed to catalyse the conversion of a large amount of substrate. Enzymes exhibit their activity under mild reaction conditions in which normal chemical catalysts hardly function at all, such as atmospheric pressure, temperatures around 30°C and pH values near neutrality. These unique

properties of enzymes are extremely useful when unstable molecules are to be converted without undesired side reactions.

On the other hand, these unique features occasionally make microbial enzymes hard to be used as convenient catalysts. A microbial cell contains many different types of enzymes which may give rise to undesired products. As for the types of reactions that can be conducted with the microorganisms, only some generalisations are possible. Thus yeasts are most suitable for the reduction of carbonyl compounds whereas fungi can introduce hydroxyl groups into a wide variety of organic compounds. Besides, bacteria have the ability to oxidise organic substrate completely to carbon dioxide and water.

Microbial transformation is an ancient art. The oldest and best known prototype is vinegar production which has been performed since the dawn of recorded history. The process has been further developed along traditional lines of fermentation without knowledge of the underlying biochemistry. It was only much later, through the discovery of the specific modifications of steroids by microorganisms

that microbial transformation obtained the significance it has today. Thousands of microbial transformations involving different types of reactions with organic compounds and natural products are now known. A wide variety of chemicals used for analysis and research in clinical chemistry, biochemistry, metabolism and pharmacology are also produced using enzymes. The current technological importance of microbial transformations or enzymatic process is well recognised in many fields of biotechnology.

Eugenol (1-hydroxy-2-methoxy-4-allyl benzene) is the main constituent of several important essential oils like clove oil, cinnamon oil and pimenta oil. Clove oil is the essential oil obtained from the leaf, stem or flower bud of the clove tree, Eugenia caryophyllata. It contains free eugenol (70-90%), eugenol acetate and caryophyllene as its main constituents. Eugenol is a faintly yellow liquid possessing a strong odour of cloves and a burning taste. It is widely used in pharmaceutical preparations, perfumes, cosmetics and for scenting soaps. The main use of eugenol is as a starting material for the production of vanillin. Isoeugenol (1-hydroxy-2-methoxy-4-propenyl benzene) occurs

in oil of ylang-ylang, nutmeg and champaca. It is also prepared by various methods of treating eugenol with potassium hydroxide. Isoeugenol is a clear liquid with an odour reminiscent of eugenol but more suave and lasting. It is one of the most useful ingredients in perfume work and serves equally well for the scenting of cosmetics and soaps. Isoeugenol on mild oxidation yields vanillin. Vanillin occurs in nature as a glucoside, which hydrolyses to vanillin and sugar. The best known natural source of vanillin is the vanillin plant, Vanilla plansfolia. Vanillin is extensively used as a flavouring agent in food industry and in the synthesis of drugs like Aldomet, L-dopa and Trimethoprim. It is also used in perfumes and metal plating industries. Vanillin is manufactured by the alkaline-air oxidation of lignin, the main component in the spent sulfite liquors from sulfite pulp mills. It can also be manufactured by the oxidation of isoeugenol. In this study, the microbial transformations of eugenol and isoeugenol were undertaken with a view to producing vanillin, an extremely useful compound.

Two microorganisms, one CUAC 20 which can grow on eugenol and the other CUAC 30 which can grow on isoeugenol,

were isolated from the soil. A third microorganism CUAC 10 which can transform both eugenol and isoeugenol in a mineral medium containing an easily assimilable carbon source like glucose was also isolated from the aerial flora. This organism was not capable of growing on eugenol or isoeugenol as a single carbon source. These micro-organisms were then purified and identified upto the generic level. The first two organisms (CUAC 20 and CUAC 30) were identified as belonging to the Pseudomonas sp. and the third one (CUAC 10) belonging to the Bacillus sp. In addition to these, a culture of Pseudomonas aeruginosa, which also grow on eugenol, was obtained as a gift from Dr.Kohji Tadasa, Shinshu University, Japan.

The fermentations of eugenol and isoeugenol were done in a mini jar fermentor. The transformation products were separated and identified using spectral data, physical properties and chemical reactions. The Bacillus sp. CUAC 10 transformed eugenol to bisdehydroeugenol. Bisdehydroeugenol on oxidation with nitrobenzene and alkali gives dehydrodivanillin. This strain CUAC 10 converted isoeugenol to dehydrodiisoeugenol and bisdehydroisoeugenol.

Dehydrodiisoeugenol on oxidation with nitrobenzene and alkali gives vanillin as the main product. The Pseudomonas sp. CUAC 20 degraded eugenol to ferulic acid, vanillin, vanillic acid and protocatechuic acid. The same products were obtained in the fermentation of isoeugenol with the Pseudomonas sp. CUAC 30. All these transformation products were further degraded with the cleavage of the aromatic ring resulting in low yield of the products. The cell free extracts of the strains CUAC 20 and CUAC 30 were prepared and transformations of eugenol/isoeugenol by the cell free extracts were studied. The degradation pathways of eugenol and isoeugenol by these strains are proposed, specifying the mode of cleavage of the aromatic ring.

Microbial transformations of eugenol and isoeugenol were attempted using immobilised microbial cells also. If whole microbial cells are immobilised directly without extracting the enzyme, the immobilised cells can be used as a solid catalyst. Thus immobilised whole cell systems have been increasingly used to prepare fine chemicals. Another advantage of this method is that for unit production of the desired compound the volume of fermentation broth is much smaller in the case of the

continuous method using immobilised cells than in the case of conventional batch fermentation methods. The results of the studies using immobilised whole cells are also presented in this thesis.

CHAPTER 2

HISTORICAL REVIEW

HISTORICAL REVIEW

2.1 INTRODUCTION

Microbial transformations have been widely used since the early days of mankind for the production of bread, dairy products and alcoholic beverages. It was in 1862 that Louis Pasteur[1] laid a scientific foundation for one of these early applications, namely the oxidation of alcohol to acetic acid by using a pure culture of Bacterium xylinium.[2] Investigations of the oxidation of glucose to gluconic acid by Acetobacter aceti[3] and of sorbitol to sorbose by Acetobacter sp.[4] were then reported. The reducing action of fermenting yeast, Saccharomyces cerevisiae was first observed by Dumas in 1874.[5] A.J.Brown obtained propionic acid from n-propanol and fructose from mannitol.[2] Numerous further microbial transformations followed.

However, most of the microbial transformations have been largely confined to reactions involving carbohydrates,[6] simple aliphatic and aromatic compounds[7-10] and steroids,[11-14] till the 1950s. In 1959, following the classification of microbial transformations by Stodola,[15] which encouraged more fundamental studies involving

different classes and types of substrates, a rapid development took place in the microbial transformations of other structural classes of compounds.

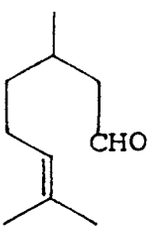
Todate an enormous amount of work has been done in this area and several excellent reviews have been published.[16-23] To avoid excessive echoing of these reviews we will limit our survey to the transformations of terpenoids and phenolic compounds only which are more relevant to our investigations.

2.2 MICROBIAL TRANSFORMATIONS OF TERPENOIDS

The vast majority of microbial transformations of terpenes involves the breakdown of these natural products and suitable strains have only been obtained by tedious adaptation tests on appropriate substrates. Also the substrates as well as the end products are usually lost as volatile compounds by the aeration used in fermentation. However, microbial techniques can be used to bring about specific changes in these compounds. Ciegler[24] and Kieslich *et al*.[25] have reviewed the work in this field.

Mayer and Neuberg were the first to report the reduction of citronellal (1) to citronellol (2) by yeast.[26]

Some strains of Acetobacter xylinium caused transformation of citronellal to citronellol and citronellic acid (3).[27]



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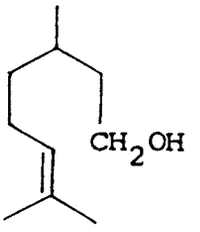

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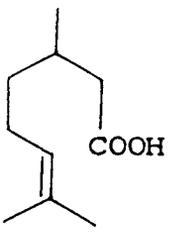

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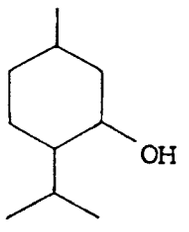
Joglekar and Dharlikar were able to prove the cyclisation of acyclic terpenes when they obtained menthol (4) in addition to citronellol (2), citronellic acid (3), dihydrocitronellol (5) and 2,8-dihydroxy-2,6-dimethyl octane (6) from citronellal by the action of Pseudomonas aeruginosa.[28,29] Linalool (7) was also cyclized to camphor (8) using a pseudomonad strain.[30]



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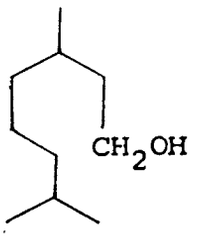

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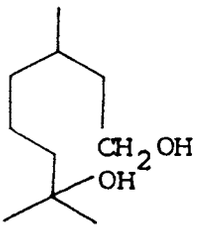

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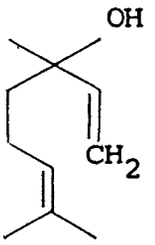

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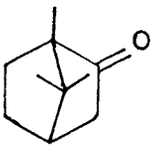

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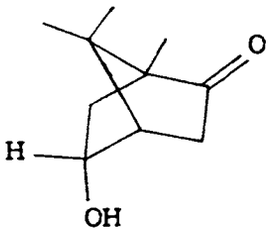
A strain of Pseudomonas putida degraded camphor to 5-endo-hydroxy camphor (9), 5-exo-hydroxy camphor (10) and 5-oxocamphor (11).[31]



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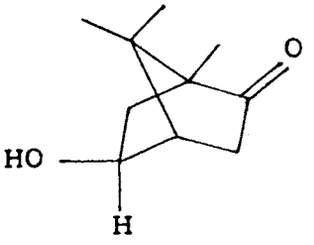

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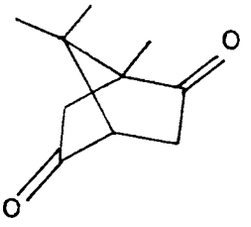
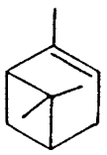


(11)

A strain of Serratia marcescens could effect the allylic oxidation of d-α-pinene (12) to d-trans-verbenol (13) as the major product, along with d-verbenone (14) and d-trans-sobrerol (15) as minor products.[32] Allylic oxygenation of α and β pinene was reported to be the most characteristic reaction of a strain of Armillariella mellea.[33]



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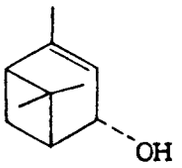

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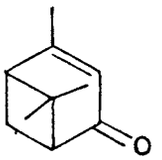

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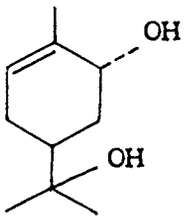

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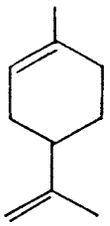
Co-oxidation of limonene (16) in a medium containing a hydrocarbon, with Corynebacterium hydrocarboclastus, yielded carvone (17) in good quantities.[34]



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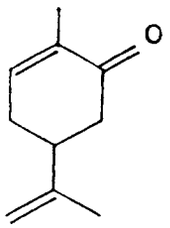

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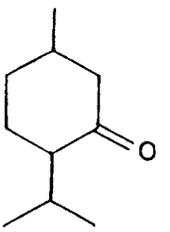
A bacterium isolated from the sewage was found to degrade (-) menthol to menthone (18), 3,7-dimethyl-6-hydroxy octanoic acid (19) and 3,7-dimethyl-6-oxo-octanoic acid (20).[35]



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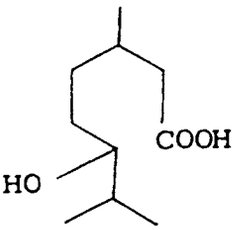

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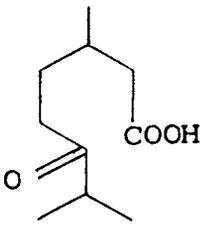
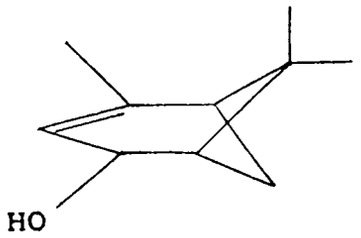


(20)

In 1990, Hunt and Borden reported the conversion of cis-verbenol (21) to verbenone (22) with the yeasts Hansenula capsulata and Pichia pinus.[36] Liu et al. have studied the stereochemistry of microbial hydroxylations of 1,4-cineole (23) with Bacillus cereus and Streptomyces griseus.[37]



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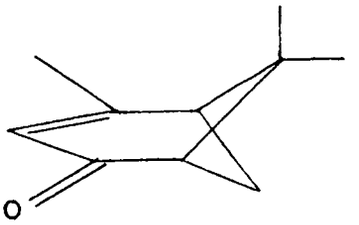

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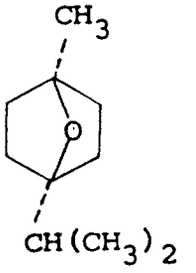
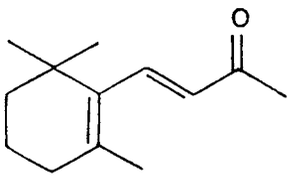


(23)

The pregrown mycelia of the fungus, Lasiodiplodia theobromae was able to transform β-ionone (24) to β-cyclohomogeraniol (25) as the main product.[38]



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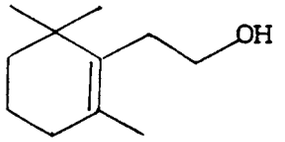
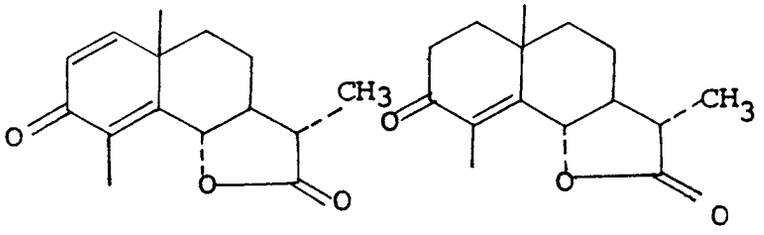


(25)

The soil microorganism Pseudomonas cichorii utilised α-santonin (26) as the sole carbon source producing 1,2-dihydro-α-santonin (27) and lumisantonin (28).[39,40] Cross and his colleagues in 1965, were able to demonstrate the conversion of gibberellin A_{12} (29) to gibberellic acid (30) and gibberellin A_{13} (31), by the fungus Gibberella fujikuroi.[41]



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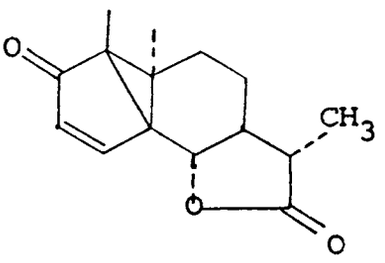

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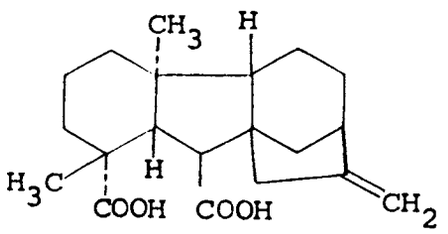

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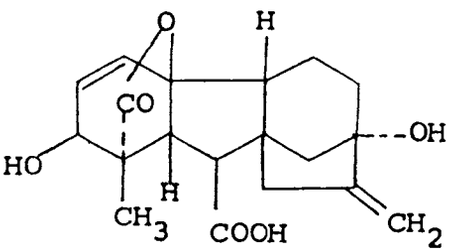

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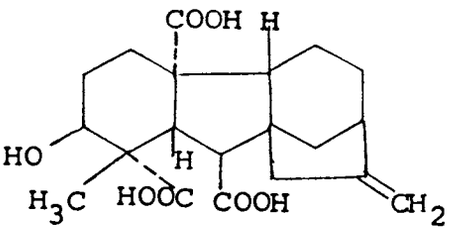
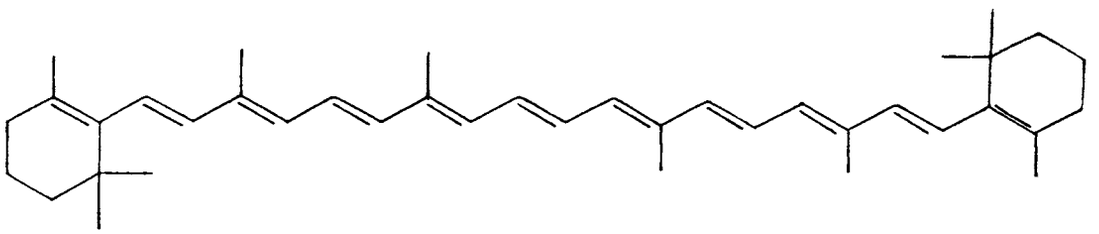


(31)

Dmitrovskii and Starikova have reported the cleavage of β-carotene (32) to vitamin A (33) by a strain of Pseudomonas aeruginosa.[42]



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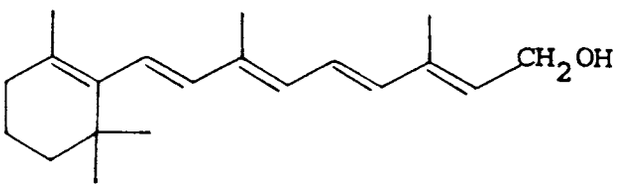


(33)

2.3 MICROBIAL TRANSFORMATIONS OF PHENOLIC COMPOUNDS

Compared to carbohydrates and steroids, not much work has been done in the transformation of phenolic compounds which may be because of their antimicrobial properties. Our present state of knowledge about the microbiological transformations of aromatic compounds, phenolic compounds in particular, has already been summarised in a number of detailed reviews.[43-47]

Because the breakdown of aromatic compounds is a vital biochemical step in the natural carbon cycle, many microorganisms are capable of cleaving the aromatic ring. Before there can be any microbial decomposition of an aromatic ring, a precondition is that the substrate must have an o- or p-dihydroxyphenol structure or it must be possible to convert it to one of these structures by hydroxylation.[16] The enzymes responsible for this hydroxylation have the character of mixed function oxidases which incorporate one oxygen atom into the substrate molecule. However, dioxygenases can also be used which introduce two vicinal oxygen atoms simultaneously. Oxidative cleavage of the aromatic ring may occur by three different mechanisms:[16]

a) ortho cleavage - The ring is opened at the bond between the C atoms which bear the hydroxy groups of an o-dihydroxyarene producing a cis-2,4-hexadieneoic acid. Catechol:oxygen-1,2-oxidoreductase (catechol 1,2-oxygenase) has been named as the enzyme responsible for ring cleavage.

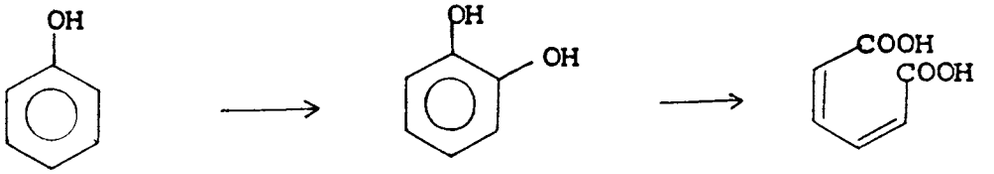
b) meta cleavage - The ring cleavage occurs at the bond between the C atoms bearing a hydroxy group and an adjacent unsubstituted carbon, producing a 2-hydroxy-6-oxo-2,4-hexadienoic acid. The ring splitting enzyme is called catechol 2,3-oxidoreductase (catechol 2,3-oxygenase.

c) p-dihydroxyphenol cleavage - The ring bond between the C atom bearing a hydroxy group and an adjacent C atom bearing a hydrogen, a carboxy group or a side chain is broken. This produces a 4-hydroxy-6-oxo-cis,cis-2,4-hexadieneoic acid from quinol and 4-hydroxy-2-oxo-cis, cis-3,5-heptadienedioic acid from 2,5-dihydroxybenzoic acid.

2.3.1 Phenol and catechol

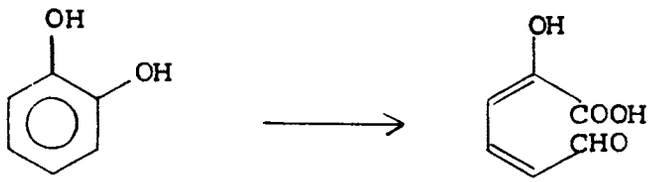
In 1952, Gale isolated a strain of bacteria which could transform phenol (34) to 1,2-dihydroxybenzene (35).[48]

Cain and coworkers reported the ring fission of catechol by o-cleavage, with a bacteria isolated from soil.[49]

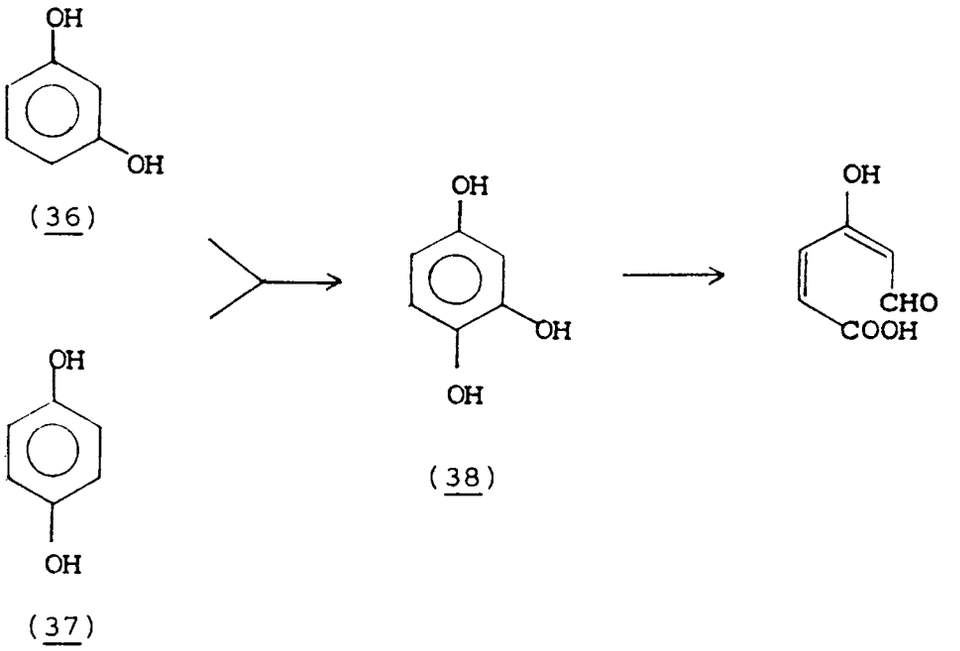


(34) (35)

In 1959, Dagley and Stopher isolated a strain of bacteria which could break the aromatic ring by m-cleavage.[50]



In 1965, Larway and Ewans hydroxylated 1,3- and 1,4-dihydroxybenzene (36, 37), with a bacterial strain and the trihydroxybenzene (38) formed was broken down by p-dihydroxyphenol cleavage.[51]



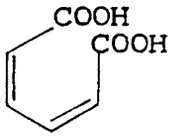
(36)

(37)

(38)

The assimilation of phenol as a sole carbon and energy source by strains of thermophilic bacteria, Bacillus stearothermophilus have been reported.[52] Some strains of yeasts can oxidise phenol to catechol which is then oxidised to cis, cis-muconic acid (39).[53] Smith and Ratledge have reported a quantitative conversion of catechol to cis, cis-muconic acid with a Nocardia sp.[54] In 1990, Gajendiran

and Mahadevan reported their studies on the utilisation of many phenolic compounds by a Rhizobium sp.[55] Certain strains of bacteria can anaerobically degrade o-phenyl-phenol (40).[56]



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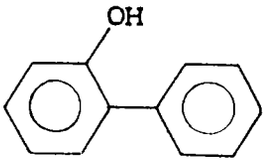
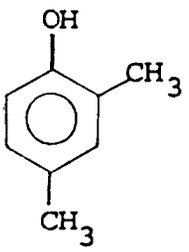


(40)

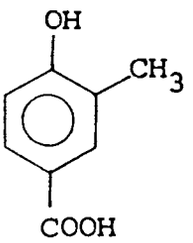
A Trichosporon sp. preferentially utilised phenol in a medium containing both phenol (200 ppm) and glucose (0.1%) until the concentration of phenol decreased to 20 ppm.[57] Hoffman and Ute Vogt have reported the studies on the induction of phenol assimilation in chemostat cultures of Candida maltosa.[58] Yi Tin Wang has studied the effects of some alkyl phenols on the anaerobic biodegradation of phenol in batch methanogenic cultures.[59]

2.3.2 Methyl substituted phenols

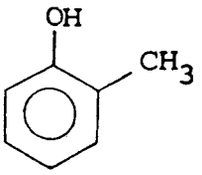
A strain of Pseudomonas sp. oxidised 2,4-dimethylphenol (41) to 4-hydroxy-3-methylbenzoic acid (42).[60] Another strain of Pseudomonas sp. metabolised 1-hydroxy-2-methylbenzene (43)[61] and 1-hydroxy-3-methylbenzene (44)[62] by m-cleavage.



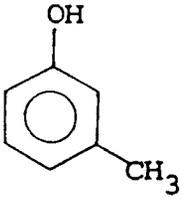
(41)



(42)

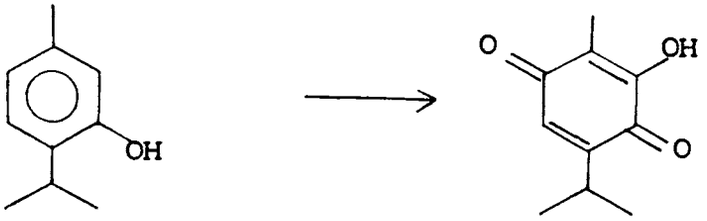


(43)



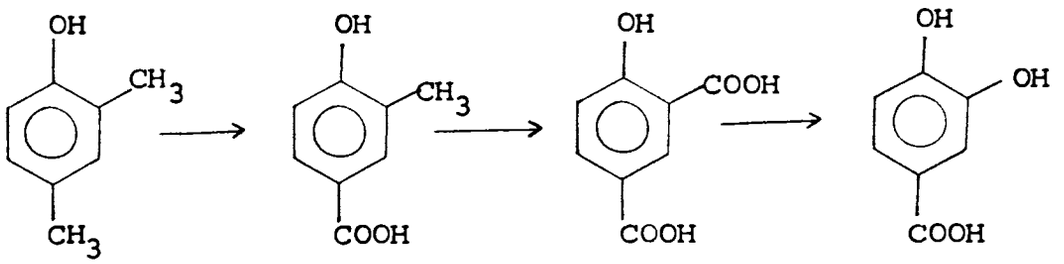
(44)

The oxidation of thymol (45) by Pseudomonas putida affords the hydroxyquinone (46) in about 10% yield.[63]



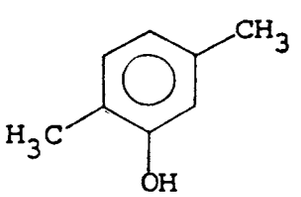
(45) (46)

In 1968 the oxidation of 2,4-xylenol (41) by cell suspensions of a fluorescent pseudomonad was reported by Hopper and Chapman.[60]

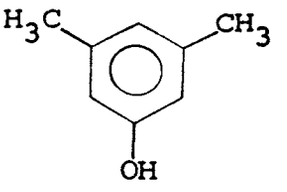


(41)

The same authors have reported the catabolism of 2,5-xylenol (47) and 3,5-xylenol (48).[64]

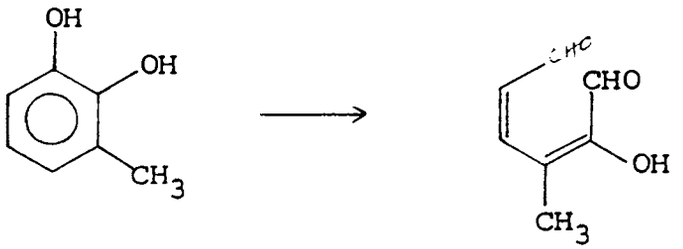


(47)



(48)

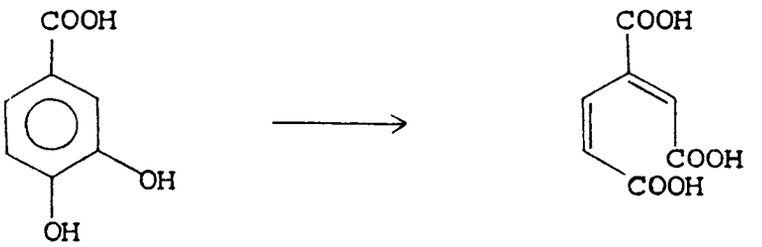
An Achromobacter sp. could oxidise 3-hydroxy-catechol (49) to 2-hydroxy-3-methylmuconic semialdehyde (50).[65] In 1979 Engelhardt, reported the co-metabolism of phenol and methyl substituted phenols by a Nocardia sp.[66]



(49) (50)

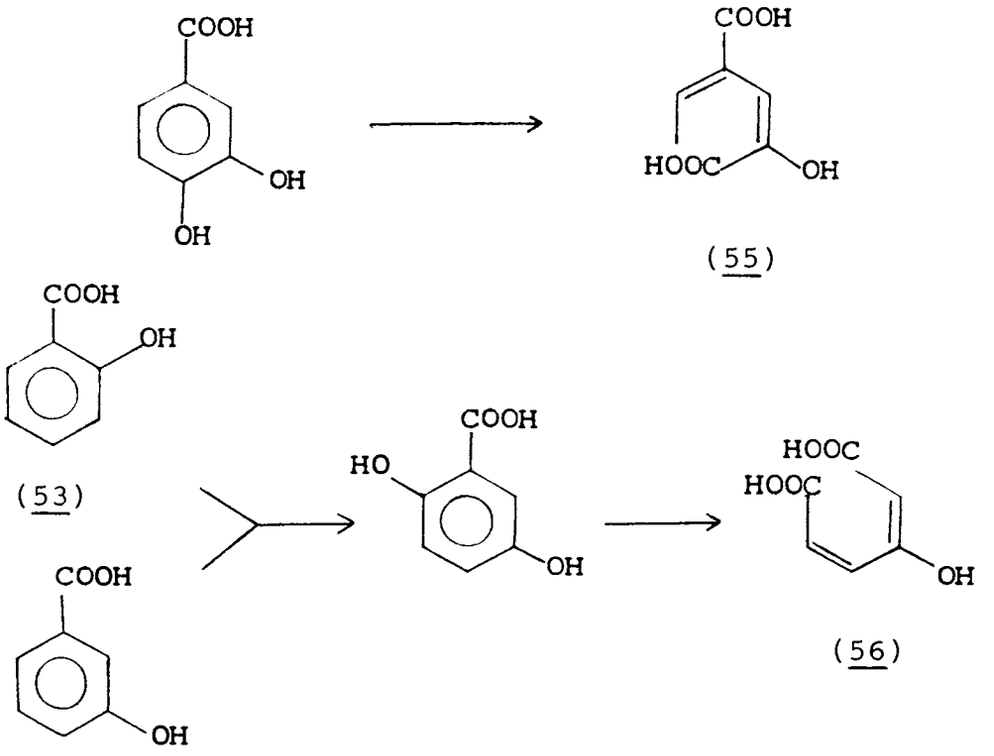
2.3.3 Carboxy substituted phenols

In 1954, McDonald and coworkers reported the o-cleavage of 3,4-dihydroxybenzoic acid (51) by a strain of bacterium to give (52).[67]

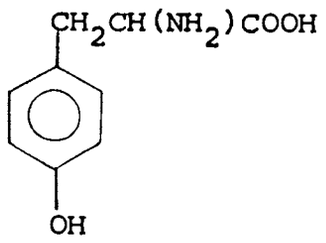


(51) (52)

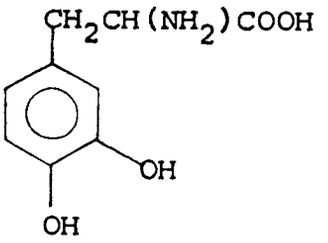
Subsequently Tripett et al. reported the m-cleavage of the same compound[68] and Hopper et al. reported the p-dihydroxy phenol cleavage of 2-hydroxy and 3-hydroxy-benzoic acids (53, 54) to give (55) and (56) respectively.[69]



In 1973, a novel microbial synthesis of L-tyrosine (57) or 3,4-dihydroxyphenyl-L-alanine (58) from pyruvic acid, ammonia and phenol or catechol, with Erwinia herbicola, was reported.[70]

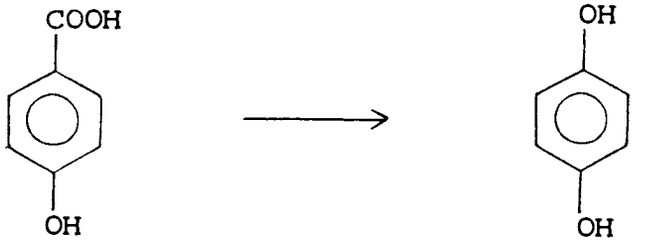


(57)



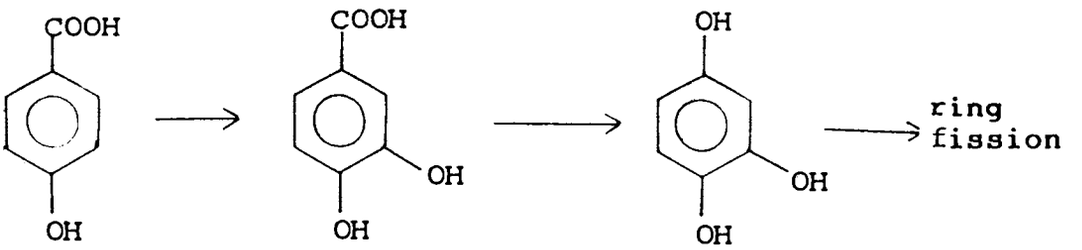
(58)

Some strains of yeasts of the genus Debaromyces converted p-hydroxybenzoic acid (59) to hydroquinone (37).[71]



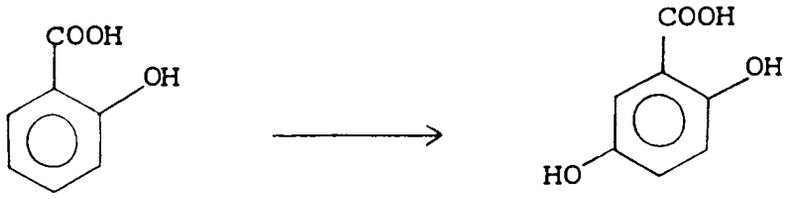
(59) (37)

A strain of Trichosporon cutaneum metabolises p-hydroxybenzoic acid via protocatechuic acid (52) and hydroxyquinol (38).[72]



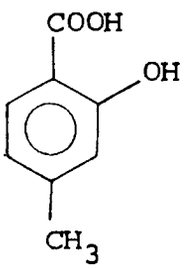
(52) (38)

A strain of Aspergillus niger transformed salicylic acid (53) to 2,3-dihydroxybenzoic acid.[73] Yano and Arima have isolated a bacteria which can convert 3-hydroxybenzoic acid to 2,5-dihydroxybenzoic acid.[74] Using a Pseudomonas sp. salicylic acid was converted to gentisic acid (60).[75] Some strains of Streptomyces sp. also metabolise salicylic acid to gentisic acid.[76]

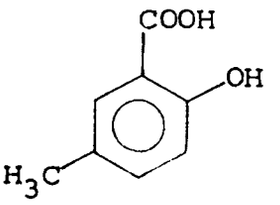


(53) (60)

In 1989, Engelberts et al. reported that a Pseudomonas sp. degraded 4- and 5-methylsalicylic acids (61, 62) by the m-cleavage pathway.[77]

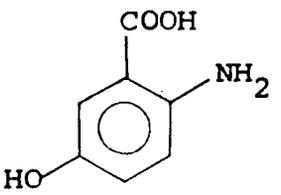


(61)



(62)

Nocardia opaca metabolises 5-hydroxyanthranilic acid (63) to gentisic acid which is degraded to maleyl- and formylpyruvates.[78] The dehydroxylation of 5-hydroxy-anthranilic acid to anthranilic acid (64) with Escherichia coli is a very rare reaction.[79]



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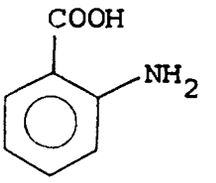
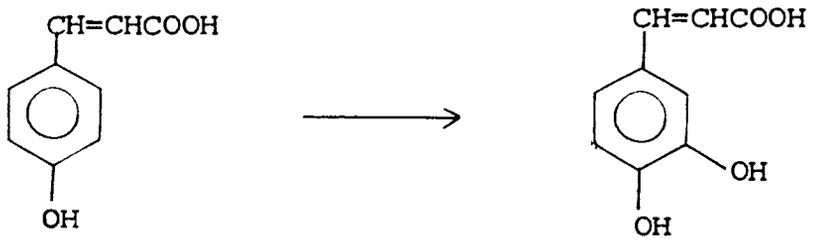


(64)

Pseudomonas testosteroni oxidises 3- and 4-hydroxybenzoic acids to 3,4-dihydroxybenzoic acid.[80] A strain of Lentinus lepideus oxidises 4-hydroxycinnamic acid (65) to 3,4-dihydroxycinnamic acid (66).[81]

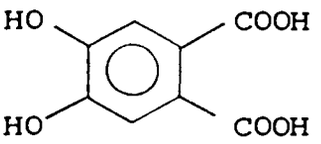


(65) (66)

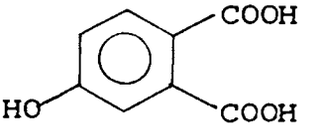
Salicylic acid can microbially undergo oxidative decarboxylation to catechol.[82] The fungus Valsa friesii can decarboxylate 6-methylsalicylic acid (67) to m-cresol.[83]

(67)

In 1959, Ribbons and Evans prepared a cell-free enzyme system from phthalate-grown pseudomonad cells which could decarboxylate 4,5-dihydroxyphthalic acid (68) under anaerobic conditions to protocatechuic acid.[84] In 1978, Nakazawa and Hayashi isolated a strain of Pseudomonas testosteroni which could convert 4-hydroxyphthalic acid (69) to 4,5-dihydroxyphthalic acid.[85]

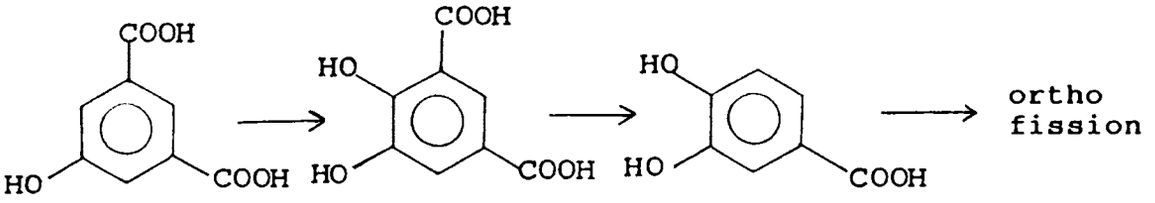


(68)



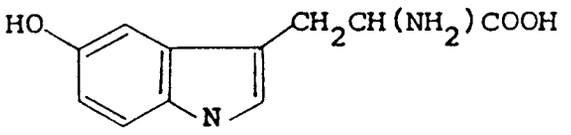
(69)

Elmorsi and Hopper have described a catabolic pathway for the utilisation of 5-hydroxyisophthalic acid (70) by a Coryneform sp. through (71).[86]

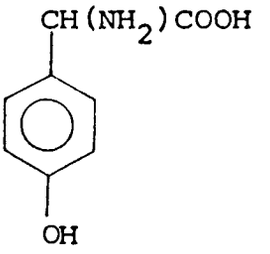


(70) (71)

In 1985, Showa Denka reported that 5-hydroxy-L-tryptophan (72) can be produced from 5-hydroxyanthranilic acid with Bacillus subtilis.[87] Vanden Tweel has studied the involvement of an enantioselective trans-aminase in the metabolism of D-3- and D-4-hydroxyphenyl glycine (73) in Pseudomonas putida.[88] Both D-3- and D-4-hydroxyphenyl-glycines are converted to the corresponding hydroxyphenyl-glyoxylates.

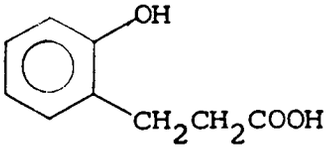


(72)

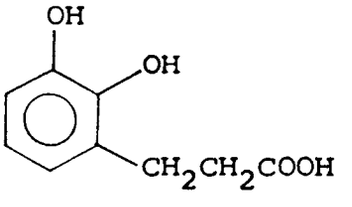


(73)

The enzyme preparation from an Arthrobacter sp. can oxidise melilotic acid (74) to its 3-hydroxylated derivative (75).[89]

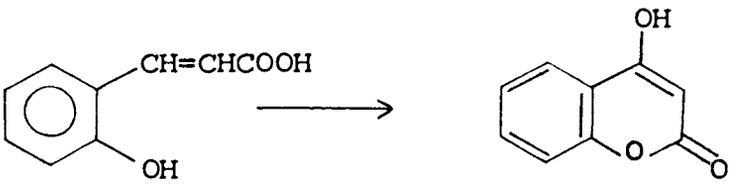


(74)

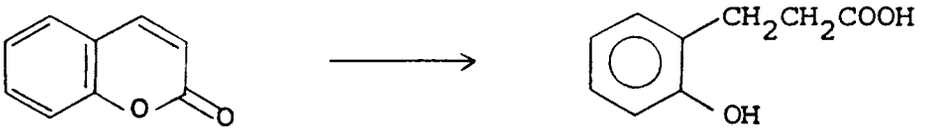


(75)

Several microorganisms oxidise o-coumaric acid (76) to 4-hydroxycoumarin (77).[90] A strain of Aspergillus niger converts coumarin (78) to melilotic acid (74).[91]

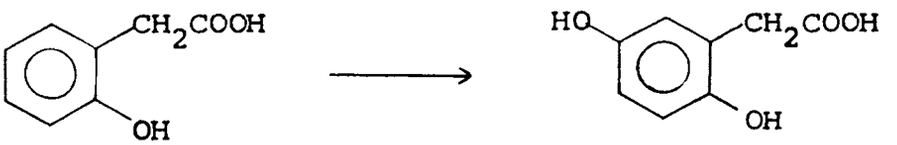


(76) (77)



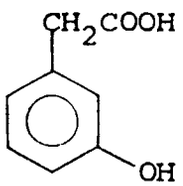
(78) (74)

Dagley et al. in 1952, oxidised o-hydroxyphenylacetic acid (79) to homogentisic acid (80) with a Vibrio sp.[92]

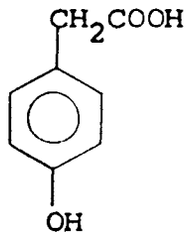


(79) (80)

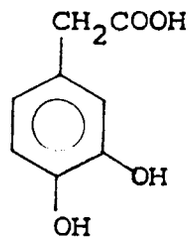
Cooper and Skinner have studied the catabolism of 3- and 4-hydroxyphenylacetic acids (81, 82).[93] Both compounds were catabolised by the same pathway with 3,4-dihydroxyphenylacetic acid (83) as the substrate for the fission of the benzene nucleus, by strains of E.coli.



(81)

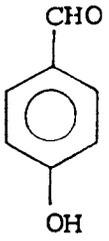


(82)

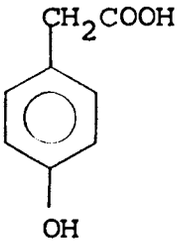


(83)

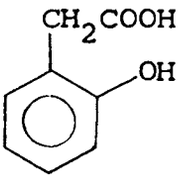
The utilisation of p-hydroxybenzaldehyde (84) and p-hydroxyphenylacetic acid as the sole carbon and energy source by Pseudomonas solanacearum was studied by Arunakumari and Mahadevan.[94] Dugan and Golovlov have proposed a pathway for the metabolism of 2-hydroxyphenylacetic acid (81) via homogentisic acid.[95]



(84)

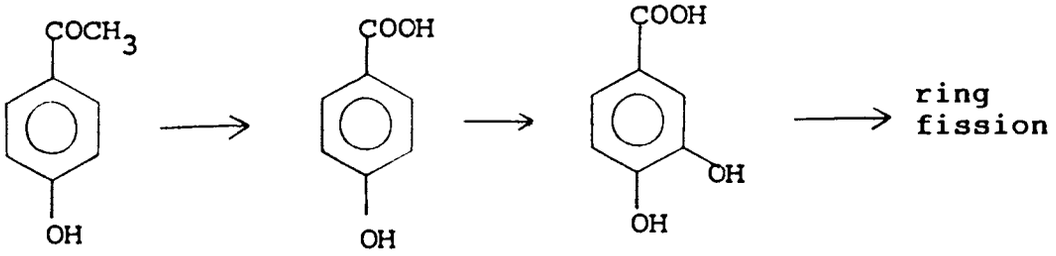


(82)



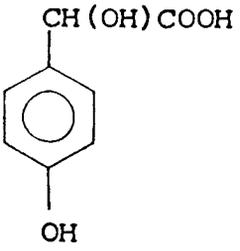
(79)

In 1985, David J.Hopper and coworkers reported the metabolism of 4-hydroxyacetophenone (85) and proposed a pathway for its degradation with an Alcaligenes sp.[96]

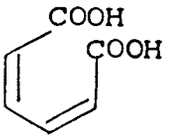


(85)

Chen and his colleagues have studied the degradation of 4-hydroxymandelic acid (86) with Rhizobium leguminosarum to cis,cis-muconic acid (39).[97]



(86)

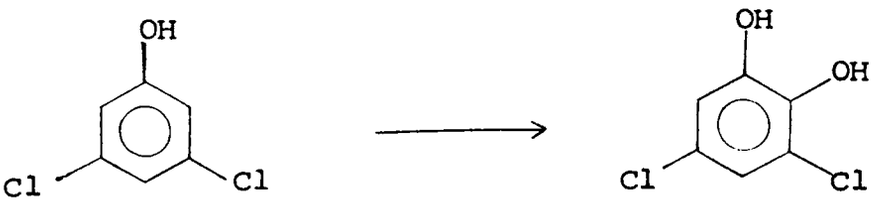


(39)

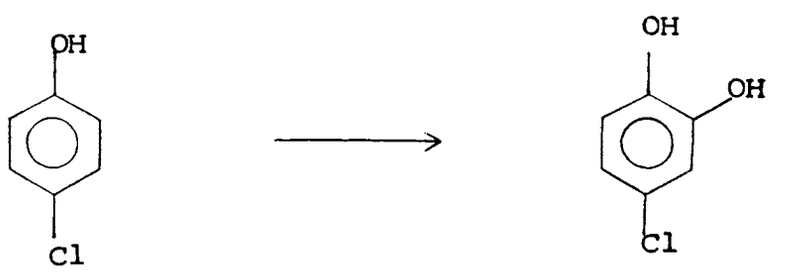
2.3.4 Halogen, nitro and amino substituted phenols

In view of their applications as herbicides, the microbial conversion of halogen substituted phenols and their derivatives has been studied in detail.

In 1968, Bollag et al. reported the following hydroxylation reactions of (87) and (88) with microorganisms.[98]

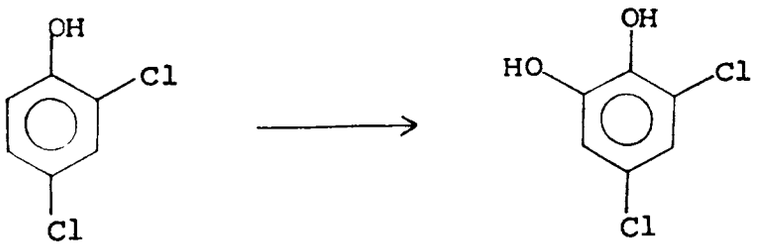


(87)

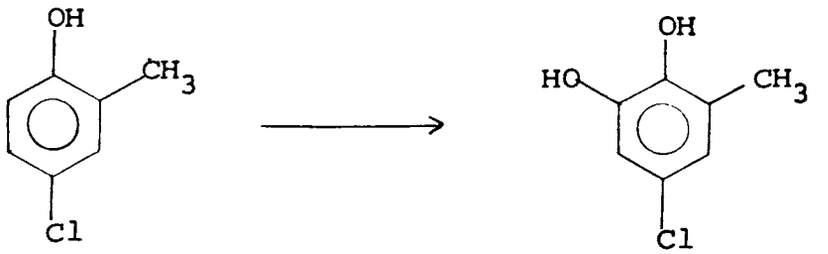


(88)

An Achromobacter strain when grown on 2,4-dichlorophenol (89) or 2-methyl-4-chlorophenol (90) oxidised it to 3,5-dichlorocatechol (91) or 3-methyl-5-chlorocatechol (92), respectively.[99]

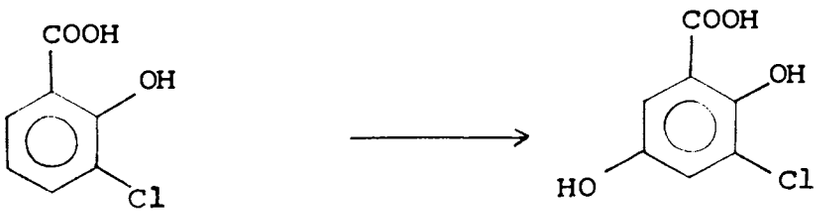


(89) (90)



(91) (92)

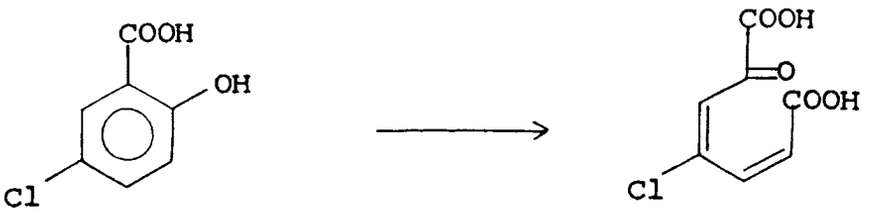
A Nocardia sp. converts 3-chlorosalicylate (93) to 3-chlorogentisate (94) which then undergoes oxygenative cleavage.[100]



(93) (94)

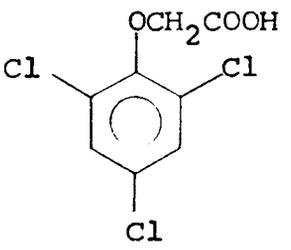
Bacillus brevis degrades 5-chlorosalicylate (95) by a pathway, which does not follow the general schemes in

which prior to ring fission the aromatic nucleus must be substituted with atleast two hydroxy groups.[101]

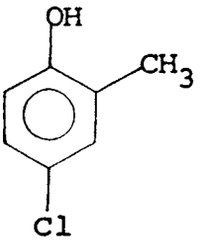


(95)

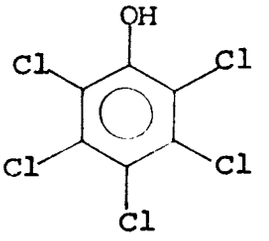
In 1974, Spokes and Walker studied the co-metabolism of chlorophenol and chlorobenzoic acid by a Pseudomonas sp. and Bacillus sp.[102] A pure culture of Pseudomonas cepacia completely or partially dehalogenates a wide variety of halophenols and 2,4,5-trichlorophenoxy acetic acid (96).[103] In 1989, Haggblom et al. reported that a strain of Rhodococcus sp. was able to o-methylate chlorophenols to the corresponding anisoles (97).[104] The degradation kinetics of pentachlorophenol (98) by certain strains of bacteria have also been reported.[105]



(96)

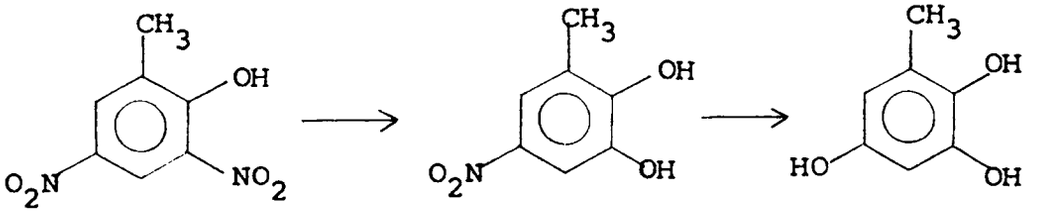


(97)



(98)

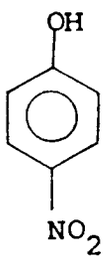
Tewfik and Evans have reported the degradation of the herbicide 3,5-dinitro-o-cresol (99) by a Pseudomonas sp.[106]



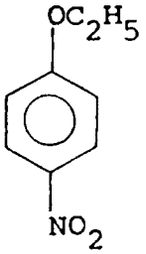
(99)

ring cleavage

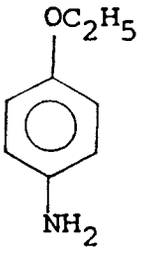
The transformations of p-nitrophenol (100) by Bacillus and Pseudomonas bacteria have been reported. p-Nitrophenol (101) and p-phenetidine (102) are the intermediate products of p-nitrophenol degradation.[107]



(100)



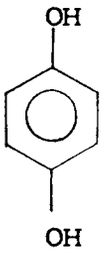
(101)



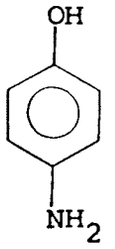
(102)

Iyayi, C.B., has reported the oxidation of p-nitrophenol to hydroquinone (58) by Penicillium chrysogenum.[108]

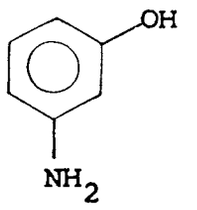
Sloane et al. has reported the metabolism of 4-aminophenol (103).[109] In 1989, Lechner and Straube described the degradation of 3-aminophenol (104) by an Arthrobacter sp. by the β-keto adipate pathway.[110]



(37)

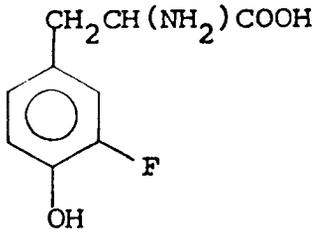


(103)

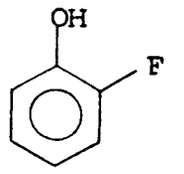


(104)

In 1987, Faleev and his colleagues prepared fluoro-derivatives of L-tyrosine (105) from fluorophenols (106), pyruvic acid and ammonia using Citrobacter intermedius cells in 7-12% poly(vinyl alcohol)cryogel as a biocatalyst.[111]



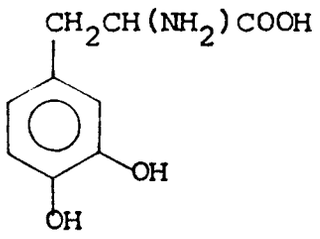
(105)



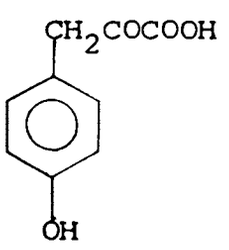
(106)

2.3.5 Tyrosine, tyramine and synephrine

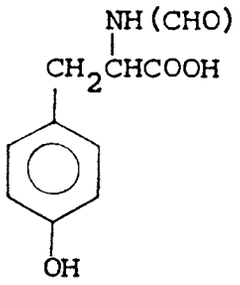
Several microorganisms oxidise suitably derivatised L-tyrosine to L-DOPA (107), which is used in the treatment of Parkinson's disease.[112] Jones and coworkers have reported that a Vibrio strain produced homogentisic acid from tyrosine via p-hydroxyphenylpyruvic acid (108).[113] Singh et al. also have reported a microbial synthesis of L-DOPA from L-tyrosine or N-formyl-L-tyrosine (109) by a pseudomonad mutant strain.[114]



(107)



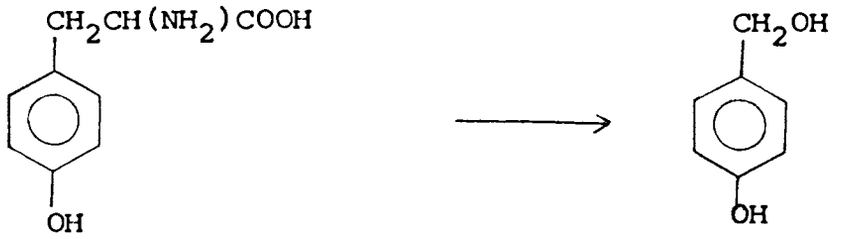
(108)



(109)

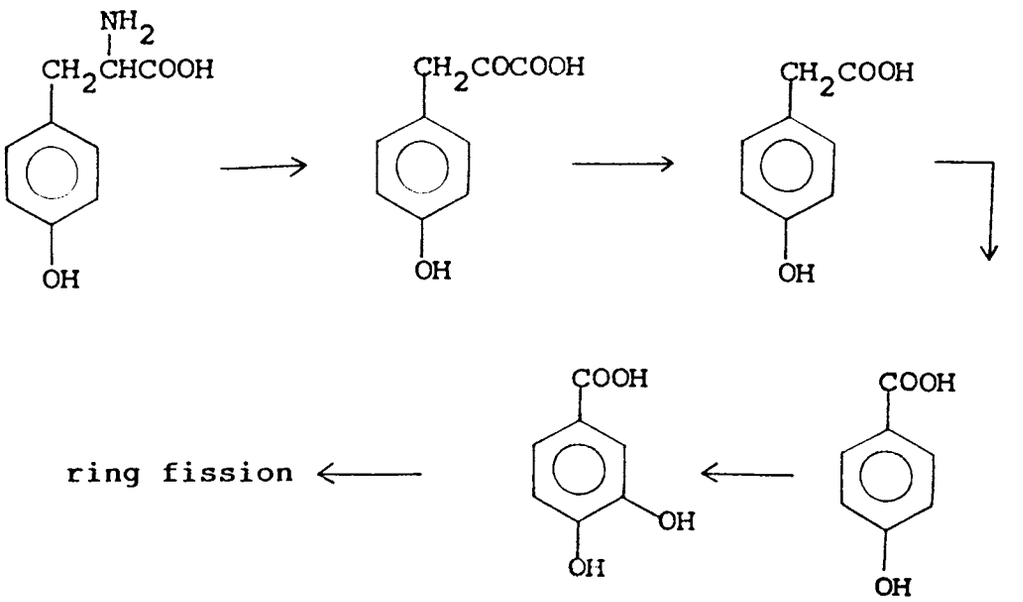
In 1970, Robert H.White prepared 4-hydroxybenzyl alcohol (110) from L-tyrosine with E.coli.[115]

The production of (110) was inhibited by the addition of thiamine to the medium.

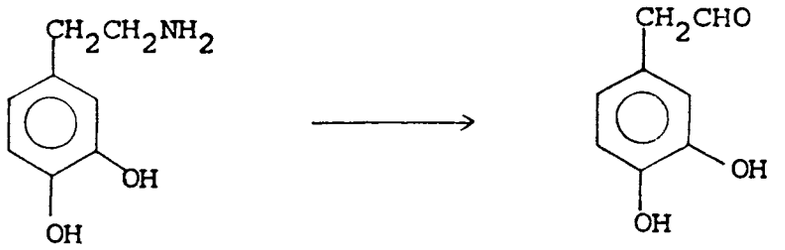


(110)

Lippoldt et al. have proposed a pathway for the degradation of tyrosine, involving p-hydroxyphenylacetic acid and p-hydroxyphenylpyurvic acid with Candida maltosa.[116]

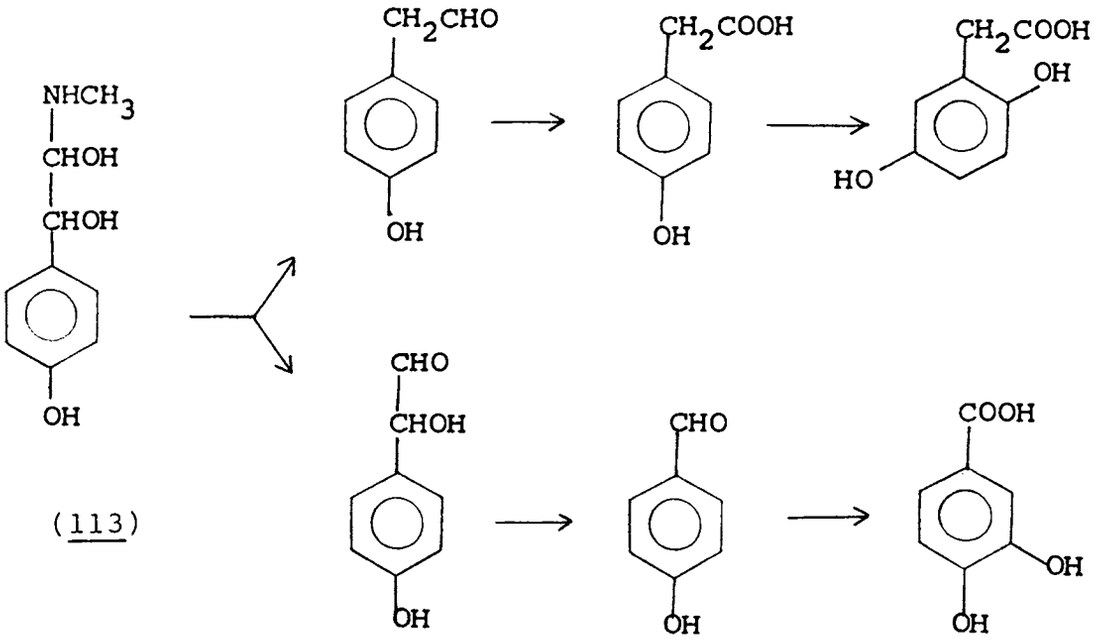


Tyramine oxidase, the enzyme from Sarcina lutea responsible for the oxidation of tyramine (111) to p-hydroxyphenylacetaldehyde (112) has been isolated and crystallised.[117]



(111) (112)

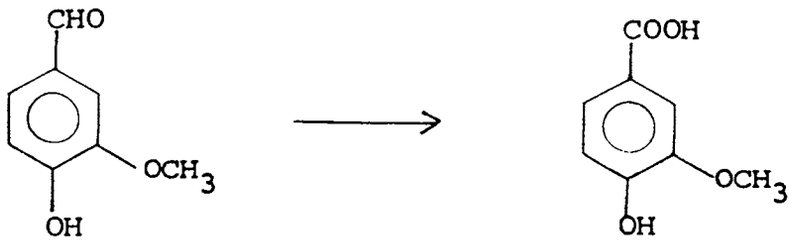
A Nocardia sp. degraded synephrine (113) by two novel routes - one involving monoamine oxidase and the other involving conversion to p-hydroxyphenylacetaldehyde by the synephrinase enzyme.[118]



(113)

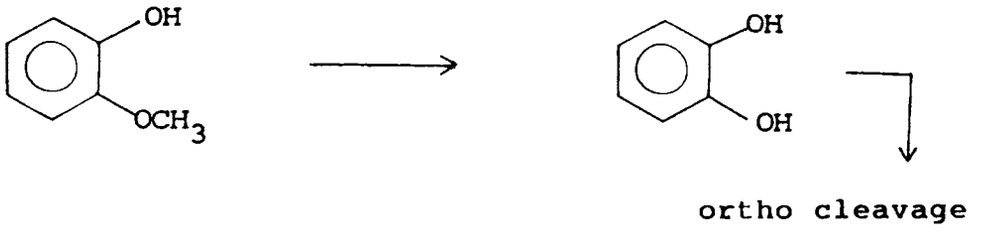
2.3.6 Methoxy substituted phenols

In 1919, Robbins and Lathrop isolated a bacterium from the soil which could oxidise vanillin (114) to vanillic acid (115).[119]



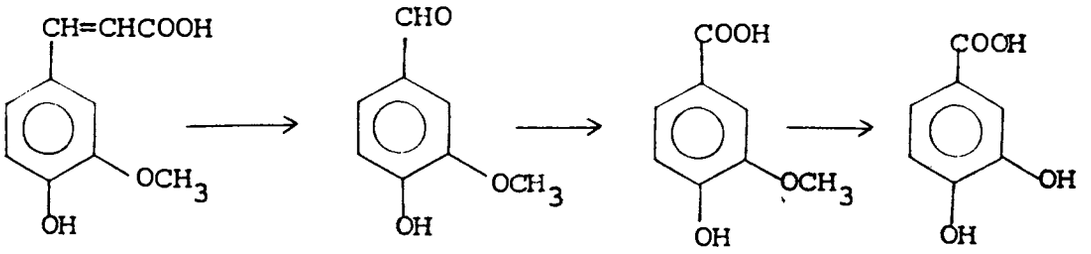
(114) (115)

A strain of Acinetobacter sp. degraded guaiacol (116) by ortho cleavage.[120]



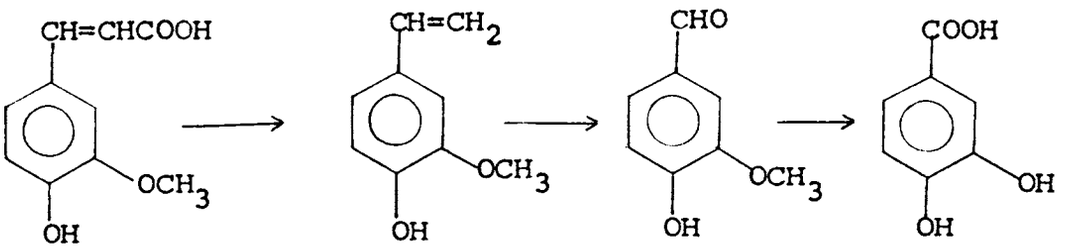
(116)

Washed cell suspensions of Pseudomonas acidovorans degraded ferulic acid (117) to protocatechuic acid via vanillin and vanillic acid.[121]



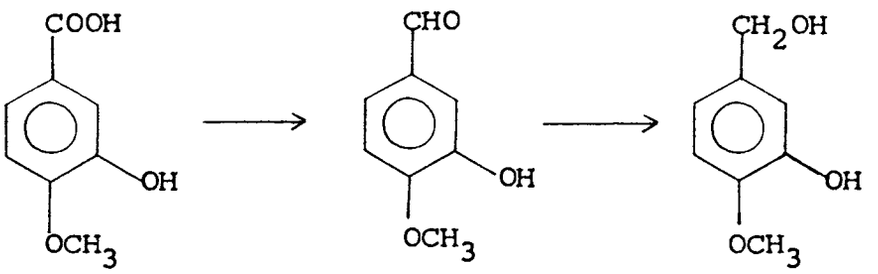
(117)

Iwahara Shojiro has studied the microbial degradation of ferulic acid by different microorganisms.[122] One strain of bacteria converted ferulic acid to vanillic acid whereas Rhodotorula and Fusarium sp. metabolised the acid completely. Nazareth and Mavinkurve have reported that a Fusarium solani strain metabolises ferulic acid to a transient intermediate, 4-vinylguaiacol, (118) which then undergoes further degradation.[123]



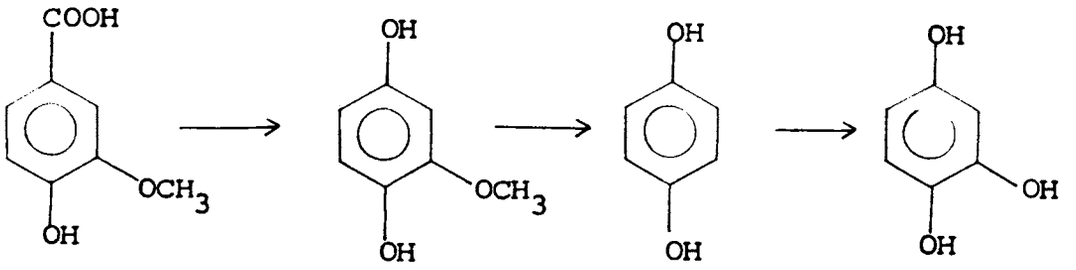
(118)

A wood destroying fungi, Polystictus versicolor can transform vanillic acid and isovanillic acid (119) to their aldehydes and alcohols.[124]

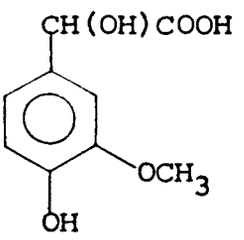


(119)

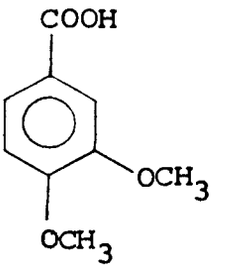
In 1983, Paul Ander *et al*. proposed a new pathway for the metabolism of vanillic acid by *Sporotrichum pulverulentum*.[125]



A strain of *Acinetobacter lwoffi* degrades 4-hydroxy-3-methoxymandelic acid (*120*) to the benzoate which is then hydroxylated to yield protocatechuic acid.[126] A mutant strain of *Pseudomonas* sp. transforms veratric acid (*121*) to vanillic acid and isovanillic acid.[127]

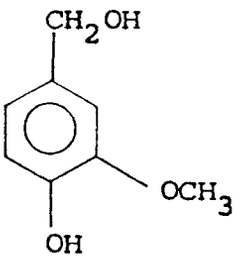


(*120*)

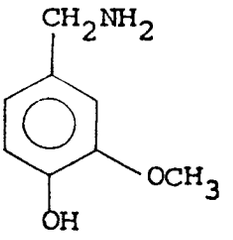


(*121*)

DeWulf and his colleagues have reported that a Saccharomyces cerevisiae strain could reduce vanillin to vanillyl alcohol (122).[128,129] In 1988, Hisae Asai et al. reported the vanillyl amine (123) metabolism in Pseudomonas fluorescens.[130] Under growing conditions the cells metabolised vanillyl amine to vanillin, vanillic acid and vanillyl alcohol. Under non-growing conditions, vanillin, vanillic acid and protocatechuic acid were formed.

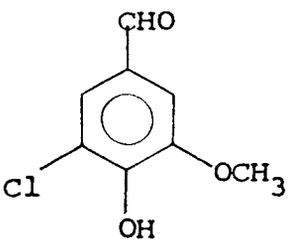


(122)

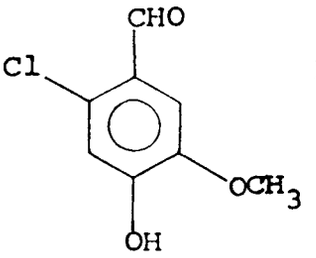


(123)

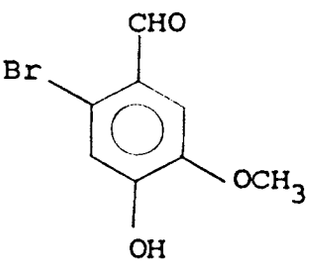
The transformations of 5- and 6-chlorovanillin (124, 125) and 6-bromovanillin (126) to the corresponding acids have been reported.[131]



(124)

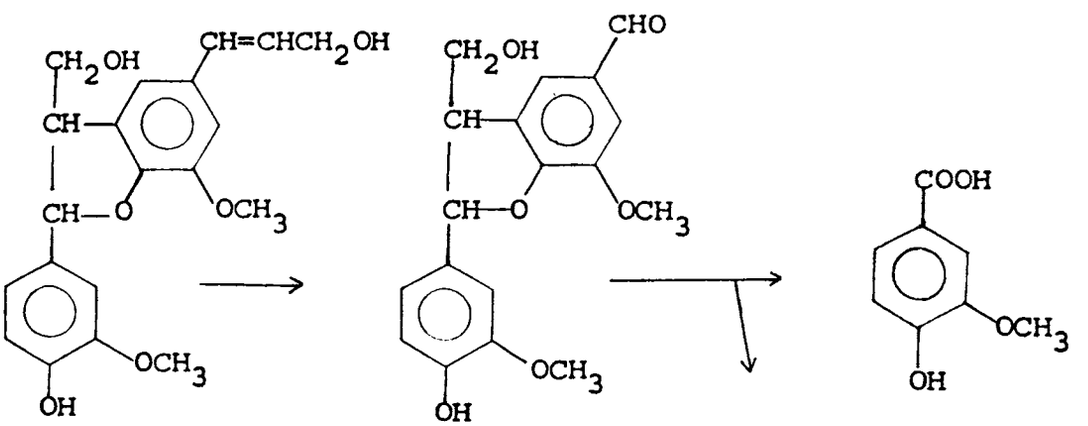


(125)

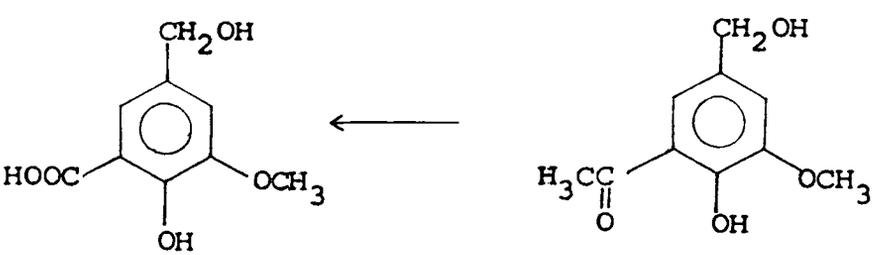


(126)

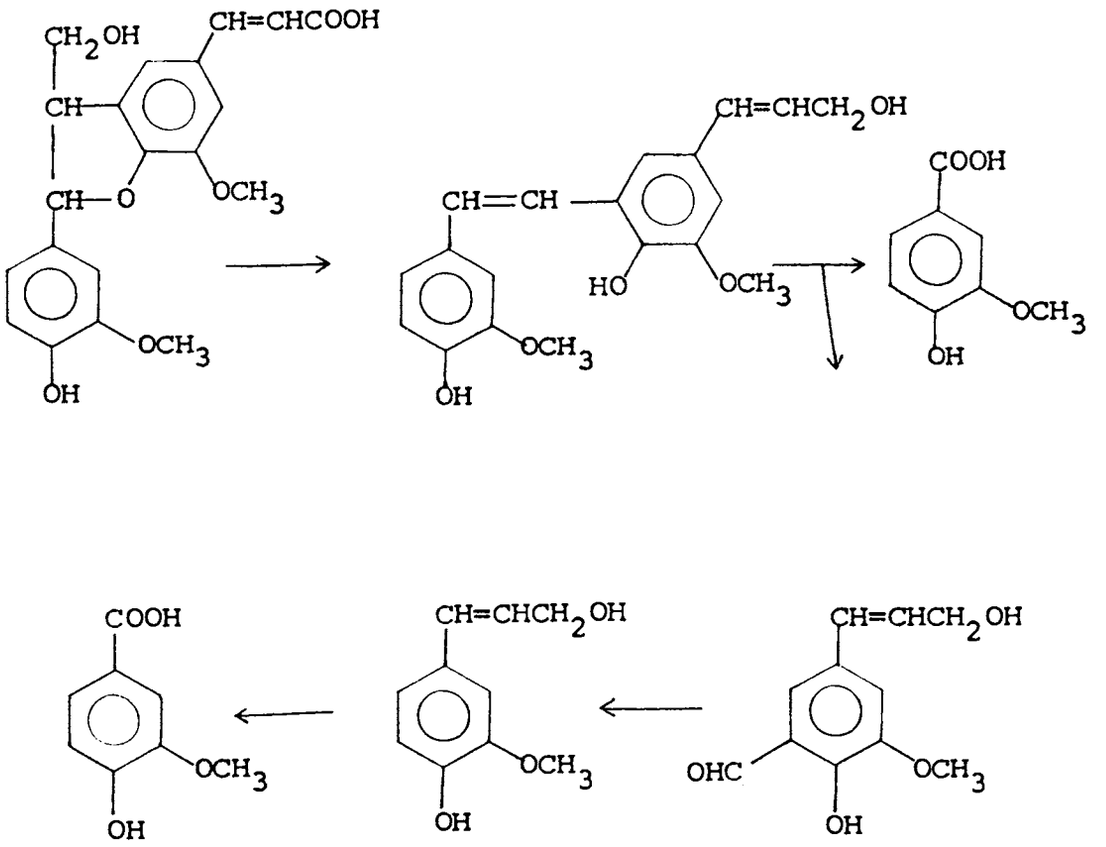
Ohta et al. have reported the partial metabolism of dehydrodiconiferyl alcohol (127) by Fusarium solani.[132]



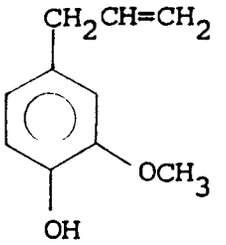
(127)



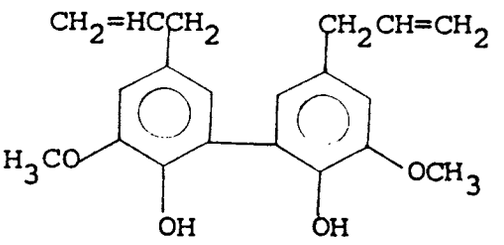
In 1988, Habu and coworkers reported the metabolic pathway of dehydrodiconiferyl alcohol by a Pseudomonas sp.[133]



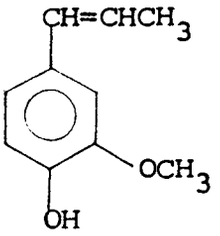
A glycerol maceration of Russula delica converted eugenol (128) to bisdehydroeugenol (129)[134,135] and isoeugenol (130) to dehydrodiisoeugenol (131).[136,137]



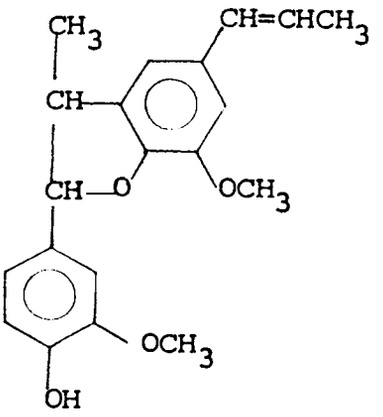
(128)



(129)



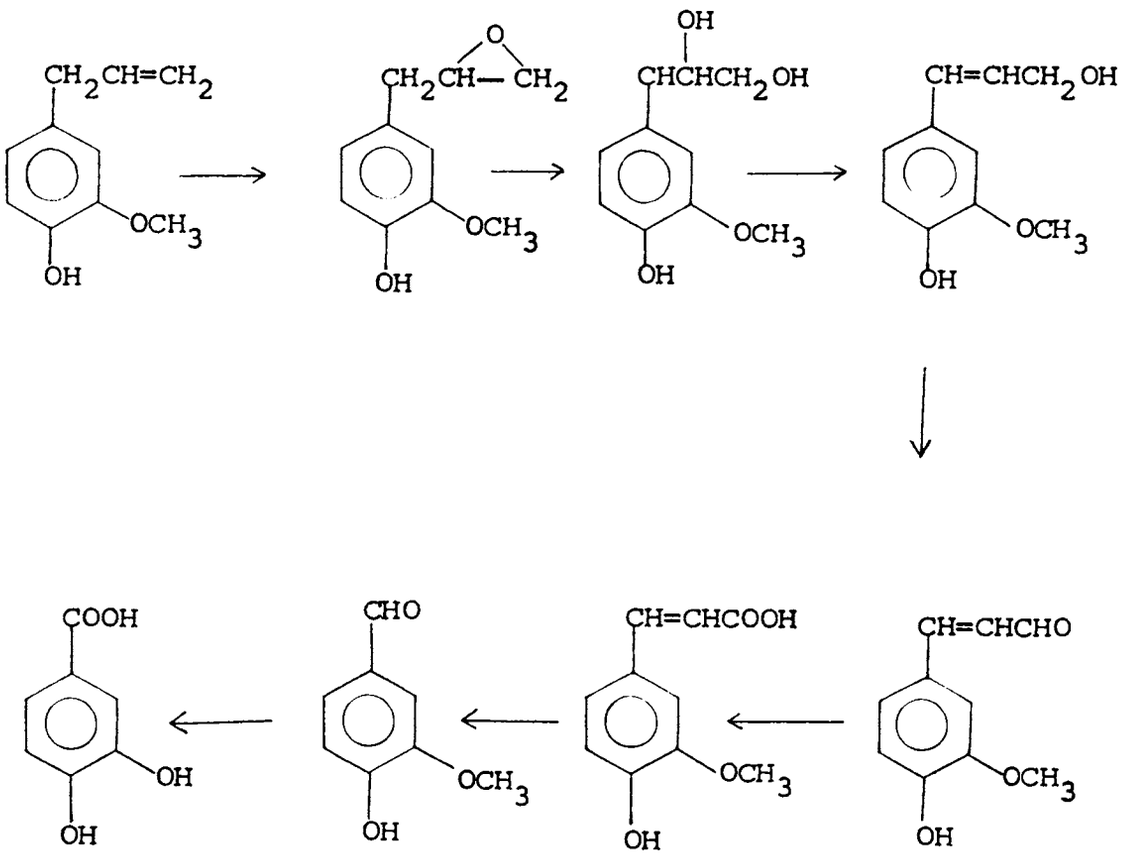
(130)



(131)

Kohji Tadasa has reported that a Corynebacterium sp. degraded eugenol to give vanillin, and vanillic, ferulic and protocatechuic acids.[138] A Pseudomonas sp. when grown on eugenol included more metabolites than with the

Corynebacterium sp., but this strain could not degrade isoeugenol, an isomer of eugenol.[139]



In 1986, Nazareth and Mavinkurve reported that eugenol was transformed to 4-vinyl guaiacol with a Fusarium solani strain, which degraded further to protocatechuic acid.[123]

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 CHEMICALS

Eugenol, eugenol acetate, bisdehydro eugenol, isoeugenol, isoeugenol acetate, bisdehydro isoeugenol, dehydrodiisoeugenol, ferulic acid, vanillin, vanillic acid and protocatechuic acid were used as the substrates. Ferulic acid was obtained as a gift from Dr.Kohji Tadasa, Shinshu University, Japan and vanillin was purchased from Sisco Research Laboratories, Bombay.

3.1.1 Isolation of eugenol from clove oil

Eugenol was extracted from commercially available clove oil by treating with potassium hydroxide.[140] Into a well cleaned 250 cc round bottom flask, 10 ml of clove oil was introduced. To this 75 ml aqueous 1 N potassium hydroxide solution was added and shaken thoroughly for 5 minutes. Then it was heated on a water bath with occasional shaking for 10 minutes. The flask was cooled and the contents of the flask were poured into a separatory funnel and the non-phenolic portion was separated. The aqueous layer was filtered through a filter paper and the

filtered solution was acidified with dilute hydrochloric acid until the mixture was strongly acid to litmus. This was transferred to a separatory funnel and extracted with ether. The ether extract was dried with anhydrous sodium sulphate, filtered and evaporated under reduced pressure to get 8.5 g of eugenol as a pale yellow liquid, boiling at 255°C.[141]

3.1.2 Preparation of eugenol acetate

Into a dry 50 ml RB flask 1.5 ml eugenol, 4 ml pyridine and 3 ml acetic anhydride were placed. It was kept for 6 hours, shaking intermittently. The mixture was then poured into ice-cold water, stirring continuously. It was extracted with ether and the ether extract was washed several times first with 1 N hydrochloric acid and then with 5% sodium bicarbonate solution. It was dried with anhydrous sodium sulphate and evaporated under vacuum to get 2.14 g of eugenol acetate. Eugenol acetate is a pale yellow liquid freezing at 30°C.[142]

3.1.3 Preparation of isoeugenol

Isoeugenol was prepared by the reaction of eugenol with potassium hydroxide.[143] In a 250 ml RB flask 10 g

eugenol was mixed with 4.5 g KOH in 10 ml of water. Water was then removed at 80-90°C under reduced pressure. Three gram diethylene glycol and 1 g triethanolamine were added to it and the mixture was heated for about 5 minutes at 160°C. The melt was cooled , run into water and neutralised with dilute sulphuric acid. It was extracted with benzene and the benzene extract was distilled under reduced pressure to yield 9 g isoeugenol as a pale yellow liquid, boiling at 266°C.[141]

3.1.4 Preparation of bisdehydroeugenol

In a 250 ml RB flask, 4 ml eugenol in 20 ml of ethanol and 6 g ferric chloride in 30 ml of water were placed and shaken well. The mixture was cooled and the precipitate was filtered and washed with 40% ethanol. It was recrystallised from ethanol to give 1.62 g bisdehydro-eugenol having a melting point of 106°C.

3.1.5 Preparation of isoeugenol acetate

Into a dry 50 ml RB flask 1.5 ml isoeugenol, 4 ml pyridine and 3 ml acetic anhydride were placed. It was kept for 6 hours shaking occasionally. The mixture was poured into ice-cold water, stirring continuously. It was filtered

and the precipitate was washed with 1 N hydrochloric acid followed by 5% $NaHCO_3$ solution to give 2.31 g of isoeugenol acetate melting at 79°C.[142]

3.1.6 Preparation of bisdehydroisoeugenol and dehydrodiisoeugenol

In a 250 ml RB flask, 5 g isoeugenol in 30 ml of ethanol and 7 g ferric chloride in 30 ml of water were placed and shaken well for 5 minutes. Ethanol was distilled off under reduced pressure and the mixture was cooled. It was transferred to a separatory funnel and extracted with ether. The ether extract was dried with anhydrous sodium sulphate, filtered and concentrated under reduced pressure. It was subjected to column chromatography as will be described under section 3.5.3. 0.70 g bisdehydroisoeugenol melting at 158°C and 1.48 g dehydrodiisoeugenol melting at 132°C were obtained. Both products were recrystallised from ethanol.

3.1.7 Preparation of vanillic acid

Vanillic acid was prepared by fusing vanillin with potassium hydroxide and sodium hydroxide under controlled conditions.[144] In a stainless steel beaker of 250 ml

capacity equipped with a stirrer and heated by an electric hot plate, 1.8 g of sodium hydroxide pellets, 1.8 g of potassium hydroxide pellets and 1 ml water were placed. The mixture was stirred well and heated to 160°C. The hot plate was turned off and 1.5 g of vanillin was added in portions at a rate sufficient to maintain the reaction temperature at about 190°C. Stirring was continued for 5 more minutes, the hot plate was removed and the mixture allowed to cool with stirring. When the temperature of the mixture came down to about 150°C, 10 ml water was added and stirred well. The mixture was then cooled to room temperature and acidified with 15 ml 6 N hydrochloric acid keeping in an ice bath and stirring. The ppt. formed was filtered, washed with water and dried. It was recrystallised from water-acetone mixture which yielded, 1.42 g vanillic acid melting at 210°C.

3.1.8 Preparation of protocatechuic acid

Protocatechuic acid was prepared by fusing vanillin with sodium hydroxide and potassium hydroxide.[145] In a stainless steel beaker of 250 ml capacity, equipped with a stirrer and heated by a hot plate 1 g NaOH pellets, 3.5 g KOH pellets and 1 ml water were placed. The mixture was stirred and heated to 160°C. Then 1.6 g vanillin was

added in portions at a rate to maintain the temperature at about 190°C, stirring simultaneously. Heat was applied until the temperature reached 250°C. Temperature was kept at 250°C for 5 minutes, the hot plate was removed and the mixture allowed to cool with stirring. At about 150°C, 10 ml water was added to dissolve the mixture. It was allowed to cool to the room temperature and acidified with 15 ml 6 N hydrochloric acid. The mixture was cooled in an ice bath for 2 hours, filtered and the precipitate was washed with ice water. It was dried and recrystallised from water-acetone mixture to yield 0.95 g protocatechuic acid melting at 200°C.

3.2 SOURCE OF MICROORGANISMS

As the stock cultures in our laboratory were found to be inactive on eugenol, microorganisms that transform eugenol were isolated from the local soil using the elective culture technique. As mentioned previously, one of the organisms used in this study, Pseudomonas aeruginosa was obtained as a gift from Dr.Kohji Tadasa.

3.2.1 Screening of soil samples

The screening of the soil samples was done in the following mineral salts medium.

NH_4NO_3	:	1 g
KH_2PO_4	:	0.9 g
Na_2HPO_4	:	0.55 g
$MgSO_4 7H_2O$	:	0.2 g
$CaCl_2 2H_2O$	:	0.1 g
$FeSO_4 7H_2O$	:	0.1 g
Tap water	:	1000 ml
pH	:	7.2

For preparing mineral broth 0.1% (v/v) eugenol/ isoeugenol was added as the carbon source (unless otherwise specified). To get a solid medium, 2% (w/v) agar was added to the above broth.

About 2 g of soil, collected from the natural habitat of clove trees was suspended in 100 ml of sterile tap water and shaken thoroughly. It was allowed to settle and 10 ml of the supernatent was transferred to 100 ml of

the mineral medium containing eugenol/isoeugenol as the sole carbon source. This was incubated at 30°C for 2 days on a rotary shaker. One ml of the broth was then transferred to another 100 ml of the mineral medium containing eugenol/ isoeugenol and incubated at 30°C. After 2 days 1 ml of the broth was pour-plated in sterile petri dishes with eugenol-mineral agar (EMA)/isoeugenol-mineral agar (IMA) medium. The plates were incubated at 30°C for 2 days. The colonies developed on the plates were isolated and purified by streaking repeatedly over fresh EMA/IMA plates. Two strains of bacteria, one growing on EMA and the other growing on IMA slants, were obtained. The pure strains designated as CUAC 20 and CUAC 30 respectively, were transferred to EMA and IMA slants respectively.

3.2.2 Isolation of the strain CUAC 10

The strain CUAC 10 was isolated as a chance contaminant in a mineral medium containing eugenol and glucose. The broth was extracted with ether, after acidi-fying it to pH 2. The ether extract was concentrated and subjected to thin layer chromatography studies, which showed the presence of one more spot other than that of eugenol.

This strain CUAC 10 was also able to transform isoeugenol in a mineral medium containing isoeugenol and glucose. But this strain was not able to grow on EMA or IMA plates. So it was purified by streaking repeatedly over nutrient agar plates and the pure strain was transferred to nutrient agar slants.

3.2.3 Maintenance and preservation of the cultures

The culture CUAC 10 was maintained on nutrient agar slants and nutrient agar under oil.[146] The cultures CUAC 20 and Pseudomonas aeruginosa were maintained on EMA slants and CUAC 30 was maintained on IMA slants. The media were sterilised by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The cultures were transferred to appropriate slants and incubated for one day before storing them in refrigerator. They were transferred to fresh slants every month to keep them viable.

3.3 IDENTIFICATION OF THE MICROORGANISMS

The isolated organisms were identified to the generic level based on their morphological and biochemical characters.[147,148]

3.3.1 Morphological characters

These tests included the cell morphology, gram staining, spore staining, chromogenesis and motility. Chromogenesis was tested by growing the cultures on nutrient agar. Motility was tested by the hanging drop method.

3.3.2 Biochemical characters

Biochemical characterisation included tests for production of indole, utilisation of citrate, reduction of nitrate, hydrogen sulphide production, gas production from glucose, oxidation-fermentation test, cytochrome oxidase activity, catalase activity and ability to produce hydrolytic enzymes namely gelatinase, amylase and caseinase.[149]

3.4 GROWTH STUDIES OF THE ISOLATED MICROORGANISMS

The medium used for the studies with the strain CUAC 10 was a mineral medium containing 1% (w/v) glucose and 0.1% (v/v) eugenol/isoeugenol (unless otherwise specified). The medium used for the studies with the strains (CUAC 20) and Pseudomonas aeruginosa was mineral medium containing 0.1% (v/v) eugenol and that used for CUAC 30 was a mineral

medium containing 0.1% (v/v) isoeugenol. The composition of the mineral medium was that described under section 3.2.1.

3.4.1 Preparation of inoculum

A loopful of the culture from the agar slants was transferred to 100 ml mineral broth and incubated at 30°C for 24 hours. The turbidity of the culture was then observed at 600 nm and the absorbance was adjusted with fresh mineral broth to give a final absorbance of 1.0. This cell suspension, in quantities of 2 ml/100 ml broth, was used as the inoculum.

3.4.2 Incubation procedure

To 10 ml quantity of mineral broth in each test tube, which was autoclaved at 15 lbs pressure for 15 minutes, 0.2 ml of the inoculum was added. These test tubes were incubated at 30°C for a period of 24 hours on a rotary shaker (unless otherwise specified). Controls were maintained for all experiments. All the experiments were done in duplicate.

3.4.3 Measurement of growth

The growth was measured in terms of turbidity. The turbidity formed in the inoculated tubes due to the growth

of the bacteria was measured at 600 nm in a UV-visible spectrophotometer (Hitachi Model 200-20) at the end of 24 hours (unless otherwise stated). Growth was expressed as optical density (OD) values.

3.4.4 Effect of concentration of eugenol/isoeugenol

The effect of concentration of eugenol/isoeugenol on the growth of the bacteria was tested by inoculating the test strain of bacteria in mineral broth adjusted to various concentrations of eugenol/isoeugenol, viz., 0.1 to 0.25% (v/v). The dispensed medium was inoculated, incubated and the growth was measured.

3.4.5 Effect of pH

The effect of pH on the growth of bacteria was tested in mineral broth prepared after incorporating the substrate at its optimal concentration and adjusting it to various levels of pH from 5 to 10. The growth was measured after inoculating the broth and incubating for 24 hours.

3.4.6 Effect of concentration of sodium chloride

By incorporating various concentrations of sodium chloride from 0 to 10% (w/v), 10 ml aliquots of mineral

broth were prepared. The growth was measured after inoculating the test strains and incubating it.

3.4.7 Effect of different nitrogen sources

Ammonium nitrate in the mineral broth was replaced with ammonium chloride, ammonium sulphate, urea, potassium nitrate and sodium nitrate in concentrations giving equal nitrogen content in the medium and 10 ml aliquots were prepared. Cultivation was carried out with the cultures and the growth was measured after incubation for 24 hours.

3.4.8 Effect of temperature

Ten ml aliquots of the mineral broth adjusted to optimal conditions were prepared and inoculated with the test strains. These were incubated at various temperatures viz., 15° to 60°C and the growth was measured.

3.5 ANALYTICAL METHODS

3.5.1 Extraction procedure

After fermentation, the broth was centrifuged at 6000 rpm for 15 minutes. The supernatent was acidified with

2 N sulphuric acid to pH 2 and exhaustively extracted with solvent ether. The extracts were pooled, dried with anhydrous sodium sulphate and concentrated under reduced pressure. The residue was subjected to chromatographic and spectrophotometric studies.

3.5.2 Thin layer chromatography

Thin layer chromatography (TLC) was carried out with silica gel plates of 0.25 mm thickness prepared on glass plates with a spreader. The plates were developed with the following solvent systems.

i) Chloroform
ii) Ethyl acetate : Hexane - 3:7
iii) Benzene : Dioxane : Acetic Acid - 90:25:4

Spots were detected either by exposing the plates to iodine vapours or by spraying them with appropriate reagents.[150]

3.5.3 Column chromatography

Column chromatography was performed with 60-120 mesh silica gel (unless otherwise specified). The eluant

used was either chloroform : hexane system or ethylacetate : hexane system, depending on the nature of the substrate as observed from TLC. As elution progressed, fractions yielding the same compound as shown by TLC were pooled and distilled under reduced pressure to get the product.

3.5.4 High pressure liquid chromatography

High pressure liquid chromatography (HPLC) 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 and refractive index detector were used.

Standard solutions of the substrate in methanol were prepared and 10 μl 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 calibration curve was drawn from which concentration of the substrate was computed.

3.6 EFFECT OF PHYSICOCHEMICAL FACTORS ON TRANSFORMATION

The effect of physicochemical factors namely pH, temperature, different nitrogen sources and concentrations of sodium chloride and glucose on the rate of transformation of eugenol/isoeugenol by the bacterial strains was studied. For the studies with the strain CUAC 10, 50 ml aliquots of mineral broth in 250 ml Erlenmeyer flasks supplemented with 1% (w/v) glucose and 0.1% (v/v) eugenol/isoeugenol (unless otherwise stated) were used. For the studies with the strains CUAC 20 and Pseudomonas aeruginosa, 50 ml aliquots of mineral broth supplemented with 0.1% (v/v) eugenol and for the studies with the strain CUAC 30, mineral broth supplemented with 0.1% (v/v) isoeugenol were used. The mineral medium used was the same as that described under section 3.2.1. The medium was autoclaved at 15 lbs pressure for 15 minutes before cultivation and preparation of inoculum was the same as described under section 3.4.1. The inoculated flasks were incubated at 30°C for specific periods of 6, 12, 18, 24 and 36 hours on a rotary shaker (220 rpm). All experiments were done in duplicate and controls were kept for all experiments.

After incubation for specific intervals of time, the broth was centrifuged and the supernatent was acidified. It was extracted with ether and the ether extract was evaporated to dryness. The residue was then estimated for residual eugenol/isoeugenol.

3.6.1 Estimation of eugenol/isoeugenol

Standard solutions of eugenol/isoeugenol in methanol were prepared and the absorbance values at 281/260 nm were measured in a spectrophotometer. Standard curves were then drawn each for eugenol and isoeugenol. From the absorbance values of the test solutions the concentrations of eugenol/isoeugenol were then estimated.

3.6.2 Effect of concentration of sodium chloride

The effect of concentration of NaCl on transformation of eugenol/isoeugenol was tested in mineral broths having various sodium chloride concentrations, viz., 0 to 1% (w/v). After inoculation the flasks were incubated for specific intervals of time for each concentration of sodium chloride. The contents of the flasks were then analysed for residual eugenol/isoeugenol.

3.6.3 Effect of concentration of glucose

Fifty ml aliquots of mineral broth were prepared by incorporating various glucose concentrations from 0.2 to 2% (w/v) in the case of studies with the strain CUAC 10 and from 0 to 1% (w/v) in the case of studies with the strains CUAC 20 and CUAC 30. The flasks containing the broth were inoculated with the test strains, incubated for specific intervals of time and analysed.

3.6.4 Effect of pH

Fifty ml aliquots of mineral broth were prepared and adjusted to pH values ranging from 5 to 9. These were inoculated, incubated and analysed for residual eugenol/ isoeugenol.

3.6.5 Effect of temperature

The flasks containing the mineral broths were inoculated and incubated at different temperatures from 15°C to 60°C. After specific intervals of time, the contents of the flasks were analysed.

3.6.6 Effect of different nitrogen sources

Ammonium nitrate in the mineral broth was replaced with ammonium chloride, ammonium sulphate, urea, potassium nitrate and sodium nitrate in concentrations giving equal nitrogen content in the medium and cultivation was carried out. After incubation for specific intervals of time, the contents of the flasks were analysed for unreacted eugenol/ isoeugenol.

3.7 GROWTH CURVE

By incorporating the optimal conditions, mineral broths were prepared for the three cultures CUAC 10, CUAC 20 and CUAC 30 and the broths were inoculated with the cultures. They were incubated and the growth was measured in terms of optical density at various intervals of time. A graph was plotted with time interval versus optical density for each culture, which formed the growth curve.

3.8 FERMENTATION CONDITIONS

The fermentation was carried out in a mini jar fermentor (Eyela model M-100). A volume of 1.5 litres of mineral broth supplemented with eugenol/isoeugenol was used

for fermentation. In the case of fermentation with the strain CUAC 10, 1% (w/v) glucose was also added to the medium. The fermentor together with the medium was sterilised at 15 lbs pressure for 20 minutes. The fermentation was conducted at pH 7 at 30°C (unless otherwise stated). Continuous aeration at a flow rate of 1 VVM was given with an air pump and a speed of 220 rpm of the impeller was used.

A loopful of the culture was transferred to 10 ml of the mineral broth and incubated for one day. This was inoculated in a 100 ml broth contained in an Erlenmeyer flask and incubated for one day in a shaker. This was again transferred to 400 ml broth in a 1000 ml shaking flask and incubated for 15 hours. The broth was then centrifuged at 10000 rpm for 20 minutes. The cells were separated, washed with physiological saline and this was used as the inoculum for fermentation.

Since eugenol/isoeugenol was strongly antibacterial, cultivations were done by the feeding culture method in which the substrate was added in stages increasing the concentration from 0.01% (v/v).

3.9 ISOLATION AND CHARACTERISATION OF TRANSFORMATION PRODUCTS

After fermentation the broth was centrifuged at 6000 rpm for 15 minutes and the cells were removed. The supernatent was acidified with 2 N sulphuric acid to pH 2. This was exhaustively extracted with solvent ether. The ether extracts were pooled, dried with anhydrous sodium sulphate and filtered. A small portion of the ether extract was subjected to thin layer chromatography with different solvent systems, which gave an idea about the nature and number of products formed during fermentation. The rest of the ether extract was then concentrated under reduced pressure. The residue was subjected to column chromatography. Chloroform-hexane or ethyl acetate-hexane was used as the eluant depending on the nature of the mixture to be separated. The fractions yielding the same product were pooled and concentrated under reduced pressure. The separated components were purified by recrystallisation from appropriate solvents.

The pure compounds were identified by determining the physical constants and chromatographic and spectrometric methods. These included comparing with authentic samples

the melting points, Rf values (TLC) in different solvent systems and UV, IR, NMR and mass spectra. Suitable derivatives of these compounds were also prepared.

3.9.1 Preparation of acetates of the transformation products

Five hundred mg of the compound was dissolved in 1.5 ml pyridine in a 50 ml clean dry round bottom flask and 1 ml acetic anhydride was added to it. The flask was kept for 6 hours shaking intermittently. The mixture was then poured into ice-cold water stirring continuously and filtered. The precipitate was washed several times with 1 N hydrochloric acid followed by 5% sodium bicarbonate solution. The product was then recrystallised from dilute ethanol and the melting point determined.

3.9.2 Preparation of methyl esters of acidic products

In a 50 ml round bottom flask, 700 mg of the acid and 2 ml absolute methanol were placed and 0.2 ml con. sulphuric acid was added cautiously with shaking.[151] A reflux condenser was fitted to the flask and it was refluxed for 3 hours. It was then cooled to room temperature and 10 ml of water was added. The excess of methanol was

distilled off on a water bath under reduced pressure. The flask was cooled and the contents were poured into a separatory funnel. The lower layer of ester was separated and washed with water followed by 5% $NaHCO_3$ solution. It was then dried with anhydrous magnesium sulphate and filtered.

3.10 TRANSFORMATION OF FERMENTATION PRODUCTS

The transformations of fermentation products were studied by cultivating the cultures in the mineral broth supplemented with the fermentation product. The mineral medium used was the same as that described under section 3.2.1. The inoculated broth was incubated on a rotary shaker at 30°C. The turbidity of the broth was periodically observed with a spectrophotometer to find out the growth of the bacteria. After incubation for different intervals of time, the broth was centrifuged, the supernatent was acidified and extracted with ether. The ether extract was concentrated under reduced pressure and subjected to analysis.

3.11 FERMENTATION OF ACETATES OF EUGENOL AND ISOEUGENOL

The acetate of eugenol/isoeugenol was fermented in the mineral medium described under section 3.2.1.

For the studies with the strain CUAC 10 the medium was supplemented with 1% (w/v) glucose and 0.1% (w/v) eugenol acetate/isoeugenol acetate. For studies with the strains CUAC 20 and Pseudomonas aeruginosa the medium was supplemented with 0.1% (v/v) eugenol acetate and for CUAC 30 the medium with 0.1% (v/v) isoeugenol acetate was used. The rest of the fermentation procedures was the same as with eugenol/isoeugenol.

3.12 MODE OF AROMATIC RING CLEAVAGE

The procedure of Ottow and Zolg was adopted for the determination of the mode of cleavage of the aromatic ring.[152] The organism was subcultured 2 times on mineral agar slants supplemented with eugenol/isoeugenol. After 2 days of incubation at 30°C, a thick loopful of the culture was suspended in 2 ml 0.05 M phosphate buffer (pH 8.0) in a test tube. Then 0.5 ml toluene and 2.5 ml of 4 mM sodium protocatechuate were added to it and it was vigorously shaken. A green colour within 3 minutes indicated a positive meta-cleavage. If no reaction occurred, the tube was incubated by shaking for 18 hours at 30°C, and 1 g of freshly prepared aqueous 1% sodium nitroprusside solution

and 0.5 ml concentrated ammonia were added. A deep purple colour within 3 minutes was regarded as a positive test for the ortho-cleavage of the substrate.

3.13 IMMOBILISATION OF WHOLE CELLS

Immobilisation of whole cells (IWC) was done by entrapment in calcium alginate beads.[153,154] Sodium alginate on treatment with calcium chloride gave calcium alginate beads.

3.13.1 Entrapment in calcium alginate

Hundred ml distilled water was warmed in a 250 ml flask and 4 g sodium alginate was added. It was heated till the start of boiling with constant stirring in order to avoid the formation of lumps. It was then removed from the heater and allowed to cool to form the sodium alginate slurry.

About 20 g of wet bacterial cells was made to a homogeneous paste with 25 ml distilled water and then the volume was made upto 100 ml.

Sodium alginate slurry and the bacterial cell slurry were mixed in 1:1 ratio and stirred well to obtain a uniform mix. It was extruded through a syringe needle into 0.1 M calcium chloride solution with stirring. The beads were cured in the same solution at room temperature for 6 hours. The gel beads were washed with distilled water and stored in buffer containing 50 mM calcium chloride.

3.13.2 Packing of the column

A glass column of 30 cm length and 2.5 cm diameter was used for the packing of the immobilised cells. The column was packed in the order of glass wool at the bottom, a perforated plate, cell immobilised aliginate beads and then again a perforated plate. The mineral broth containing eugenol was continuously passed into the column and the eluant was collected periodically.

3.13.3 Activation of immobilised cell beads

About 30 g of immobilised cell beads was packed in the column. The mineral broth containing eugenol/isoeugenol was passed into the column and incubated at 30°C for 24 hours. The broth was eluted off and the beads were

washed by passing freshly prepared saline solution continuously through the column. This column was then used for further studies.

3.13.4 Effect of temperature and pH on transformation by IWC

Fifty ml aliquots of the mineral broth containing the substrate adjusted to different pH values were prepared and passed into the column. These were incubated at 30°C and eluted and analysed at different intervals of time. Similarly 50 ml aliquots of the mineral broth adjusted to the optimum pH were prepared and passed into the column. These were incubated at different temperatures from 15°C to 60°C, eluted and analysed.

3.14 ENZYME STUDIES

Cell free extracts (CFE) of the cultures were prepared and were used as the source of enzymes.

3.14.1 Preparation of cell free extracts

The organism was subcultured two times and then inoculated in 400 ml of the mineral broth containing eugenol/isoeugenol. It was incubated for 15-18 hours and

centrifuged at 10,000 rpm for 20 minutes. The cells were separated, washed twice with 0.05 M phosphate buffer (pH 7.0) and the cell paste was frozen at 0°C. The frozen cell paste together with two weights of alumina was ground for 15 minutes with a chilled mortar and pestle.[155] The thick paste was diluted to 10 ml with buffer and the alumina, unruptured cells and cell walls were removed by centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatent solution was used as the crude enzyme preparation.

3.14.2 Effect of pH and temperature on transformation by CFE

Five ml aliquots containing 0.05 ml substrate, 0.5 ml CFE and 4.45 ml 0.05 M phosphate buffer adjusted to different pH values were incubated for 3 hours at 30°C and then analysed. Similarly 5 ml aliquots adjusted to the optimum pH were incubated at different temperatures for 3 hours and then analysed.

3.14.3 Transformations with CFE

Five ml aliquots containing 0.05 ml substrate, 0.50 ml CFE and 4.45 ml of 0.05 M phosphate buffer adjusted to optimum pH were incubated at the optimum temperature and periodically analysed by TLC and HPLC.

The formation of aromatic ring cleavage products γ-carboxy-α-hydroxy-cis, cis-muconic acid semialdehyde and β-carboxy-cis, cis-muconic acid were monitored spectrophotometrically by measuring the absorption at 410 nm and 290 nm respectively.[156,157] Thus to a cuvette containing 2 ml of 0.05 M phosphate buffer, 0.1 ml CFE and 0.4 ml of 1 mM sodium protocatechuate solution were added and the absorption was measured at 410/290 nm.

RESULTS AND DISCUSSION

CHAPTER 4

MICROBIAL TRANSFORMATIONS OF EUGENOL

MICROBIAL TRANSFORMATIONS OF EUGENOL

4.1 INTRODUCTION

Eugenol was extracted from clove oil and purified by column chromatography. A strain of bacteria which could metabolise eugenol was isolated from soil by enrichment culture technique. This strain, designated as CUAC 20, showed good growth in a mineral broth containing eugenol as the sole carbon source. Another bacteria, designated as CUAC 10, which fermented eugenol in a mineral broth containing glucose or any other nutrient broth was also isolated from the atmosphere. The strains were identified as belonging to Pseudomonas sp. and Bacillus sp. respectively, from their morphological and biochemical characters (Tables 4.1(a) and 4.1(b)).

4.2 STUDIES WITH THE BACILLUS sp. CUAC 10

As this strain was not able to grow in a mineral medium containing eugenol as the sole carbon source, studies were conducted in a mineral broth containing 1.0% (w/v) glucose in addition to 0.1% (v/v) eugenol (unless otherwise specified).

4.2.1 Growth studies

4.2.1.1 Effect of concentration of eugenol

The results of the studies of the effect of concentration of eugenol on the growth of the bacteria are presented in Table 4.2.1. As the concentration of eugenol was increased, there was a corresponding decrease in the growth of the bacteria. This effect was much less upto 0.12% (v/v) eugenol concentration. But with further increase in the concentration of eugenol, the growth was retarded.

4.2.1.2 Effect of concentration of sodium chloride

The results, presented in Table 4.2.2, indicated that the bacteria had no affinity for sodium chloride. On the contrary addition of NaCl resulted in decreased growth of the organism.

4.2.1.3 Effect of different nitrogen sources

The results, presented in Table 4.2.3, indicated that urea favoured maximum growth of the culture. Ammonium salts also favoured good growth, but when they were replaced by alkali metal nitrates, poor growth resulted.

4.2.1.4 Effect of pH

Table 4.2.4 shows the results of the effect of pH on the growth of the organism. Though the bacteria tolerated a wide range of pH from 6.5 to 8.5, optimum pH value was 7.0 to 7.5.

4.2.1.5 Effect of temperature

As shown by the results presented in Table 4.2.5, the bacteria favoured a wide range of temperatures from 30 to 45°C. It could even tolerate temperatures upto 60°C, but the optimum temperature was 30 to 37°C.

4.2.2 Effect of physico-chemical factors on transformation of eugenol

4.2.2.1 Effect of concentration of sodium chloride

The results of the studies of the effect of concentration of sodium chloride on the transformation of eugenol are presented in Table 4.2.6. Addition of sodium chloride did not affect the transformation much. In all the cases tested, more than 45 per cent of eugenol was transformed in 24 hours. After that there was not much reaction.

4.2.2.2 Effect of concentration of glucose

The results presented in Table 4.2.7 indicated that the optimum concentration of glucose was 1% (w/v). When the concentration of glucose was decreased to 0.5% (w/v), the rate of transformation was also decreased. When the concentration of glucose was increased to 1.5% (w/v), the rate of transformation of eugenol during the first 18 hours was comparatively low. After that the rate of transformation increased so that more than 45 per cent eugenol was fermented in 24 hours.

4.2.2.3 Effect of different nitrogen sources

Ammonium sulphate favoured more transformation of eugenol, as can be seen from Table 4.2.8. Though the organism recorded maximum growth with urea as the nitrogen source, the transformation rate of eugenol was comparatively less with urea. The rate of transformation was the least when potassium nitrate was used.

4.2.2.4 Effect of pH

The results of the effect of pH on the transformation of eugenol are presented in Table 4.2.9.

The maximum conversion of eugenol was recorded, when the pH was 7.0. A pH of 7.5 also favoured very good conversion. When the pH was decreased to 6.0, there was a considerable decrease in the conversion rate of eugenol.

4.2.2.5 Effect of temperature

The results shown in Table 4.2.10 suggested that the optimum temperature was 30°C. But there was no appreciable decrease in the transformation of eugenol upto 45°C. The transformation of eugenol at 37°C was almost as good as that at 30°C. Decreasing the temperature to 15°C drastically reduced the transformation.

4.2.3 Growth curve

By incorporating the optimal conditions the growth of the culture was monitored at different intervals of time to obtain the growth curve (Fig.4.2.1). The bacteria was in lag phase during the first four hours. Then the logarithmic phase of growth started and it attained almost full growth at 15 hours. The stationary phase started at 18 hours and lasted for about 3 hours. Thus the organism was found to be a fast growing one.

4.2.4 Fermentation of eugenol

Based on the growth studies and the studies on the effect of various physico-chemical parameters on the transformation of eugenol, the optimal conditions for the fermentation of eugenol with the Bacillus sp. CUAC 10 were obtained. By incorporating these optimal conditions, eugenol was fermented in a mini jar fermentor.[158] The mineral medium described under section 3.2.1 supplemented with 1.0% (w/v) glucose and 0.1% (v/v) eugenol was used for the fermentation.

As fermentation proceeded, 10 ml samples were withdrawn from the fermentor and analysed. During the first 10 hours, the formation of bisdehydroeugenol (BDHE) was not considerable. By 18 hours about 38% of BDHE was formed. The concentration of BDHE increased to a maximum of 45.4% yield at the end of 24 hours. Thereafter no further transformation was recorded.

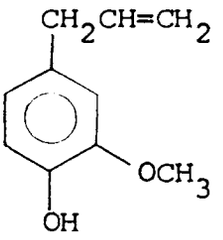
4.2.5 Isolation and identification of bisdehydroeugenol

The fermentation broth was centrifuged, acidified and extracted with solvent ether. The ether extract was evaporated under reduced pressure and subjected to column

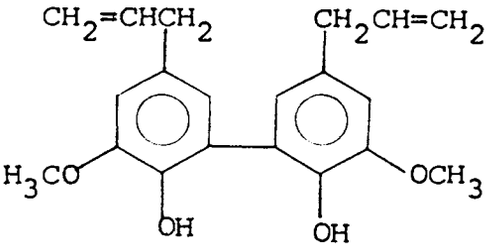
chromatography using chloroform : hexane (3:1) solvent system as eluant. The product, obtained as white solid, was recrystallised from chloroform : hexane mixture.

The pure product E_{20} had a melting point of 106°C. The NMR spectrum (Fig.4.2.3) of this compound was very similar to the NMR spectrum of eugenol (Fig.4.2.2). However, on closer examination it was found that there were only two aromatic protons corresponding to each $-OCH_3$ group in the product molecule. In the case of eugenol, the number of aromatic protons corresponding to each $-OCH_3$ group is three. The absence of one aromatic proton in the NMR spectrum suggested that there might have been an oxidative coupling at that position to give a dimer. The molecular weight determination by mass spectrometry and elemental analysis supported this view. The actual position of coupling was also deduced from an analysis of the NMR spectrum. Both of the aromatic protons appeared at δ 6.75 indicating similar environment and no coupling between them. Hence the bond formation must have taken place either through position 5 or 6 of the eugenol molecule (1). Knowing that oxidative coupling of phenols takes place either at the para or ortho position,[159] the

position of coupling may be inferred as 6. Hence the structure (2), 3,3'-diallyl-5,5'-dimethoxy-6,6'-dihydroxy biphenyl is suggested for the product obtained in this process.



(1)



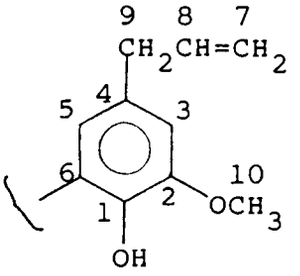
(2)

Eugenol on treatment with ferric chloride gave the same product with identical melting points and Rf values in different solvent systems. The structure of the product obtained by $FeCl_3$ treatment had already been established as (2), bisdehydroeugenol.[134,160]

The acetylated derivative of (2) was prepared by reacting it with acetic anhydride in pyridine. The NMR spectrum of the acetate showed that the compound was

diacetylated. The diacetate had $-COCH_3$ protons at δ 2.1, $-OCH_3$ protons at δ 3.85 and aromatic protons at δ6.75, thus confirming the structure of (2).

The ^{13}C NMR spectrum of the product E_{20} (Fig.4.2.4) showed signals for 10 carbons only thus proving the symmetrical structure of the molecule. The $-OCH_3$ carbon and $-CH_2-$ carbon appeared at 56.087 ppm (quartet) and 39.982 ppm (triplet) respectively and the sp^2 hybrid carbons appeared between 110 ppm and 147 ppm. The chemical shift of the carbon through which dimerisation has taken place is 124.423 ppm (singlet) indicating that it is adjacent to the carbon bearing the hydroxyl group. The assignment of chemical shifts of all the carbons are as shown in the figure below:



C_1 - 147.237 ppm (s)
C_2 - 140.907 " (s)
C_3 - 123.117 " (d)
C_4 - 131.921 " (s)
C_5 - 110.686 " (d)
C_6 - 124.423 " (s)
C_7 - 115.725 " (t)
C_8 - 137.659 " (d)
C_9 - 39.982 " (t)
C_{10}- 56.087 " (q)

4.2.6 Fermentation of eugenol acetate

Eugenol acetate was fermented in the mineral medium described under section 3.2.1 supplemented with 1.0% (w/v) glucose and 0.10% (v/v) eugenol acetate. After fermentation the broth was centrifuged, acidified and extracted with ether. On analysis of the ether extract, it was found that eugenol and BDHE were the products of the fermentation. The yield of BDHE was 47.5% and that of eugenol was 49.1% at the end of 24 hours. After that there was no further increase in the concentration of BDHE and only traces of eugenol acetate remained.

4.2.7 Fermentation of transformation product

Bisdehydroeugenol was fermented in a mineral medium containing 0.1% (w/v) BDHE and 1% (w/v) glucose. The Bacillus sp. CUAC 10 was found not able to ferment BDHE, as no transformation product was detected in the fermentation broth. This indicated that BHDE was the end product of fermentation of eugenol with the strain CUAC 10.

4.2.8 Immobilisation of whole cells

From the studies on the transformation of eugenol with immobilised whole cells, it was found that the optimum pH was 8.0 and the optimum temperature was the same as that of intact cells, ie., 30°C, for the transformation of eugenol. The retention time was 12 hours, when about 42.5% of eugenol was found to be converted. The immobilised cells were active even after continuous use for one week. After 10 days of operation the activity was reduced to almost 50% of the initial activity.

4.2.9 Discussion

The organism, isolated from the atmospheric flora was identified to belong to the Bacillus sp. from the morphological studies. The organism was a fast growing one. The growth of the organism was retarded by the addition of eugenol to the medium since eugenol was strongly antibacterial. This effect was quite negligible upto 0.07% (v/v) of eugenol concentration and was more pronounced from a concentration of 0.10% (v/v). Subsequent addition of eugenol caused a considerable decrease in the growth of the bacteria. The strain did not show any affinity for sodium chloride. Of the different nitrogen

sources tested urea had a positive effect on the growth of the bacteria. This might be due to the fact that urea served as an additional carbon source for the growth of the bacteria. The optimal pH value was 7 to 7.5 though it showed good growth at pH 6.5 and 8.0 also. The bacteria tolerated a wide range of temperatures from 30 to 45°C though the optimum temperature was 30 to 37°C.

The studies on the effect of various physico-chemical factors on the transformation of eugenol led to the optimal conditions. Addition of sodium chloride did not enhance the transformation of eugenol. The optimal concentration of glucose was 1% (w/v). When the concentration of glucose was decreased, the transformation rate of eugenol was also found to be decreased. This might be because of the poor growth of the bacteria for want of easily assimilable carbon source. The decrease in the rate of transformation of eugenol when the concentration of glucose was increased to 2% (w/v) could be due to the fact that the products of metabolism of glucose slightly inhibited the transformation of eugenol. Of the nitrogen sources tested though urea favoured an increase in growth of the bacteria, the transformation of eugenol was slightly decreased with urea. The maximum conversion was noted when

ammonium sulphate was used as the nitrogen source. The optimal pH value was 7.0 to 7.5 and temperature 30 to 37°C. It was interesting to note that the transformation was not much affected when the temperature was increased upto 45°C.

As mentioned previously the culture was a fast growing one in the medium tested. During the first 12 hours the rate of transformation was very low. Only when the culture entered the logarithmic phase of growth, the rate of transformation of eugenol increased. About 49% of eugenol was fermented by 24 hours and thereafter there was no appreciable reaction.

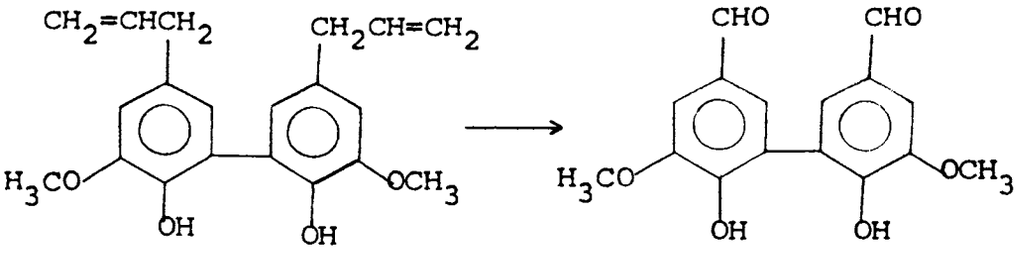
Thus the above studies led to the optimal conditions for the transformation of eugenol by the Bacillus sp. CUAC 10. These conditions were incorporated in the transformation of eugenol in the fermentor and the yield of bisdehydroeugenol was 45.4%.

When the acetate of eugenol was fermented, the yield of BDHE was found to increase to 47.5%. The bacteria first hydrolysed eugenol acetate to eugenol which was then converted to BDHE. It is possible that when the phenolic

compound, eugenol was produced in situ from its acetate its concentration would be comparatively lower and hence the toxic effects of it would not affect the growth of the bacteria.[161]

Of the various methods of immobilisation of microbial cells, the entrapping method was used in this study. In the studies for obtaining optimal pH, a shift of 1 unit to the alkaline range was observed in comparison with that of intact cells and the optimum temperature was 30°C. Though the yield of BDHE with immobilised cells was less than that with intact cells, the retention time was less and the column of immobilised cells could be reused for more than one week of continuous operation.

The oxidation of BDHE with nitrobenzene and alkali gives dehydrodivanillin.[162]



4.3 STUDIES WITH THE PSEUDOMONAS sp. CUAC 20

This strain was able to grow in a mineral medium containing eugenol as the sole carbon source. All the studies were conducted in the mineral medium described under section 3.2.1 supplemented with 0.1% (v/v) eugenol (unless otherwise stated).

4.3.1 Growth studies

4.3.1.1 Effect of concentration of eugenol

The results of the effect of concentration of eugenol on the growth of the bacteria, presented in Table 4.3.1 indicate that higher and lower concentrations of the substrate did not enhance the growth. The optimum concentration was 0.1% (v/v) of eugenol. When the concentration of eugenol was increased to 0.15% (v/v) there was a slight decrease in growth. Further addition of eugenol resulted in very poor growth.

4.3.1.2 Effect of concentration of sodium chloride

The organism showed a slight increase in the growth with the addition of sodium chloride upto 0.5% (w/v)

concentration. Further addition of sodium chloride resulted in a poor growth of the bacteria, as can be seen from Table 4.3.2.

4.3.1.3 Effect of different nitrogen sources

Table 4.3.3 shows the result of the studies on the effect of different nitrogen sources on the growth of the bacteria. Of all the compounds tested, urea favoured more growth. The least growth was observed with the alkali metal nitrates.

4.3.1.4 Effect of pH

The results presented in Table 4.3.4 indicated that the optimum pH was 7.0. The strain could also tolerate pH 7.5 with fairly good growth. The growth was drastically reduced with decrease or increase of pH.

4.3.1.5 Effect of temperature

As can be seen from Table 4.3.5 the optimum temperature was 30°C though the organism showed good growth at 37°C also. Subsequent increase in the temperature caused a considerable decrease in the growth.

4.3.2 Effect of physico-chemical factors on transformation of eugenol

4.3.2.1 Effect of concentration of sodium chloride

The results of the studies of the effect of concentration of sodium chloride on the transformation of eugenol are presented in Table 4.3.6. Addition of sodium chloride upto 0.5% (w/v) slightly increased the transformation of eugenol. Further increase of sodium chloride concentration resulted in a decrease in the transformation rate.

4.3.2.2 Effect of concentration of glucose

The addition of glucose adversely affected the transformation of eugenol as can be seen from Table 4.3.7. The effect was more pronounced with an increase in the concentration of glucose. When 1% (w/v) glucose was added, the rate of transformation was considerably decreased during the first 18 hours. But by 36 hours more than 90% of eugenol was degraded.

4.3.2.3 Effect of different nitrogen sources

The results presented in Table 4.3.8 indicated that urea favoured more transformation. When urea was used

as the nitrogen source about 60% of eugenol was degraded by 18 hours and the reaction was almost over by 24 hours. The rate of transformation with the other compounds was almost the same.

4.3.2.4 Effect of pH

The optimum pH was 7.0. When the pH was changed to 7.5, the transformation of eugenol was affected considerably during the initial stages of fermentation as can be seen from Table 4.3.9. But the amount of eugenol remaining at the end of 48 hours was almost the same as that at pH 7.0.

4.3.2.5 Effect of temperature

The results presented in Table 4.3.10 indicated that the optimum temperature was 30°C. Decreasing the temperature below 30°C or increasing it above 37°C affected the transformation considerably.

4.3.3 Growth curve

The optimal conditions obtained from the above studies were incorporated and the growth of the organism

was monitored at different intervals of time to obtain the growth curve (Fig.4.3.1). The initial lag phase of growth lasted for about 6 hours. The logarithmic phase of growth started by 15 hours. After attaining full growth, the stationary phase lasted for about 3 hours, from 21 to 24 hours. Then the death phase occurred.

4.3.4 Fermentation of eugenol

Eugenol was fermented in a mini jar fermentor incorporating the optimal parameters obtained from the studies on the growth of the organism and the effect of various physico-chemical parameters on the transformation.[163] The mineral medium used for fermentation was supplemented with 0.11% (v/v) eugenol.

4.3.4.1 Time course of formation of products

During the course of fermentation, 10 ml samples of the broth were withdrawn from the fermentor and analysed for residual eugenol and concentration of transformation products. Ferulic acid, vanillin and vanillic acid were measured by determining the optical density at 310, 347 and 293 nm, respectively. Upto 12 hours the transformation

rate was very low. After that a considerable amount of eugenol started transforming. By 24 hours the reaction was almost complete with more than 90% of transformation. After that there was not much reaction and traces of eugenol remained. Vanillin was formed in measurable amounts from 12 hours. The concentration of vanillin increased to a maximum of 4.62% yield at 17 hours and thereafter decreased to almost zero by the end of 36 hours. The other products were also formed along with vanillin. The concentration of ferulic acid attained a maximum value of 6.25% yield at 20 hours and that of vanillic acid attained a maximum of 8.9% yield at about 24 hours. The time course of formation of products is given in Table 4.3.8.

4.3.4.2 Experiments for increased production of vanillin

1. Fermentation of eugenol was carried out in the fermentor with different rates of aeration, viz., 0.5, 1.0, 1.5 and 2.0 volumes of air per volume of medium per minute (VVM). It was found that when the rate of aeration was decreased to 0.5 VVM the rate of transformation was also decreased. Even after 24 hours,

about 30% eugenol was left unreacted and correspondingly the formation of vanillin was also decreased. When the flow rate was increased to 1.5 or 2 VVM, the rate of transformation was increased. The reaction was almost complete by 21 to 22 hours, but the concentration of vanillin was decreased.

2. Fermentation of eugenol was carried out in the fermentor with aeration at a rate of 1 VVM. At 17 hours 0.5% (w/v) of glucose was added to the fermentation broth when the concentration of vanillin was maximum. This was done to find out whether the organism will opt for an easily assimilable carbon source like glucose so that the vanillin formed was not further metabolised. Ten ml samples of the fermentation broth were then withdrawn from the fermentor and analysed at different time intervals. It was observed that after the addition of glucose the rate of transformation of eugenol was decreased. But there was no increase in the concentration of vanillin. Even after 24 hours more than 20% of eugenol was left unreacted. After 48 hours only traces of eugenol remained.

4.3.5 Isolation and identification of products

The fermentation broth was centrifuged to remove the cells and the supernatent was acidified with 2 N sulphuric acid and extracted with ether. The ether extract was re-extracted with 5% aqueous sodium bicarbonate solution to remove strongly acidic products.

The ether layer containing non-acidic products was dried with anhydrous sodium sulphate and concentrated under reduced pressure. It was then subjected to column chromatography with chloroform : hexane (3:1) as the eluant. The product was obtained as a white solid. It was recrystallised from chloroform : hexane mixture. The pure product E_{30} had a melting point of 81°C. With 2,4-dinitrophenyl hydrazine-H_2SO_4, it showed positive colouration. The IR spectrum of the product indicated the presence of hydroxyl (3560-3520 cm^{-1}), aldehyde (1680-1710 cm^{-1}) and aromatic (1615-1600 cm^{-1}) groups. The NMR spectrum (Fig.4.3.2) showed methoxy protons at δ 3.95, aromatic protons at δ 6.95-7.55 and aldehydic protons at δ 9.85. These spectra completely matched those of vanillin. The Rf value (TLC) in different solvent systems was also the same as that of vanillin. Thus the product was identified as 4-hydroxy-3-methoxy benzaldehyde (vanillin).

The sodium bicarbonate layer was acidified with 2 N sulphuric acid to pH 2 and extracted again with ether. The ether layer was dried with anhydrous sodium sulphate and concentrated under reduced pressure. A small portion of it was subjected to thin layer chromatography which showed the presence of three products. This mixture was then chromatographed in a silica gel column with ethyl acetate : hexane (3:1) as the eluant. Three products (A, B & C) were obtained. They were recrystallised from water-acetone mixture which gave white solids.

The product A melted at 170°C. The methyl ester of the acid was prepared (melting point 63°C). The NMR spectrum of the ester showed the presence of methoxy group, methine group, aromatic protons and hydroxyl group indicating that it may be 4-hydroxy-3-methoxycinnamic acid (ferulic acid). The IR spectrum of the product (Fig.4.3. 3) was identical with that of authentic ferulic acid.

The product B had a melting point of 210°C. The IR spectrum of the product (Fig.4.3. 4) completely matched that of authentic vanillic acid. The methyl ester of the product (melting point 108°C) was prepared. The NMR

spectrum of the ester showed the presence of methoxy, aromatic and hydroxyl protons. From these results the product was identified as 4-hydroxy-3-methoxybenzoic acid (vanillic acid).

The product C melted at 200°C. A mixed melting point with authentic protocatechuic acid was not depressed. The NMR spectrum of the methyl ester of the product (Fig.4.3. 5) showed signals for aromatic protons, hydroxyl groups and $-OCH_3$ group. Thus the compound was identified as 3,4-dihydroxybenzoic acid (protocatechuic acid).

4.3.6 Fermentation of transformation products

Ferulic acid, vanillin, vanillic acid and protocatechuic acid were found to be assimilated by the organism Pseudomonas sp. CUAC 20. When mineral broths supplemented with each of these compounds were inoculated with the organism and incubated, an increase in turbidity was observed indicating the growth of the organism. When the broths were extracted with ether and analysed by TLC, it was found that these compounds were further metabolised, indicating that they were not the end-products.

4.3.7 Fermentation of eugenol acetate

Eugenol acetate was fermented in the mineral medium described under section 3.2.1. Eugenol, ferulic acid, vanillin, vanillic acid and protocatechuic acid were the products of fermentation of eugenol acetate. Eugenol acetate was metabolised slowly during the initial hours of fermentation. Only after 15 hours the rate of transformation increased. But by 24 hours almost complete eugenol acetate was utilised. It took two more hours for eugenol to be almost completely used up. There was not any considerable difference in the amount of vanillin formed. The maximum concentration of vanillin was noticed at 19 hours.

4.3.8 Mode of aromatic ring cleavage

From the fermentation of transformation products of eugenol it was found that protocatechuic acid (PCA) was the final aromatic compound in the degradation pathway of eugenol, but it was not the end product. The ring fission mechanism was determined by the method described under section 3.13.4 which revealed that PCA was cleaved by ortho-fission.

4.3.9 Immobilisation of whole cells

Culture cells 18 hours old, were immobilised in calcium alginate beads. The optimum pH and temperature for the transformation of eugenol with the immobilised cells were obtained as 7.5 and 30°C, respectively. The retention time was 18 hours and the immobilised cells were found to be active for more than one week.

4.3.10 Enzyme studies

Cell free extracts (CFE) of the culture were prepared and was used as the source of enzymes. Studies with CFE at different pH values and temperatures revealed that the optimum values were pH 8.0 and temperature 30°C.

CFE transformed eugenol to ferulic acid, vanillin, vanillic acid and protocatechuic acid. CFE was also found to be active on ferulic acid, vanillin, vanillic acid and protocatechuic acid. Protocatechuate solution on incubation with CFE was found to strongly absorb at 290 nm indicating the ortho-cleavage of PCA to form β-carboxy-cis,cis-muconic acid.

4.3.11 Discussion

The organism, isolated from the local soil, was identified to belong to Pseudomonas sp. based on the morphological and biochemical characters. This strain Pseudomonas sp. CUAC 20 was different from the bacterial strains, Corynebacterium sp. and Pseudomonas aeruginosa, earlier reported.[138,139] Pseudomonas aeruginosa was a green pigment-producing strain. Moreover it was reported to include a few more metabolites besides those produced by the present strain.

The growth studies with the organism led to the optimal conditions for the growth of the strain. The growth of the bacteria increased with the increase in concentration of eugenol upto 0.1% (v/v). After that there was a decline in growth and the effect was more pronounced from 0.15% (v/v) onwards. This might be due to the antibacterial property of eugenol. The growth of the organism was slightly increased by the addition of sodium chloride upto 0.5% (w/v). Among the different nitrogen sources tested, urea favoured better growth. It was also seen that the optimum pH and temperature for the growth of the organism were 7.0 and 30°C, respectively.

Addition of sodium chloride at a rate of 0.5% (w/v) slightly increased the transformation of eugenol, which might be due to the increased growth of the bacteria at this concentration of NaCl. The inclusion of glucose as an additional carbon source had not much effect upto 0.5% (w/v). Further increase of concentration of glucose resulted in a decrease in the rate of transformation of eugenol. Then the organism took more time to assimilate eugenol completely. This might be due to the reason that glucose was a more easily assimilable carbon source than eugenol. The organism showed more transformation of eugenol when urea was used as the nitrogen source. Neutral pH and a temperature of 30°C were the optimal parameters for the increased transformation of eugenol.

The rate of transformation of eugenol during the first eight hours was very low. Thereafter there was a considerable increase in the transformation and about 50% of eugenol was reacted by 18 hours. The reaction was almost complete by 24 hours. The yield of vanillin was maximum, ie., 4.62% at 17 hours. After that it decreased apparently because vanillin was further oxidised to vanillic and protocatechuic acids. PCA was subsequently metabolised by the cleavage of the aromatic ring.

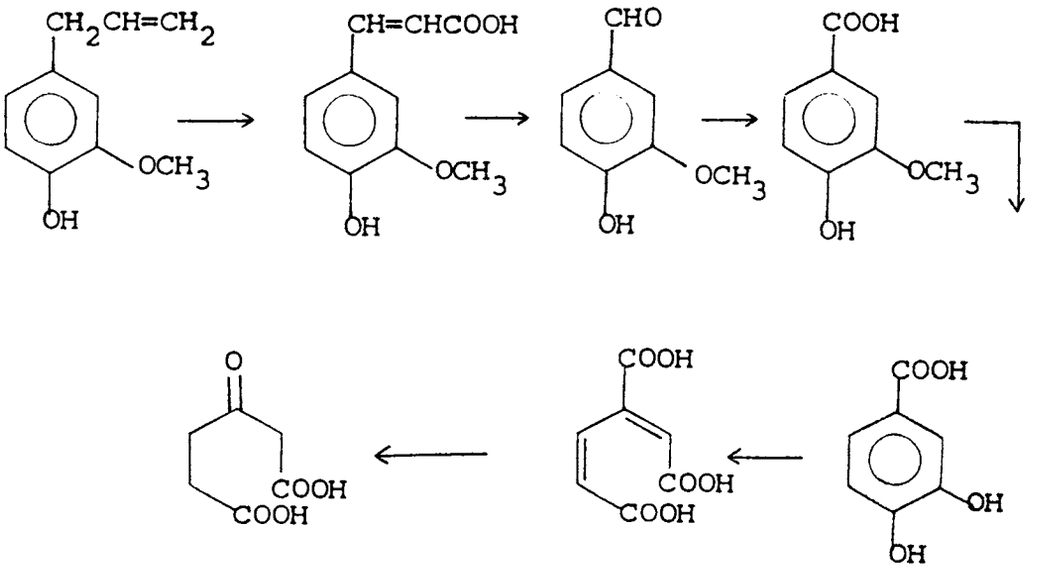
The present strain Pseudomonas sp. CUAC 20 metabolised eugenol acetate also. Eugenol acetate was first hydrolysed to eugenol which then was subsequently metabolised. There was not much difference in the yield of the products.

The organism was tested for its ability to metabolise isoeugenol by cultivating it in the mineral medium with isoeugenol as the sole carbon source. It was found that the organism was not able to grow in the broth which ruled out the possibility of isoeugenol being an intermediate in the degradation pathway of eugenol to ferulic acid.

Fermentation of eugenol with immobilised cells and cell free extracts did not improve the yield of the products. The transformation products viz., ferulic acid, vanillin, vanillic acid and protocatechuic acid were all transformed further when incubated with cell free extracts. This showed that they were not the end products of fermentation. The experiment to find the ring fission mechanism revealed that the aromatic ring was cleaved by ortho fission. Further, the presence of the enzyme

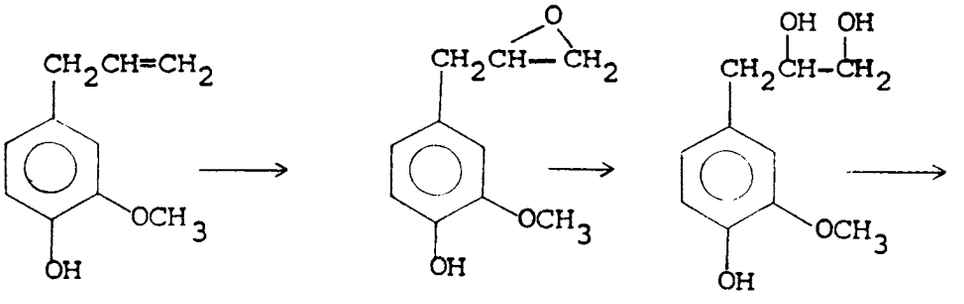
responsible for ortho-fission in the organism, proto-catechuic acid-3,4-oxygenase was confirmed by adding protocatechuate to CFE and monitoring the absorbance at 290 nm. PCA undergoes ortho-cleavage to form β-carboxy-cis, cis-muconic acid (CMA) which absorbs strongly at 290 nm. CMA was metabolised subsequently to β-keto adipic acid, which gave a deep purple colour in the colorimetric test for the determination of aromatic ring cleavage mechanism.[164]

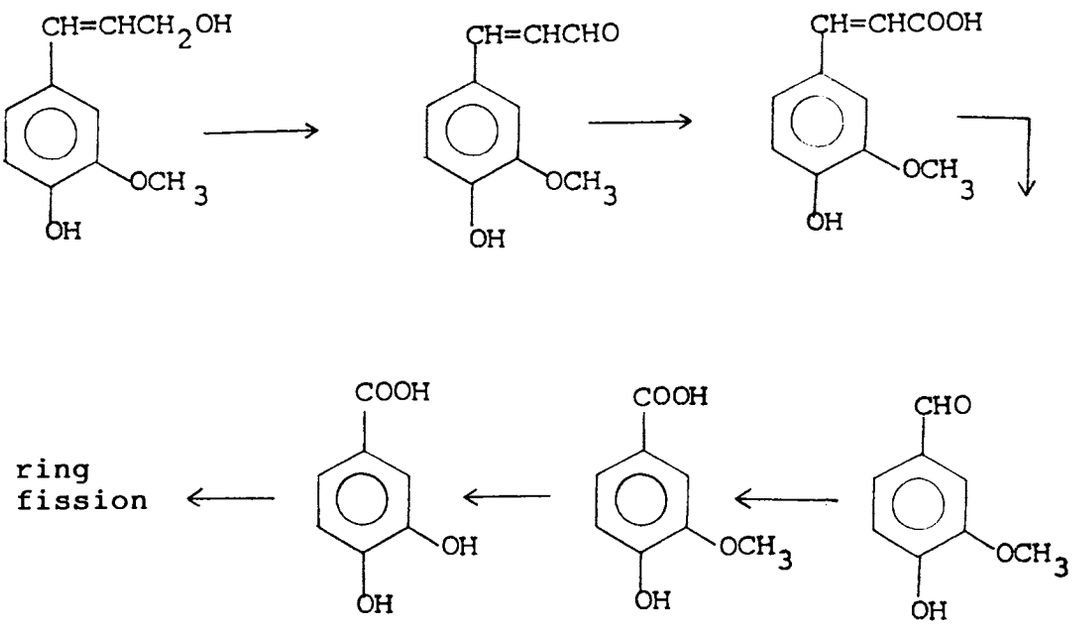
From the above results the following pathway is proposed for the degradation of eugenol by the Pseudomonas sp. CUAC 20.



4.4 STUDIES WITH PSEUDOMONAS AERUGINOSA

The fermentation of eugenol with Pseudomonas aeruginosa was carried out in the mineral medium described under section 3.2.1 supplemented with 0.1% (v/v) eugenol. The methods of cultivation and incubation were the same as those followed for Pseudomonas sp. CUAC 20. The fermented broth was centrifuged, acidified and extracted with solvent ether. The ether extract was concentrated and subjected to thin layer chromatography. The thin layer chromatogram was similar to that reported by Tadasa and Kayahara.[139] They had proposed the following pathway for eugenol metabolism by Pseudomonas aeruginosa.





Eugenol was fermented in the mini jar fermentor with Pseudomonas aeruginosa and 10 ml samples of fermentation broth were withdrawn and analysed periodically. It was found that fermentation was complete by 22 hours. The maximum concentration of vanillin was at 16 hours (yield 1.47%), after which the concentration was found to decrease.

Table 4.1(a): Identification tests for CUAC 10

Tests	Results
Form and arrangement	Long straight rods; no characteristic arrangement
Gram reaction	Positive
Spore formation	Positive
Motility	Positive
Chromogenesis in nutrient agar	Negative
Production of indole	Negative
Hydrogen sulphide production	Negative
Gas production from glucose	Positive
Oxidation-fermentation test	Oxidative
Citrate utilisation	Positive
Nitrate reduction	Negative
Cytochrome oxidase	Negative
Catalase	Positive
Gelatinase	Negative
Amylase	Positive
Caseinase	Negative

Table 4.1(b): Identification tests for CUAC 20

Tests	Results
Form and arrangement	Small rods; no characteristic arrangements
Gram reaction	Negative
Spore formation	Negative
Motility	Positive
Chromogenesis in nutrient agar	Negative
Production of indole	Negative
Hydrogen sulphide production	Negative
Gas production from glucose	Positive
Oxidation-fermentation test	Oxidative
Citrate utilisation	Negative
Nitrate reduction	Positive
Cytochrome oxidase	Positive
Catalase	Positive
Gelatinase	Positive
Amylase	Positive
Caseinase	Negative

Table 4.2.1: Effect of concentration of eugenol on growth

Engenol (%, v/v)	0	0.01	0.03	0.05	0.07	0.1	0.12	0.15	0.17	0.2
Optical density	0.995	0.991	0.975	0.954	0.930	0.892	0.863	0.737	0.576	0.353

Table 4.2.2: Effect of concentration of sodium chloride on growth

Sodium chloride (%, w/v)	0	0.5	1.0	2	3	5	7	10
Optical density	0.886	0.885	0.840	0.754	0.651	0.542	0.404	0.213

Table 4.2.3: Effect of different nitrogen sources on growth

Nitrogen source	NH_4NO_3	NH_4Cl	$(NH_4)_2SO_4$	$(NH_2)_2CO$	KNO_3	$NaNO_3$
Optical density	0.886	0.877	0.875	0.920	0.836	0.858

Table 4.2.4: Effect of pH on growth

pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Optical density	0.280	0.544	0.816	0.882	0.878	0.831	0.766	0.499

Table 4.2.5: Effect of temperature on growth

Temperature (0°C)	15	30	37	45	56	70
Optical density	0.287	0.891	0.882	0.845	0.683	0.532

Table 4.2.6: Effect of concentration of sodium chloride on transformation

Sodium chloride (%, w/v)	Residual Eugenol (%, v/v)					
	0 hr	6	12	18	24	36
0	100	98.00	93.86	56.20	51.25	51.04
0.5	100	97.26	93.65	57.13	52.06	51.77
1.0	100	99.16	95.90	60.14	53.24	53.00

Table 4.2.7: Effect of concentration of glucose on transformation

Glucose (%, w/v)	Residual Eugenol (%, v/v)					
	0 hr	6	12	18	24	36
0.5	100	98.90	96.14	64.10	56.43	55.37
1.0	100	98.28	94.25	58.39	51.10	51.08
1.5	100	98.80	97.05	65.18	53.30	52.32
2.0	100	98.95	98.60	69.74	58.63	57.85

Table 4.2.8: Effect of different nitrogen sources on transformation

Nitrogen source	Residual Eugenol (%, v/v)					
	0 hr	6	12	18	24	36
NH_4NO_3	100	96.12	93.80	56.65	51.60	50.80
NH_4Cl	100	97.00	94.16	57.90	52.23	51.89
$(NH_4)_2SO_4$	100	96.16	93.20	56.13	50.19	48.84
$(NH_2)_2CO$	100	97.00	94.18	57.95	52.91	52.60
KNO_3	100	97.63	95.80	58.50	53.86	53.85
$NaNO_3$	100	97.45	95.65	58.07	53.25	52.63

Table 4.2.9: Effect of pH on transformation

pH	Residual Eugenol (%, v/v)					
	0 hr	6	12	18	24	36
6.0	100	98.70	98.14	76.05	67.71	66.08
6.5	100	98.81	96.00	60.50	54.95	54.78
7.0	100	97.19	93.14	56.88	51.13	51.00
7.5	100	97.79	95.17	58.12	52.51	51.16
8.0	100	93.08	95.73	59.93	54.65	54.25

Table 4.2.10: Effect of temperature on transformation

Temperature (°C)	Residual Eugenol (%, v/v)					
	0 hr	6	12	18	24	36
15	100	99.10	98.71	89.90	84.75	83.76
30	100	98.00	94.10	56.78	51.14	50.83
37	100	98.11	95.00	57.80	52.01	51.51
45	100	98.25	96.00	58.13	53.70	52.68
56	100	98.40	98.19	60.23	55.18	55.05

Table 4.2.11: Growth curve

Time (hr)	0	2	4	6	9	12	15	18	21	24
Optical density	0	0.018	0.024	0.117	0.390	0.760	0.862	0.886	0.886	0.881

Table 4.3.1: Effect of concentration of eugenol on growth

Eugenol (%, v/v)	0	0.02	0.05	0.07	0.10	0.12	0.15	0.17	0.20	0.25
Optical density	0	0.215	0.476	0.658	0.726	0.713	0.687	0.608	0.459	0.190

Table 4.3.2: Effect of concentration of sodium chloride on growth

Sodium chloride (%, w/v)	0	0.5	1.0	1.5	2	3	5	7	10
Optical density	0.718	0.743	0.742	0.699	0.638	0.550	0.459	0.351	0.200

Table 4.3.3: Effect of different nitrogen sources on growth

Nitrogen source	NH_4NO_3	NH_4Cl	$(NH_4)_2SO_4$	$(NH_2)_2CO$	KNO_3	$NaNO_3$
Optical density	0.730	0.726	0.720	0.748	0.716	0.712

Table 4.3.4: Effect of pH on growth

pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Optical density	0.303	0.547	0.683	0.731	0.720	0.676	0.527	0.311

Table 4.3.5: Effect of temperature on growth

Temperature 0°C	15	30	37	45	56	70
Optical density	0.300	0.729	0.702	0.584	0.437	0.225

Table 4.3.6: Effect of concentration of sodium chloride on transformation

NaCl (%, w/v)	Residual Eugenol (%, v/v)					
	0 hr	12	18	24	36	48
0	100	82.00	48.01	6.43	2.62	0.89
0.5	100	81.47	46.76	4.11	1.10	0.22
1.0	100	81.40	47.81	6.27	2.11	1.04
1.5	100	84.73	49.18	8.95	5.02	3.99

Table 4.3.7: Effect of concentration of glucose on transformation

Glucose (%, w/v)	Residual eugenol (%, v/v)					
	0 hr	12	18	24	36	48
0.2	100	82.16	48.11	6.46	2.62	0.98
0.5	100	83.76	49.40	7.78	3.00	0.99
0.7	100	88.81	52.50	8.80	3.52	1.85
1.0	100	95.05	59.16	14.65	8.48	3.87

Table 4.3.8: Effect of different nitrogen sources on transformation

Nitrogen Source	Residual Eugenol (%, v/v)					
	0 hr	12	18	24	36	48
NH_4NO_3	100	81.87	47.92	6.10	2.44	0.68
NH_4Cl	100	82.19	48.15	6.67	2.81	1.05
$(NH_4)_2SO_4$	100	83.01	48.94	6.96	3.05	1.00
$(NH_4)_2CO$	100	80.89	40.67	1.69	0.18	--
KNO_3	100	83.18	48.83	6.86	3.17	1.11
$NaNO_3$	100	83.76	49.06	7.00	3.22	1.10

Table 4.3.9: Effect of pH on transformation

pH	Residual Eugenol (%, v/v)					
	0 hr	12	18	24	36	48
6.5	100	85.30	60.25	16.48	10.74	7.81
7.0	100	81.90	48.07	6.38	2.58	0.45
7.5	100	84.76	52.40	10.51	4.48	2.60
8.0	100	88.63	63.74	20.00	13.30	10.25

Table 4.3.10: Effect of temperature on transformation

Temperature (°C)	Residual Eugenol (%, v/v)					
	0 hr	12	18	24	36	48
15	100	93.78	88.16	82.98	70.48	58.40
30	100	82.16	47.45	6.25	2.40	0.22
37	100	84.35	50.68	11.72	5.06	4.12
45	100	86.98	72.83	49.00	39.01	36.76
56	100	90.07	80.78	66.70	58.71	52.00

Table 4.3.11: Growth curve

Time (hr)	0	3	6	9	12	15	18	21	24	36
Optical density	0	0.016	0.056	0.128	0.240	0.387	0.593	0.720	0.718	0.709

Table 4.3.12: Time course of formation of products

Product	Concentration (mg/100 ml)					
	0 hr	12	18	24	30	36
Ferulic acid	0	4.7	6.8	4.4	2.6	1.9
Vanillin	0	2.1	5.0	3.2	1.4	0.5
Vanillic acid	0	3.3	7.6	9.8	6.2	3.8

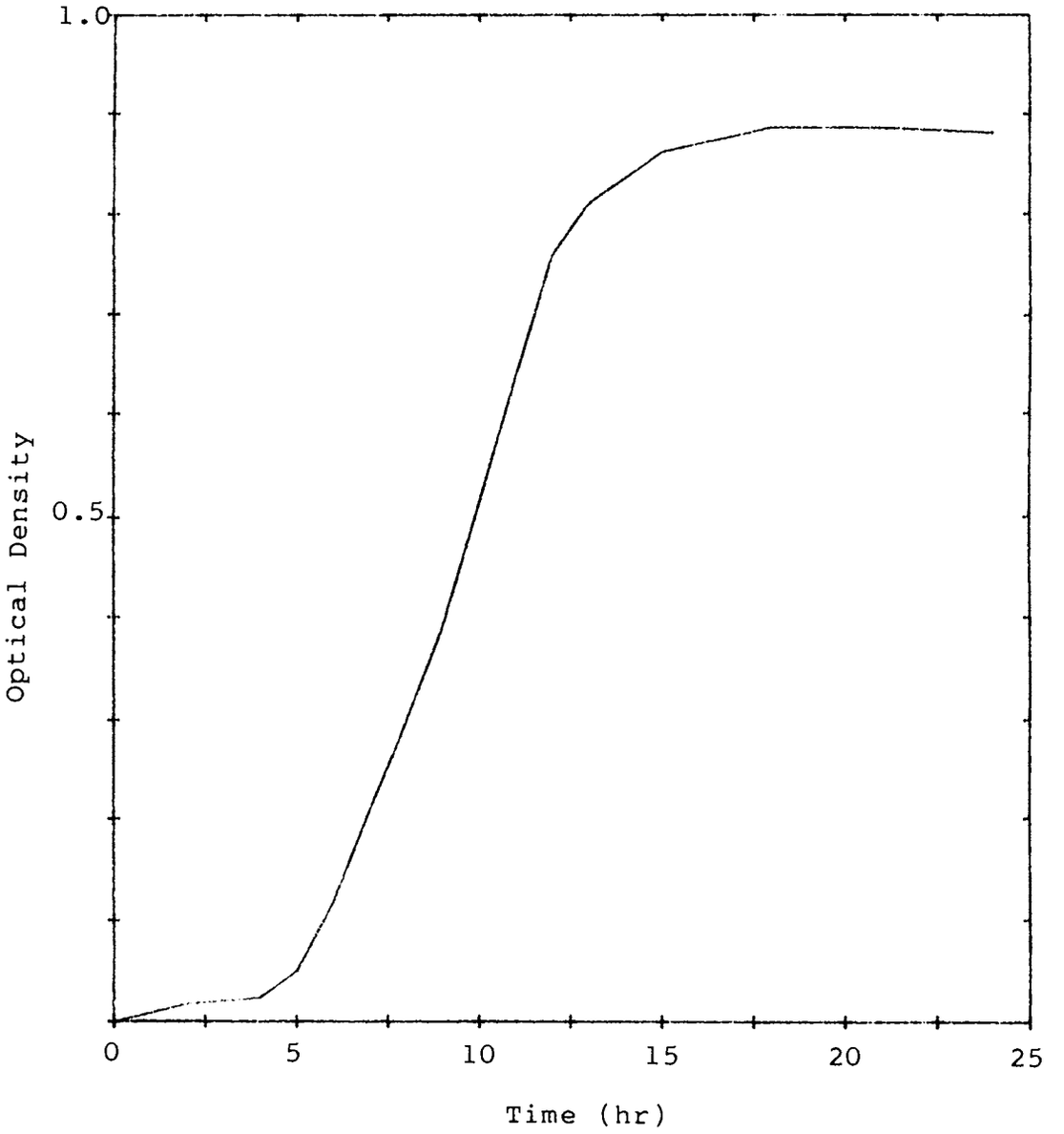


Fig.4.2.1: Growth curve of CUAC 10 in eugenol medium

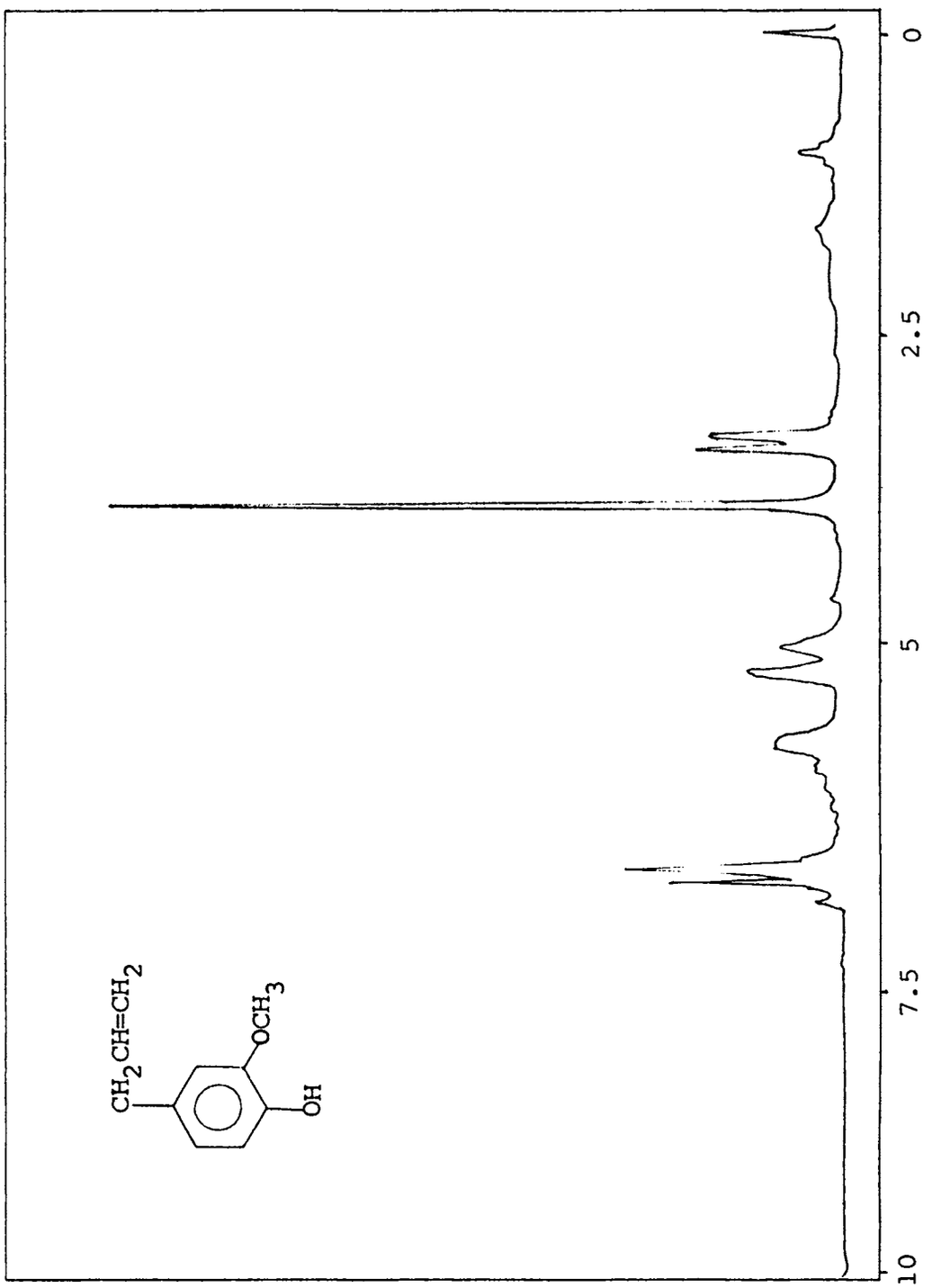


Fig.4.2.2: NMR spectrum of eugenol

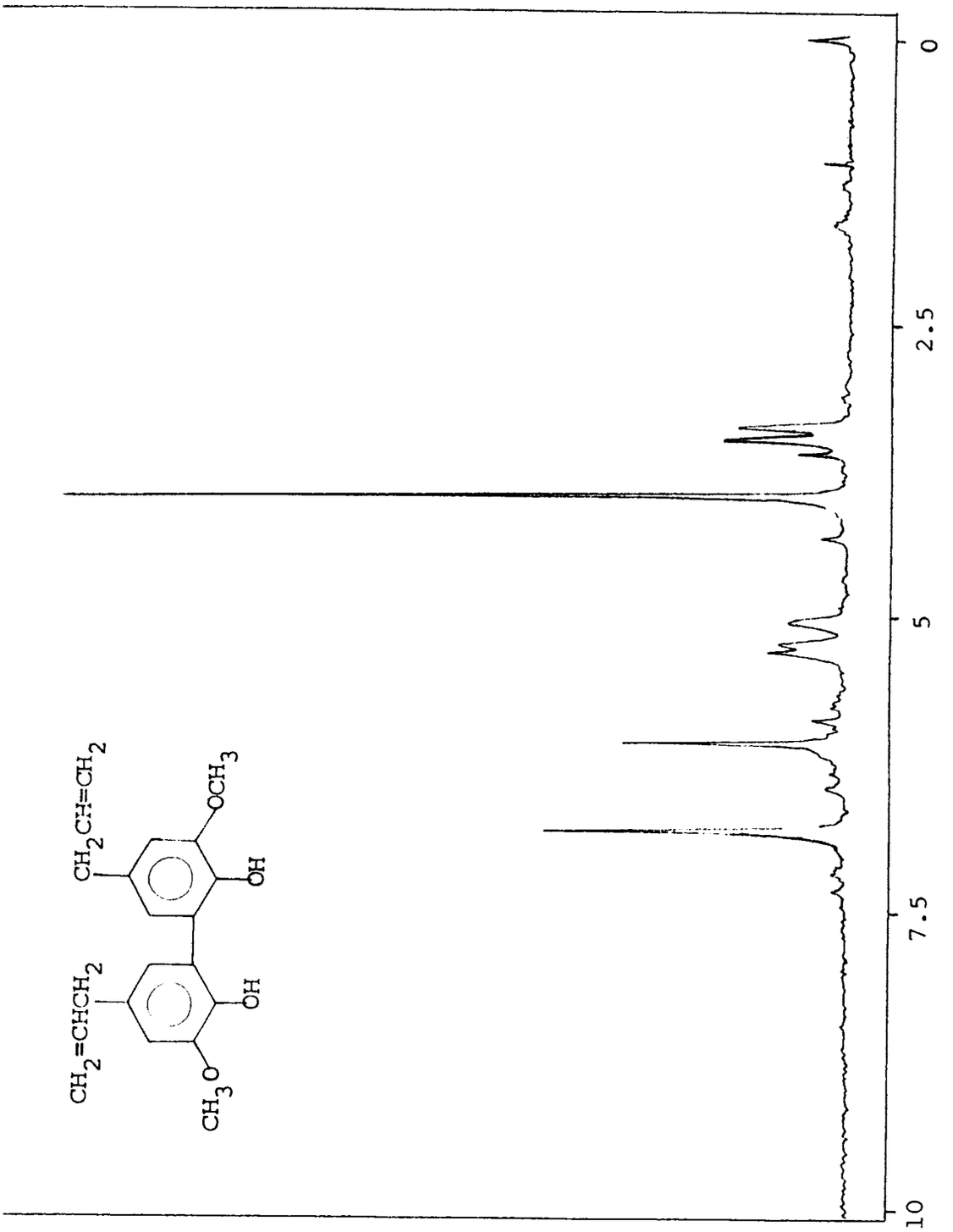
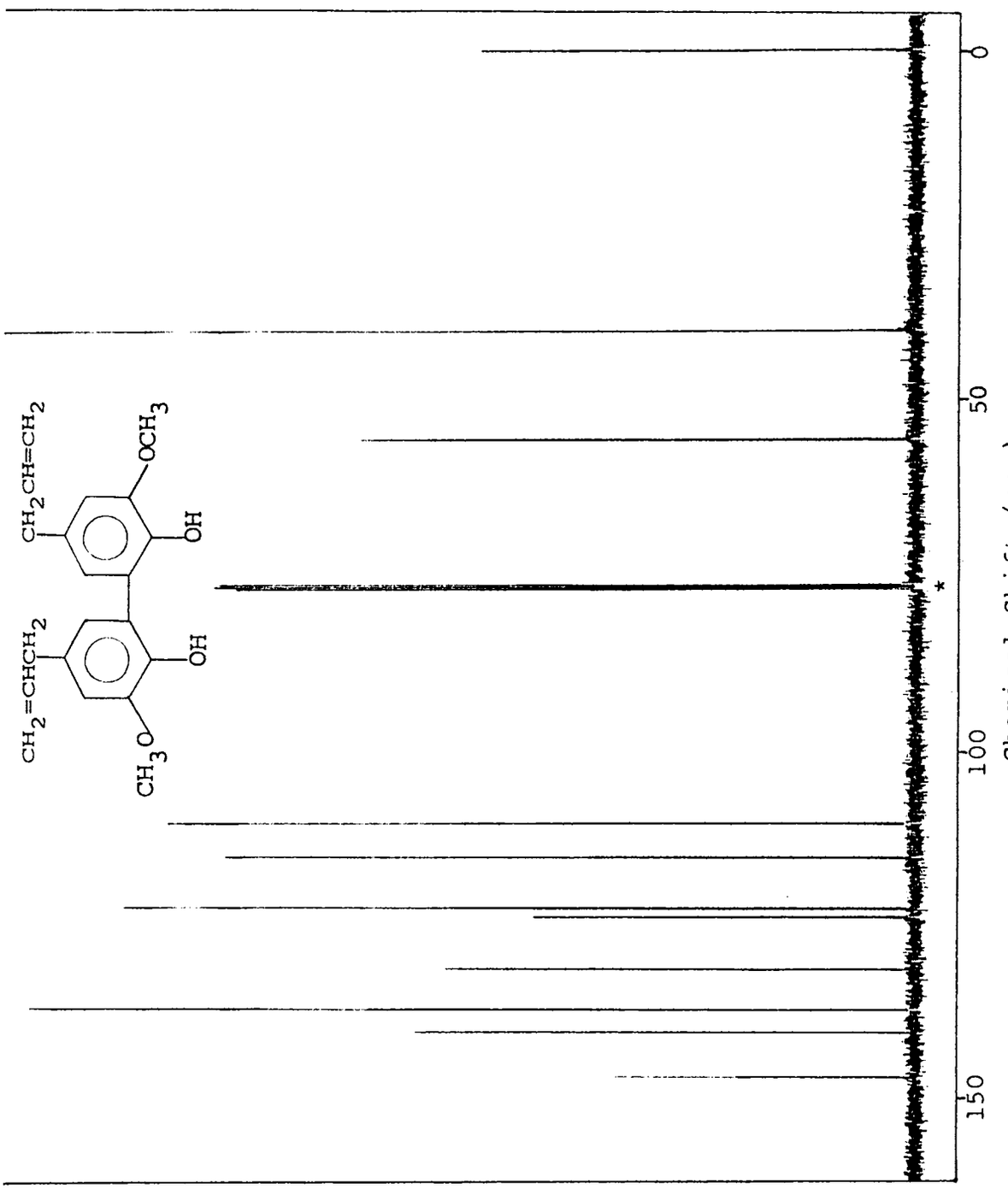


Fig.4.2.3: NMR spectrum of product E_{20}



* Signals due to the solvent, $CDCl_3$

Fig.4.2.4: ^{13}C NMR spectrum of product E_{20}

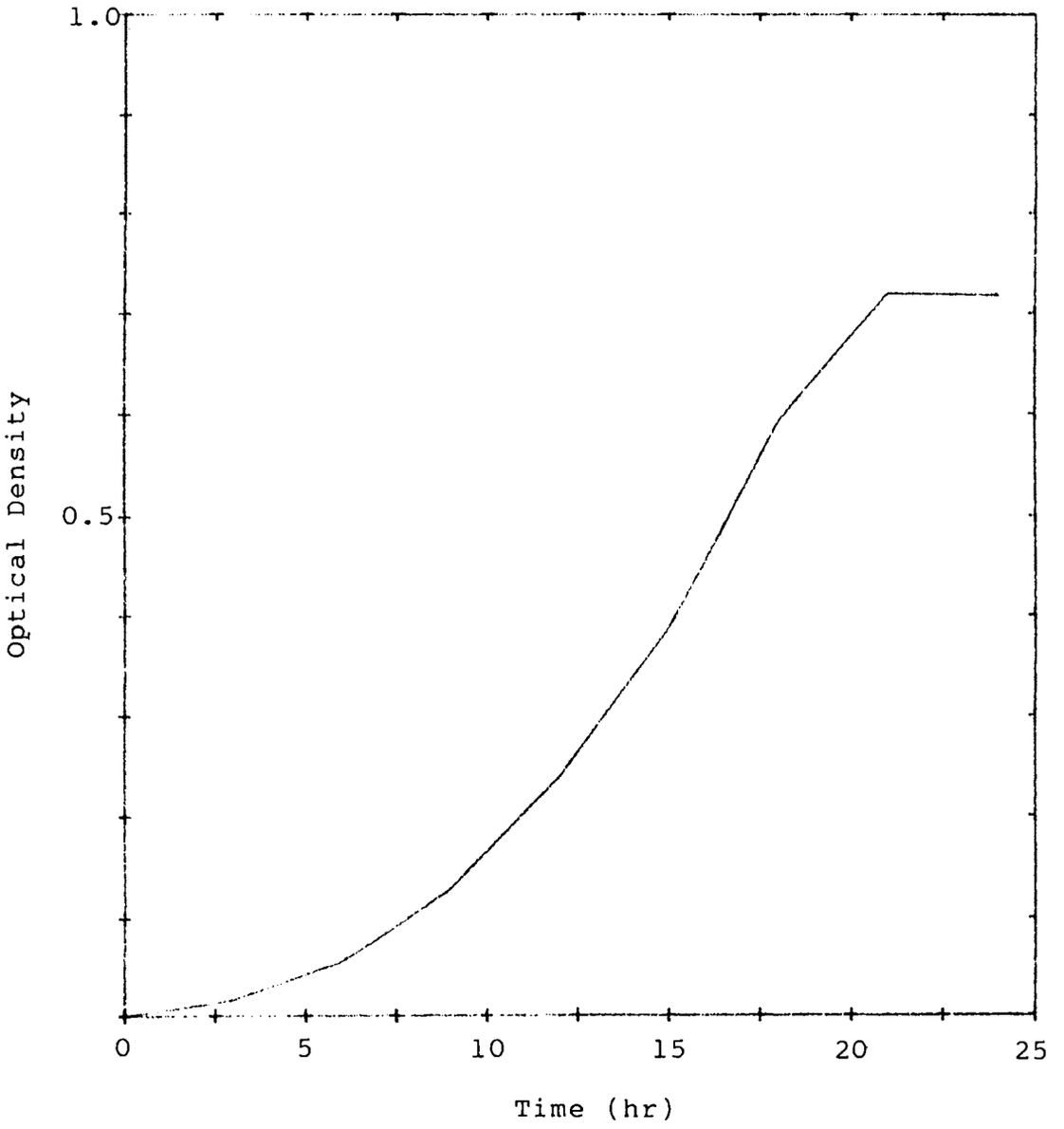


Fig.4.3.1: Growth curve of CUAC 20

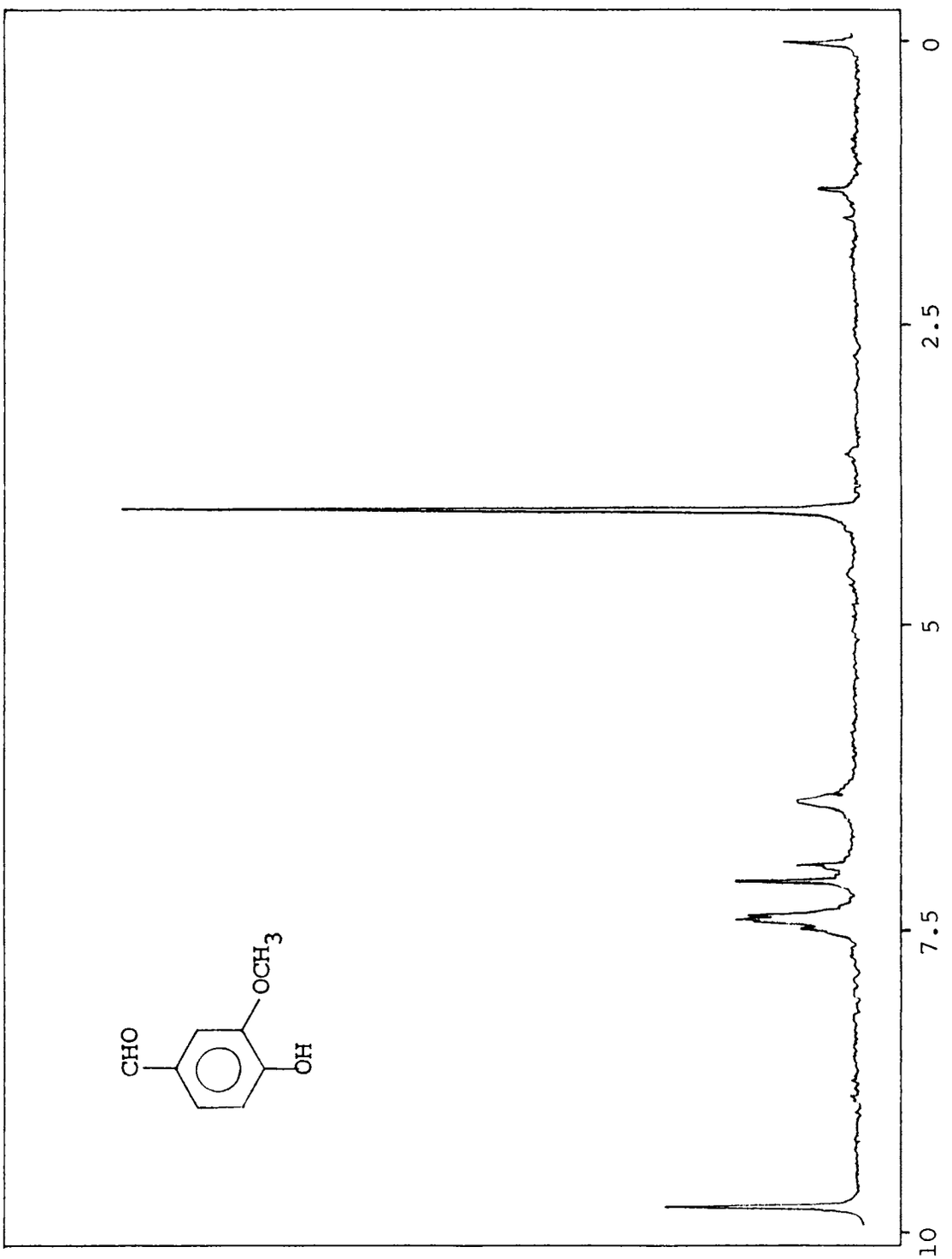


Fig.4.3.2: NMR spectrum of product E_{30}

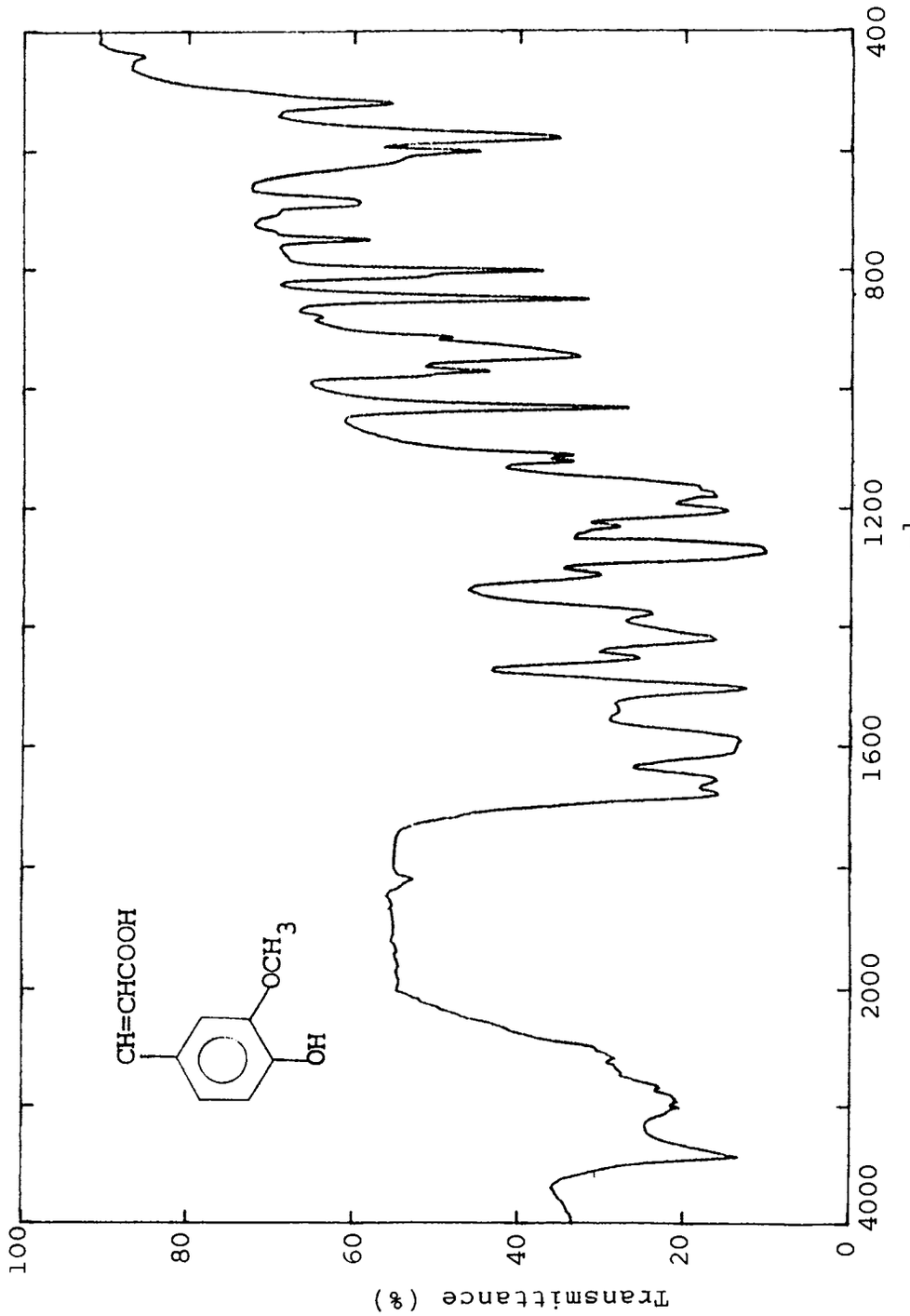


Fig.4.3.3: IR spectrum of product A

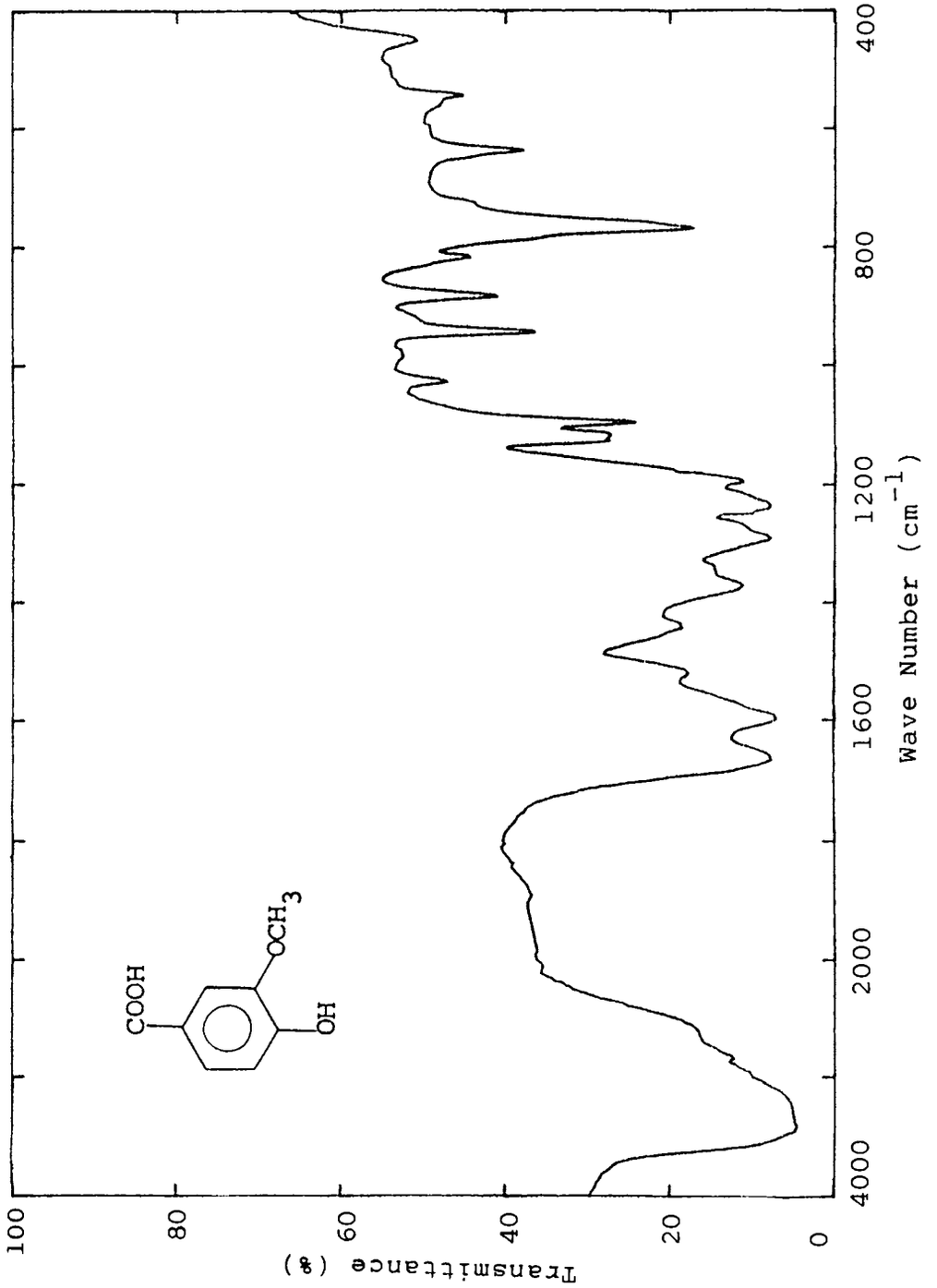


Fig.4.3.4: IR spectrum of product B

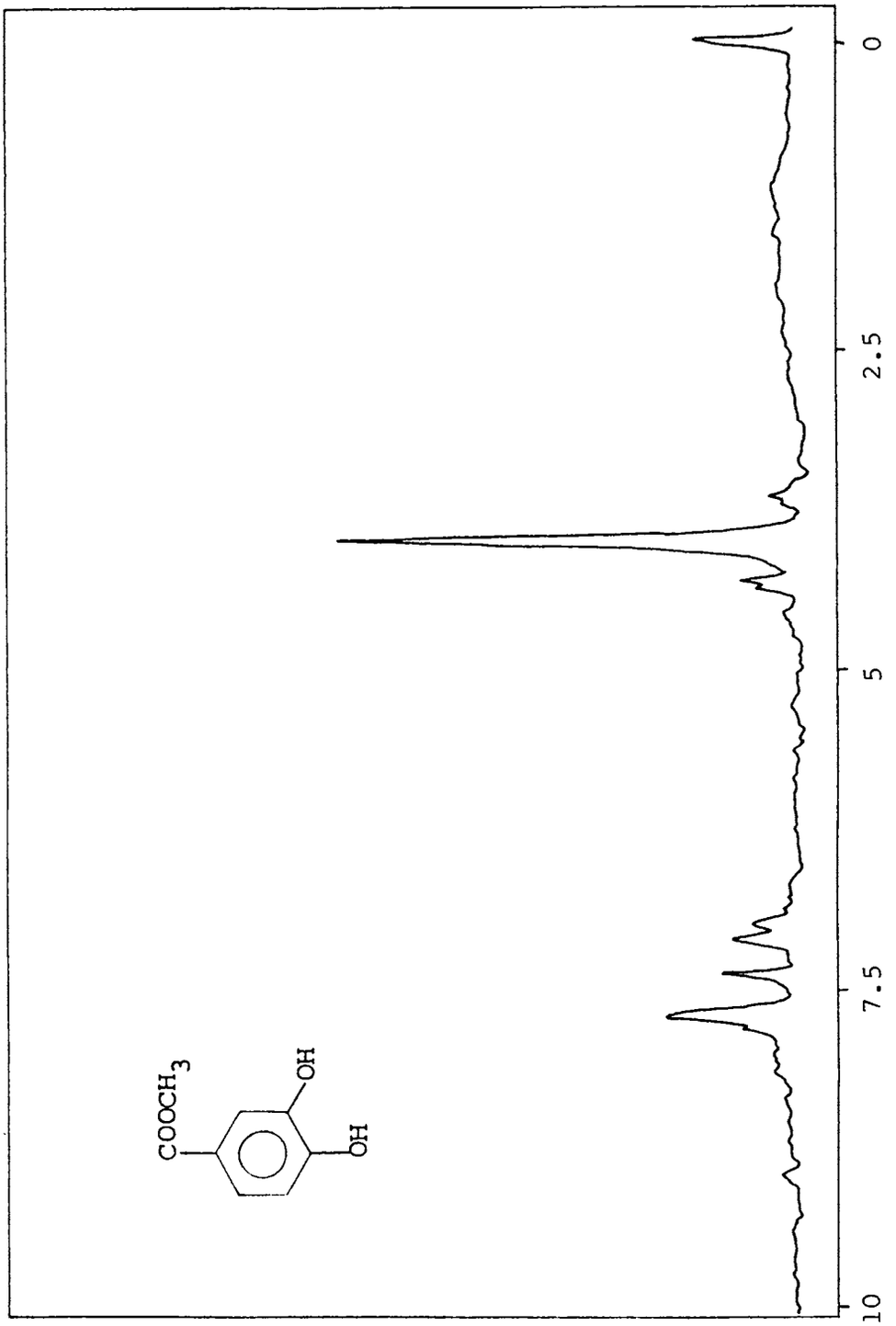


Fig.4.3.5: NMR spectrum methyl ester of product C

CHAPTER 5

MICROBIAL TRANSFORMATIONS OF ISOEUGENOL

MICROBIAL TRANSFORMATIONS OF ISOEUGENOL

5.1 INTRODUCTION

Isoeugenol was purchased and was purified by column chromatography using silica gel with hexane as the eluant. It was also prepared from eugenol by treatment with potassium hydroxide. One of the cultures isolated and identified during the studies on eugenol, Bacillus sp. CUAC 10 was found to transform isoeugenol in a mineral medium containing glucose or any other nutrient. Another strain of bacteria designated as CUAC 30 which could metabolise isoeugenol was isolated from the soil by the enrichment culture technique. This strain was able to grow in a mineral medium containing isoeugenol as the sole carbon source. It was identified as belonging to Pseudomonas sp. from its morphological and biochemical characters (Table 5.1). The strain Pseudomonas sp. CUAC 10 isolated during the studies on eugenol was found not able to metabolise isoeugenol.

5.2 STUDIES WITH THE BACILLUS Sp. CUAC 10

This strain was not able to grow in a mineral broth containing isoeugenol as the sole carbon source.

So the studies on the fermentation of isoeugenol by the Bacillus sp. CUAC 10 were conducted in a mineral medium containing 1% (w/v) glucose and 0.1% (v/v) isoeugenol (unless otherwise stated).

5.2.1 Growth studies

As the effects of concentration of sodium chloride, different nitrogen sources, pH and temperature on the growth of this strain were studied earlier in the case of fermentation of eugenol, these were not done here. However the effect of concentration of isoeugenol on the growth of the organism was studied and the results are presented in Table 5.2.1. As in the case of eugenol, an increase in the concentration of isoeugenol resulted in a retarding growth of the organism, but the effect was comparatively less. The retarding effect was more pronounced with concentrations above 0.15% (v/v) isoeugenol concentration.

5.2.2 Effect of physico-chemical factors on the transformation of isoeugenol

5.2.2.1 Effect of concentration of sodium chloride

The results of the studies on the effect of concentration of sodium chloride on the transformation of

isoeugenol are presented in Table 5.2.2. The addition of sodium chloride had not much effect on the transformation upto 0.5% (w/v). But further addition of NaCl caused a decrease in transformation.

5.2.2.2 Effect of concentration of glucose

The optimum concentration of glucose for the maximum conversion of isoeugenol was 1% (w/v), as can be seen from the results presented in Table 5.2.3. When the concentration of glucose was decreased to 0.5% (w/v), there was a corresponding decrease in the transformation of isoeugenol. When the concentration was increased to 2% (w/v) also, there was a slight decrease in the rate of transformation of isoeugenol.

5.2.2.3 Effect of different nitrogen sources

Compared to other nitrogen sources tested ammonium sulphate favoured more conversion of isoeugenol, as Table 5.2.4 indicated. When the ammonium salts were replaced by alkali metal nitrates, the transformation was comparatively low.

5.2.2.4 Effect of pH

The results of the effect of pH on transformation of isoeugenol, presented in Table 5.2.5, indicated that the optimum pH was 7.0. When the pH of the broth was reduced to 6.0, there was a sudden decrease in the transformation of isoeugenol. The decrease in the rate of transformation was less when the pH was raised to 8.0.

5.2.2.5 Effect of temperature

As presented in Table 5.2.6, the results indicated that the optimum temperature was 30°C. Though there was a drastic decrease in the rate of transformation when the temperature was decreased to 15°C, there was no appreciable decrease in the transformation when the temperature was raised upto 45°C.

5.2.3 Growth curve

The growth of the culture was monitored at different intervals of time, after incorporating the optimal conditions. The growth curve (Fig.5.2.1) showed that the culture was in a lag phase during the first

four hours. Then the logarithmic phase of growth started. The culture was in a stationary phase from 18 to 21 hours, after which was the death phase. Thus the growth curve was very similar to that when eugenol was used as the substrate for fermentation.

5.2.4 Fermentation of isoeugenol

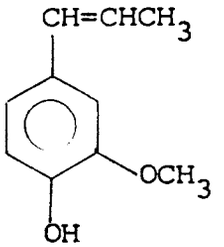
The studies on the effect of different physico-chemical factors on the growth of the bacteria and transformation of isoeugenol led to the optimal parameters for the fermentation of isoeugenol by the Bacillus sp. CUAC 10. Isoeugenol was fermented in a mini jar fermentor, incorporating these optimal parameters.[165] The mineral medium was supplemented with 1% (w/v) glucose and 0.11% (v/v) isoeugenol. During the course of fermentation, 10 ml samples of the fermentation broth were withdrawn from the fermentor periodically and analysed. At the end of 18 hours about 41% dehydrodiisoeugenol (DHDI) and 25.5% bisdehydroisoeugenol (BDHI) were formed. The yields of DHDI and BDHI were increased to 46.5% and 28.7% respectively at the end of 24 hours. There was not any considerable transformation after that.

5.2.5 Isolation and identification of bisdehydroisoeugenol and dehydrodiisoeugenol

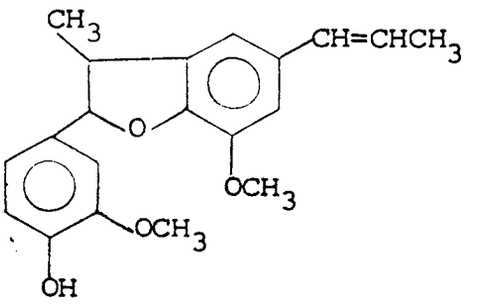
The fermentation broth was centrifuged and the supernatent was acidified and extracted with ether. The ether extract was dried with anhydrous sodium sulphate and evaporated under reduced pressure. A small portion of it was subjected to thin layer chromatography, which showed the presence of two spots in addition to the spot of isoeugenol. The rest of the residue from the ether extract was chromatographed over a column of silica gel using chloroform : hexane (3:1) as the eluant. Two products (IE_{20} and IE_{30}) were obtained as white solids which were recrystallised from chloroform-hexane mixture.

The product IE_{20}, the less polar of the two, had a melting point of 133°C. The NMR spectrum (Fig.5.2.3) of this compound was significantly different from the NMR spectrum of isoeugenol (Fig.5.2.2). The number of aromatic protons corresponding to each $-OCH_3$ group in the product molecule was less compared to isoeugenol (1). In the case of isoeugenol the number of aromatic protons corresponding to each $-OCH_3$ group is three. The molecular weight of the product was determined as 326 by mass spectrometry.

This together with the absence of one aromatic proton in the product molecule revealed that there was an oxidative coupling of two isoeugenol molecules. The elemental analysis also agreed with this view. In the NMR spectrum the aromatic protons appeared at δ 6.8 to 7.1, methoxy group at δ 3.9 and hydroxy group at δ 5.65. Signals were also observed for -CH=CH- group (δ 6.3), CH_3-C=C- group (δ 1.8) and CH_3-C- group (δ 1.5). From these results and since the oxidative coupling of phenols are 2,2'-couplings or 2,4'-couplings,[166] the structure (2), 4-(2,3-dihydro-7-methoxy-3-methyl-5-propenyl-2-benzofuryl)guaiacol is proposed for the product IE_{20}.



(1)



(2)

Isoeugenol on treatment with ferric chloride gave two products one of which had the same melting point and Rf value (TLC) as that of IE_{20}. The structure of this product obtained by $FeCl_3$ treatment had already been established as (2), dehydrodiisoeugenol.[137,167]

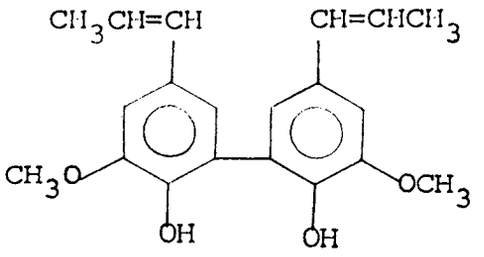
The acetylated derivative of (2), melting point 114°C, was prepared by reacting it with acetic anhydride in pyridine. The NMR spectrum of the acetate revealed that the compound was monoacetylated. The monoacetate had $-COOCH_3$ group at δ 2.3, two $-OCH_3$ groups absorbing at δ 3.8 and δ 3.9 and aromatic protons at δ 6.8 to 7.1.

The ^{13}C NMR spectrum of the product IE_{30} is shown in Fig.5.2.4. Signals were observed for the two terminal $-CH_3$ carbons (17.547 and 18.397 ppm, quartet), two $-OCH_3$ carbons (55.935 and 55.965 ppm, quartet) and $-\overset{|}{C}H-\overset{|}{C}H-$ carbons (45.628 and 93.806 ppm, doublet) and the sp^2 hybrid carbons appeared between 108 ppm and 146 ppm thus confirming the structure of (2).

The product IE_{30} had a melting point of 158°C. The NMR spectrum of the product (Fig.5.3.5) is similar to the NMR spectrum (Fig.5.2.2) of isoeugenol. But the number of aromatic protons corresponding to each $-OCH_3$ group in the product molecule is only two whereas in the case of isoeugenol the number of aromatic protons corresponding to each $-OCH_3$ group is three. The absence of one aromatic proton in the NMR spectrum suggested that there might have been an oxidative coupling at that position to give a dimer. The molecular weight determined by mass spectroscopy and elemental analysis supported this assumption. The NMR spectrum showed aromatic protons at δ 6.55-6.75, methoxy group at δ 3.80 and hydroxy group at δ 5.50. Signals were also observed for -CH=CH- group (δ 6.15) and CH_3-C=C- group (δ 1.80). Since the oxidative coupling of phenols takes place either at the ortho or para position, the position of coupling may be 6 of the isoeugenol molecule. Thus the structure (3), 3,3'-dipropenyl-5,5'-dimethoxy-6,6'-dihydroxy biphenyl is proposed for the product IE_{30}.

Isoeugenol on treatment with ferric chloride gave IE_{20} together with another product with identical melting point and Rf value (TLC) as that of IE_{30}. Phenolic compounds on treatment with one-electron oxidants such as iron (III) species give dimers formed by 2,2'-coupling and/or 2,4'-coupling.[166] Thus this product formed by the treatment of isoeugenol with $FeCl_3$ is (3), bisdehydro-isoeugenol.

The NMR spectrum of the acetylated derivative of (3), prepared by reacting it with pyridine in acetic anhydride, showed that the compound was diacetylated. The diacetate had $-COOCH_3$ group at δ 2.30, $-OCH_3$ group at δ 3.75 and aromatic protons at δ 6.65-6.90.



(3)

5.2.6 Fermentation of isoeugenol acetate

Isoeugenol acetate was fermented in the mineral medium described under section 3.2.1, supplemented with 1% (w/v) glucose and 0.1% (w/v) isoeugenol acetate. The fermentation was conducted in the mini jar fermentor and as fermentation proceeded, 10 ml samples of the broth were withdrawn periodically and analysed. After fermentation the broth was centrifuged to remove the cells and the supernatent was extracted with ether after acidifying. The ether extract was concentrated and subjected to analysis. It was found that isoeugenol, DHDI and BDHI were the products of fermentation of isoeugenol acetate with the Bacillus sp. CUAC 10. Though the rate of transformation during the initial hours of fermentation was very low, by 18 hours more than 60% of isoeugenol was fermented. The reaction was almost over by 24 hours and only traces of isoeugenol acetate remained. The yields of dehydrodi-isoeugenol, bisdehydroisoeugenol and isoeugenol at the end of 24 hours were 49%, 30.4% and 12.3% respectively.

5.2.7 Fermentation of transformation products

Two 100 ml aliquots of mineral medium, one containing 1% (w/v) glucose and 0.1% (w/v) DHDI and the

other containing 1% (w/v) glucose and 0.1% (w/v) BDHI, were inoculated with the Bacillus sp. CUAC 10 and incubated. At different intervals of time 10 ml samples were withdrawn from the flasks asceptically and analysed. No transformation products were detected in the fermentation broths which proved that DHDI and BDHI were the end products of fermentation of isoeugenol.

5.2.8 Immobilisation of whole cells

Fifteen hours old culture cells of the Bacillus sp. CUAC 10 were immobilised in calcium alginate beads and the optimal parameters for the transformation of isoeugenol with the immobilised cells were found out. The optimal pH was 8.0 and the optimal temperature 30°C. The retention time was 8 hours, at which time about 70% of isoeugenol was transformed. The immobilised cells were found to be active for more than one week.

5.2.9 Discussion

As mentioned previously this organism was isolated from the aerial flora and was identified to belong to the Bacillus sp. The growth studies of the strain were

conducted in a mineral medium containing isoeugenol as the sole carbon source. The growth of the bacteria was retarded by the addition of isoeugenol, as in the case of eugenol, but the effect was not so pronounced. When the concentration of isoeugenol in the medium was increased from 0.15% (v/v), the growth was much affected resulting in a poor growth of the organism. The effects of concentration of sodium chloride, different nitrogen sources, pH and temperature on the growth of the organism were not studied here as these studies were conducted in the case of eugenol.

The studies on the effect of various physico-chemical factors on the transformation of isoeugenol led to the optimal parameters. As in the case of eugenol, the addition of sodium chloride did not affect the transformation of isoeugenol. The optimal concentration of glucose was 1% (w/v). An increase in concentration of glucose above 1.5% (w/v) caused a decrease in the transformation of isoeugenol whereas a decrease in concentration of glucose also resulted in decreasing the rate of transformation. At higher concentrations of glucose, the products of metabolism of glucose might have inhibited the transformation

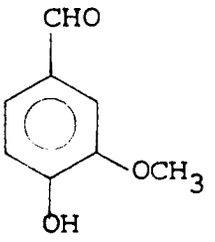
of isoeugenol. At lower concentrations of glucose the decrease in the transformation might be due to the poor growth of bacteria for lack of easily assimilable carbon source. The optimal pH value and temperature were 7.0 and 30°C. It was interesting to note that the transformation of isoeugenol was not much adversely affected at temperatures upto 45°C.

The culture attained full growth by 18 hours. During the first 10 hours of fermentation, the rate of transformation was very low. But by 24 hours the reaction was almost over with more than 80% of isoeugenol undergoing transformation. The final yields of DHDI and BDHI were 46.5% and 28.7% respectively. The fact that the culture was not able to ferment DHDI and BDHI proved that these compounds were the end products of fermentation of isoeugenol with the present strain.

When isoeugenol acetate was fermented with the Bacillus sp. CUAC 10, it was first hydrolysed to isoeugenol, which was then converted to DHDI and BDHI. As in the case with eugenol acetate, the yields of DHDI and BDHI increased when isoeugenol acetate was used as the substrate.

The studies on the transformation of isoeugenol with immobilised whole cells revealed that the optimum pH and temperature were 8.0 and 30°C. The yields of DHDI and BDHI with the immobilised cells were slightly less than that with intact cells. But the immobilised cell system has the advantage that it can be reused.

Dehydrodiisoeugenol on oxidation with nitrobenzene and alkali gives vanillin (4) as the major product along with vanillic acid (5) and 5-carboxy vanillin (6) as minor products.[162,168] The methyl ether of DHDI on oxidation with potassium permanganate gives veratric acid (7) and with nitric acid gives 4,5-dinitro veratrole (8) and trinitro veratrole (9).[169]



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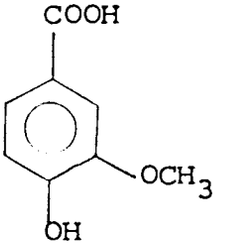

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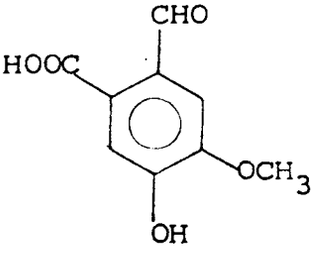

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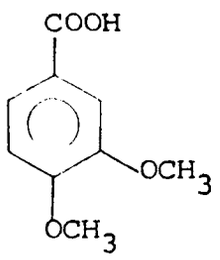

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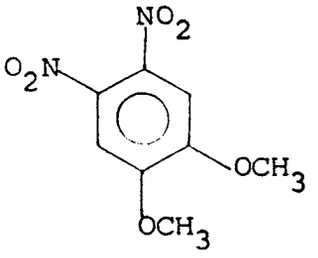

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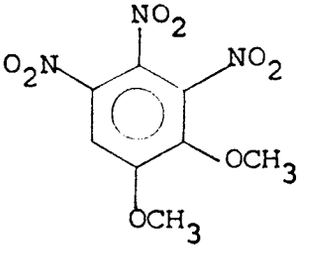


(9)

Cousin and Herissey had reported that the oxidising ferment of Russula delica converted isoeugenol to DHDI. In this study, the Bacillus sp. CUAC 10 transformed isoeugenol to dehydrodiisoeugenol and bisdehydroisoeugenol.

5.3 STUDIES WITH THE PSEUDOMONAS Sp. CUAC 30

This strain, isolated from the local soil, was able to grow in a mineral medium containing isoeugenol as the sole carbon source. Thus the studies were conducted in the mineral medium described under section 3.2.1, supplemented with 0.10% (v/v) isoeugenol (unless otherwise stated).

5.3.1 Growth studies

As the culture took 36 hours to attain full growth, the optical density readings were taken 36 hours after inoculation.

5.3.1.1 Effect of concentration of isoeugenol

The results of the effect of concentration of isoeugenol on the growth of the bacteria are given in Table 5.3.1. The optimum concentration of isoeugenol was 0.07% (v/v). As the concentration of isoeugenol increased the growth was also increased till 0.07% (v/v). Subsequent addition of isoeugenol resulted in a poor growth, the effect being more pronounced from 0.12% (v/v) onwards.

5.3.1.2 Effect of sodium chloride

The growth of the organism showed a slight increase when 0.5% (w/v) of sodium chloride was added to the medium. But further addition of NaCl caused a decrease in the growth of the organism, as can be seen from Table 5.3.2.

5.3.1.3 Effect of different nitrogen sources

The results presented in Table 5.3.3 indicated that when ammonium chloride was used as the nitrogen source there was an increase in growth. To a lesser extent urea also favoured an increase in growth of the culture.

5.3.1.4 Effect of pH

As Table 5.3.4 indicated, neutral pH favoured more growth. When the pH was decreased to 5.5, there was a sudden decrease in the growth of the organism. Raising the pH also resulted in poor growth.

5.3.1.5 Effect of temperature

The results presented in Table 5.3.5 indicated that the optimum temperature was 30°C, though there was fairly good growth at 37°C. But further increase in temperature caused a decrease in the growth of the bacteria.

5.3.2 Effect of physico-chemical factors on transformation of isoeugenol

5.3.2.1 Effect of concentration of sodium chloride

The results of the studies on the effect of concentration of sodium chloride on the transformation of isoeugenol are given in Table 5.3.6. There was no considerable difference in the transformation when sodium chloride was added to the medium upto 1% (w/v) concentration. But further addition caused a decrease in the rate of transformation.

5.3.2.2 Effect of concentration of glucose

The results presented in Table 5.3.7 indicated that when 0.2% (w/v) glucose was added to the medium, the rate of transformation of isoeugenol increased slightly during the initial stages of cultivation. But when the concentration of glucose was increased to 1% (w/v) there was a decrease in the transformation.

5.3.2.3 Effect of different nitrogen sources

As Table 5.3.8 indicated when potassium nitrate was used as the nitrogen source there was an increase in

the rate of transformation of isoeugenol. Urea also favoured a better transformation than the ammonium salts used.

5.3.2.4 Effect of pH

The results of the effect of pH on the transformation of isoeugenol presented in Table 5.3.9 indicated that the transformation was much affected by change in pH of the medium. The optimum pH was 7.0.

5.3.2.5 Effect of temperature

As can be seen from Table 5.3.10 the optimum temperature was 30°C, though there was fairly good transformation at 37°C also. But further change in temperature adversely affected the transformation of isoeugenol.

5.3.3 Growth curve

The growth of the culture was measured as optical density values periodically, incorporating the optimal conditions (Fig.5.3.1). The initial lag phase of growth

of the culture lasted for about 8 hours. The logarithmic phase of growth started by 12 hours. At 30 hours the culture attained full growth and the stationary phase lasted for 6 hours. Thus the culture was not a fast growing one, compared to Pseudomonas sp. CUAC 20.

5.3.4 Fermentation of isoeugenol

From the studies on the effect of various physico-chemical parameters on the growth of the culture and transformation of isoeugenol, the optimal conditions were obtained. Incorporating these optimal conditions, isoeugenol was fermented in a mini jar fermentor. The medium was supplemented with 0.10% (v/v) isoeugenol.

During the course of fermentation 10 ml samples of fermentation broth were withdrawn from the fermentor periodically and analysed for residual isoeugenol and transformation products. Ferulic acid, vanillin and vanillic acid were measured by determining the optical density at 310 nm, 347 nm and 293 nm respectively, as mentioned previously. At 12 hours only about 10% isoeugenol had reacted. After that there was a steady increase in the

rate of transformation and by 24 hours only less than 35% isoeugenol remained. The reaction was almost over by 36 hours. The time course of formation of products is given in Table 5.3.12. The yield of vanillin was a maximum of 6.3% at 24 hours. After that the concentration of vanillin decreased. The yields of ferulic acid and vanillic acid were maximum (8.2% and 9% respectively) at 25 and 28 hours respectively, after which the concentration of both acids decreased.

5.3.5 Isolation and identification of products

The fermentation broth was centrifuged to remove the cells and the supernatent was acidified with 2 N sulphuric acid and extracted with ether. The ether extract was re-extracted with 5% aqueous sodium bicarbonate solution to remove strongly acidic products.

The ether layer containing non-acidic products was concentrated under reduced pressure after drying with anhydrous sodium sulphate. It was then chromatographed over a column of silica gel with chloroform : hexane (3:1) as the eluant. The product, obtained as a white solid, was recrystallised from chloroform-hexane mixture. It had a

melting point of 81°C. It showed positive colouration with 2,4-dinitrophenyl hydrazine-H_2SO_4. The IR spectrum of the product indicated the presence of hydroxyl group (3560-3520 cm^{-1}), aldehyde group (1680-1710 cm^{-1}) and aromatic ring (1615-1600 cm^{-1}). The IR and NMR spectra completely matched those of vanillin. Thus the product was identified as 4-hydroxy-3-methoxybenzaldehyde (vanillin).

The sodium bicarbonate layer was acidified with 2 N sulphuric acid to pH 2 and extracted again with ether. The ether layer was dried with anhydrous sodium sulphate and concentrated under reduced pressure. A small portion of it was subjected to thin layer chromatography which revealed the presence of three products. The mixture was then subjected to column chromatography with ethyl acetate : hexane (3:1) as the eluant. Three products (A, B & C) were obtained which when recrystallised from water-acetone mixture gave white solids.

The product A melted at 170°C and its methyl ester melted at 63°C. The NMR spectrum of the ester showed the presence of $-COOCH_3$ group, $-OCH_3$ group, -CH=CH-group, -OH group and aromatic protons, which led to the conclusion

inoculated with Pseudomonas sp. CUAC 30 and incubated an increase in turbidity was observed indicative of the growth of the organism. The fermentation broth was extracted with ether and on analysis of the ether extract it was found that these compounds were metabolised. This showed that these compounds were intermediates in the degradation pathway of isoeugenol.

5.3.7 Fermentation of isoeugenol acetate

Isoeugenol acetate was fermented in the mineral medium described under section 3.2.1, supplemented with 0.10% (w/v) isoeugenol acetate. The products of fermentation were isoeugenol, ferulic acid, vanillin, vanillic acid and protocatechuic acid. Only traces of isoeugenol were found in the broth after fermentation. The yields of the other products were the same as in the case of fermentation of isoeugenol.

5.3.8 Mode of aromatic ring cleavage

The studies on the fermentation of isoeugenol and transformation products of isoeugenol suggested that protocatechuic acid was the final aromatic compound in the

metabolic pathway of isoeugenol. But PCA was further metabolised by the strain. The experiment to find the mechanism of aromatic ring cleavage revealed that PCA was cleaved by meta-fission.

5.3.9 Immobilisation of whole cells

Culture cells, 24 hours old, were immobilised in calcium alginate beads. The optimum pH and temperature for the transformation of isoeugenol with the immobilised whole cells were 7.5 and 30°C respectively. The retention time was 25 hours when about 79% of isoeugenol was found to be transformed but there was no increase in the quantity of the products formed.

5.3.10 Enzyme studies

The cell free extract of the culture was used as the source of enzymes. The optimum pH and temperature for the transformation of isoeugenol were 8.0 and 30°C respectively. Isoeugenol, ferulic acid, vanillin, vanillic acid and protocatechuic acid were all degraded by the cell free extracts. When CFE was added to protocatechuate solution it was found to strongly absorb at 410 nm indicating the meta-cleavage of PCA to form γ-carboxy-α-hydroxy-cis,cis-muconic acid semialdehyde.

increased to 2% (w/v), the transformation was found to decrease. Very low concentrations of glucose slightly increased the rate of transformation of isoeugenol. But when the concentration of glucose was increased to 1% (w/v), the transformation was adversely affected. This might be due to the reason that the products of metabolism of glucose, which was an easily assimilable carbon source, affected the transformation of isoeugenol. Of the different nitrogen sources tested, though ammonium chloride and urea favoured more growth of the organism, the rate of transformation of isoeugenol was more when potassium nitrate was used. The optimum pH and temperature for maximum conversion of isoeugenol were 7.0 and 30°C respectively. The bacteria was very sensitive to pH and temperature since a change in these parameters affected the transformation considerably.

The present strain was not a fast growing one as it took 30 hours to attain maximum growth. The rate of transformation of isoeugenol during the initial 10 hours was very low. After 12 hours the rate of transformation increased and by 36 hours the reaction was complete. The concentration of ferulic acid and vanillin were maximum at

25 and 24 hours respectively, when 8.2% ferulic acid and 6.3% vanillin yielded. After that the concentration decreased as they were further oxidised to vanillic and protocatechuic acids. PCA was subsequently metabolised by the cleavage of the aromatic ring.

This strain assimilated isoeugenol acetate also. Isoeugenol acetate was first hydrolysed to isoeugenol which was subsequently metabolised to ferulic acid, vanillin, vanillic acid and protocatechuic acid. But the bacteria was not able to assimilate eugenol or eugenol acetate.

The cell free extracts of the culture degraded ferulic acid, vanillin, vanillic acid and protocatechuic acid, which proved that these compounds were intermediates in the degradation pathway of isoeugenol. The test to determine the aromatic ring cleavage mechanism showed that PCA was metabolised by the meta-cleavage of the ring. The presence of the enzyme catalysing the meta-cleavage of PCA in the organism, protocatechuic acid 4,5-oxygenase, was further confirmed by the strong absorption at 410 nm when protocatechuate solution was added to the cell free extract. PCA then, undergoes meta-fission to form

γ-carboxy-∝-hydroxy-cis,cis-muconic acid semialdehyde (CHMS) which absorbs at 410 nm. CHMS turns yellow spontaneously under alkaline conditions in the colorimetric test for the determination of aromatic ring cleavage mechanism.[170]

From the above results, the following pathway is proposed for the degradation of isoeugenol by the Pseudomonas sp. CUAC 30.

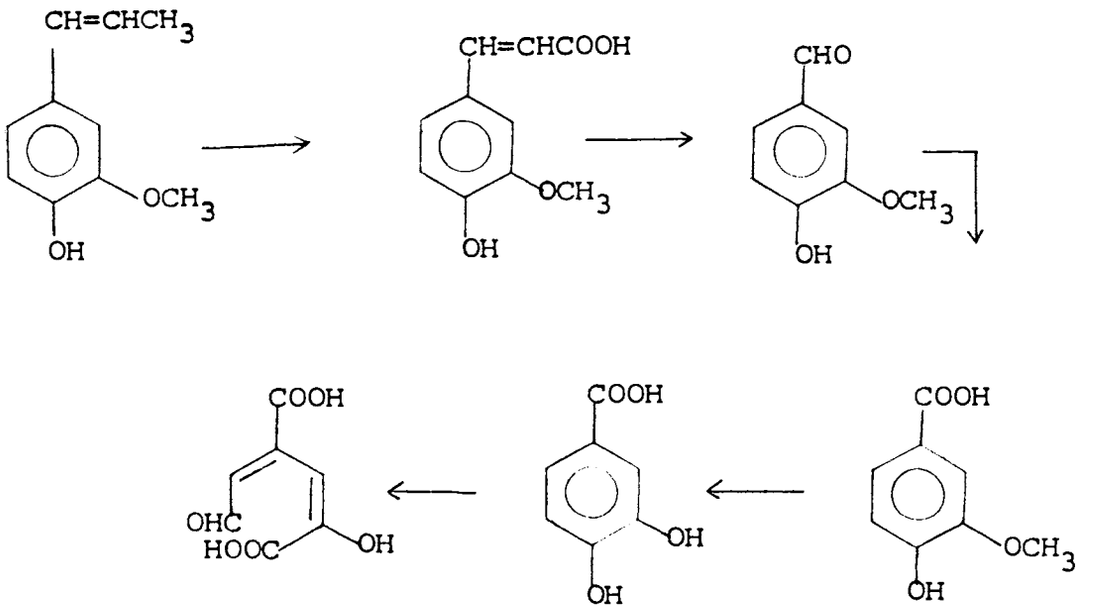


Table 5.1: Identification tests for the strain CUAC 30

Tests	Results
Form and arrangement	Small rods; no characteristic arrangement
Motility	Positive
Chromogenesis in nutrient agar	Pale green colour
Gram reaction	Negative
Spore formation	Negative
Production of indole	Negative
H_2S production	Negative
Oxidation-fermentation test	Oxidative
Gas production from glucose	Positive
Citrate utilisation	Negative
Nitrate reduction	Negative
Cytochrome oxidase	Positive
Catalase	Positive
Gelatinase	Negative
Amylase	Positive
Caseinase	Negative

Table 5.2.3: Effect of glucose on transformation of isoeugenol

Glucose (%, w/v)	Residual Ioseugenol (%, v/v)					
	0 hr	6	12	18	24	36
0.5	100	97.78	88.65	43.64	33.18	30.19
1.0	100	96.20	84.05	27.43	18.92	18.07
1.5	100	97.00	86.64	32.57	22.76	20.13
2.0	100	98.16	88.72	40.05	29.83	27.61

Table 5.2.4: Effect of different nitrogen sources on transformation of isoeugenol

Nitrogen source	Residual Isoeugenol (%, v/v)					
	0 hr	6	12	18	24	36
NH_4NO_3	100	96.28	84.00	28.21	19.25	18.12
NH_4Cl	100	96.19	85.17	30.41	22.66	20.50
$(NH_4)_2SO_4$	100	96.42	84.19	27.12	18.04	16.83
$(NH_2)_2CO$	100	97.08	86.37	32.21	25.17	23.45
KNO_3	100	96.92	87.15	32.14	26.32	24.26
$NaNO_3$	100	97.00	86.76	32.43	25.84	24.07

Table 5.2.5: Effect of pH on transformation of isoeugenol

pH	Residual Isoeugenol (%, v/v)					
	o hr	6	12	18	24	36
6.0	100	97.73	88.50	48.83	42.76	41.22
6.5	100	97.18	85.91	35.42	30.99	29.78
7.0	100	96.40	83.77	28.06	18.92	18.06
7.5	100	96.28	84.18	27.95	22.74	21.85
8.0	100	96.53	84.37	30.16	26.13	25.44

Table 5.2.6: Effect of temperature on transformation of isoeugenol

Temperature (°C)	Residual Isoeugenol (%, v/v)					
	0 hr	6	12	18	24	36
15	100	99.00	95.23	84.52	72.57	68.39
30	100	95.98	83.73	28.06	19.14	18.18
37	100	96.52	84.17	28.81	21.96	21.65
45	100	96.64	84.87	31.25	25.41	24.73
56	100	97.43	85.53	36.17	30.94	30.12

Table 5.2.7: Growth curve

Time (hr)	0	2	4	6	9	12	15	18	21	24
Optical density	0	0.022	0.038	0.186	0.515	0.827	0.901	0.921	0.920	0.914

Table 5.3.1: Effect of concentration of isoeugenol on growth

Isoeugenol (%, v/v)	0	0.01	0.03	0.05	0.07	0.10	0.12	0.15	0.17	0.20
Optical density	0	0.071	0.206	0.329	0.457	0.441	0.394	0.308	0.235	0.102

Table 5.3.2: Effect of concentration of sodium chloride on growth

Sodium chloride (%, w/v)	0	0.5	1.0	2	3	5	7	10
Optical density	0.444	0.452	0.450	0.428	0.392	0.310	0.206	0.901

Table 5.3.3: Effect of different nitrogen sources on growth

Nitrogen source	NH_4NO_3	NH_4Cl	$(NH_4)_2SO_4$	$(NH_2)_2CO$	KNO_3	$NaNO_3$
Optical density	0.445	0.468	0.447	0.462	0.438	0.434

Table 5.3.4: Effect of pH on growth

pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Optical density	0.157	0.316	0.408	0.440	0.426	0.400	0.326	0.228

Table 5.3.5: Effect of temperature on growth

Temperature	15	30	37	45	56	70
Optical density	0.289	0.444	0.421	0.354	0.260	0.128

Table 5.3.6: Effect of sodium chloride on transformation of isoeugenol

Sodium chloride (%, w/v)	Residual Isoeugenol (%, v/v)					
	0 hr	12	18	24	36	48
0	100	88.17	66.00	31.82	2.11	0.95
0.5	100	88.45	65.57	31.63	2.08	1.13
1.0	100	89.51	66.39	32.76	2.90	1.48
2.0	100	91.10	68.42	34.91	6.82	5.15

Table 5.3.7: Effect of concentration of glucose on transformation of isoeugenol

Glucose (%, w/v)	Residual Isoeugenol (%, v/v)					
	0 hr	12	18	24	36	48
0.2	100	87.46	63.21	29.87	2.16	0.89
0.5	100	88.22	66.17	32.42	2.83	1.15
1.0	100	99.26	68.91	35.44	6.75	5.38

Table 5.3.8: Effect of different nitrogen sources on transformation of isoeugenol

Nitrogen Source	Residual Isoeugenol (%, v/v)					
	0 hr	12	18	24	36	48
NH_4NO_3	100	88.11	65.86	32.02	1.95	0.98
NH_4Cl	100	88.47	66.23	32.71	2.15	1.12
$(NH_4)_2SO_4$	100	89.24	66.81	33.25	2.83	1.79
$(NH_2)_2CO$	100	87.15	65.14	30.99	1.74	0.58
KNO_3	100	87.04	63.42	29.76	0.82	0
$NaNO_3$	100	87.55	64.67	30.90	1.50	0.71

5.3.9: Effect of pH on transformation of isoeugenol

pH	Residual Isoeugenol (%, v/v)					
	0 hr	12	18	24	36	48
6.5	100	90.97	70.18	40.83	14.50	11.83
7.0	100	88.32	65.78	32.17	2.06	1.16
7.5	100	88.81	67.00	35.38	5.42	4.09
8.0	100	90.14	70.13	40.00	14.52	11.25

Table 5.3.10: Effect of temperature on transformation of isoeugenol

Temperature (°C)	Residual Isoeugenol (%, v/v)					
	0 hr	12	18	24	36	48
15	100	96.67	88.39	79.22	61.73	49.68
30	100	88.30	65.84	32.13	1.98	0.83
37	100	88.82	67.19	34.85	5.07	3.74
45	100	90.93	76.45	50.00	33.18	20.00
56	100	94.98	84.32	73.77	55.15	44.62

Table 5.3.11: Growth curve

Time (hr)	0	6	10	15	18	21	24	30	36	48
Optical density	0	0.037	0.092	0.189	0.297	0.364	0.425	0.443	0.443	0.431

Table 5.3.12: Time course of formation of products

Product	Concentration (mg/100 ml)					
	0 hr	12	18	24	30	36
Ferulic acid	0	3.2	5.8	7.9	5.5	3.3
Vanillin	0	1.1	3.9	6.3	5.4	1.6
Vanillic acid	0	0.7	3.8	8.3	7.8	5.1

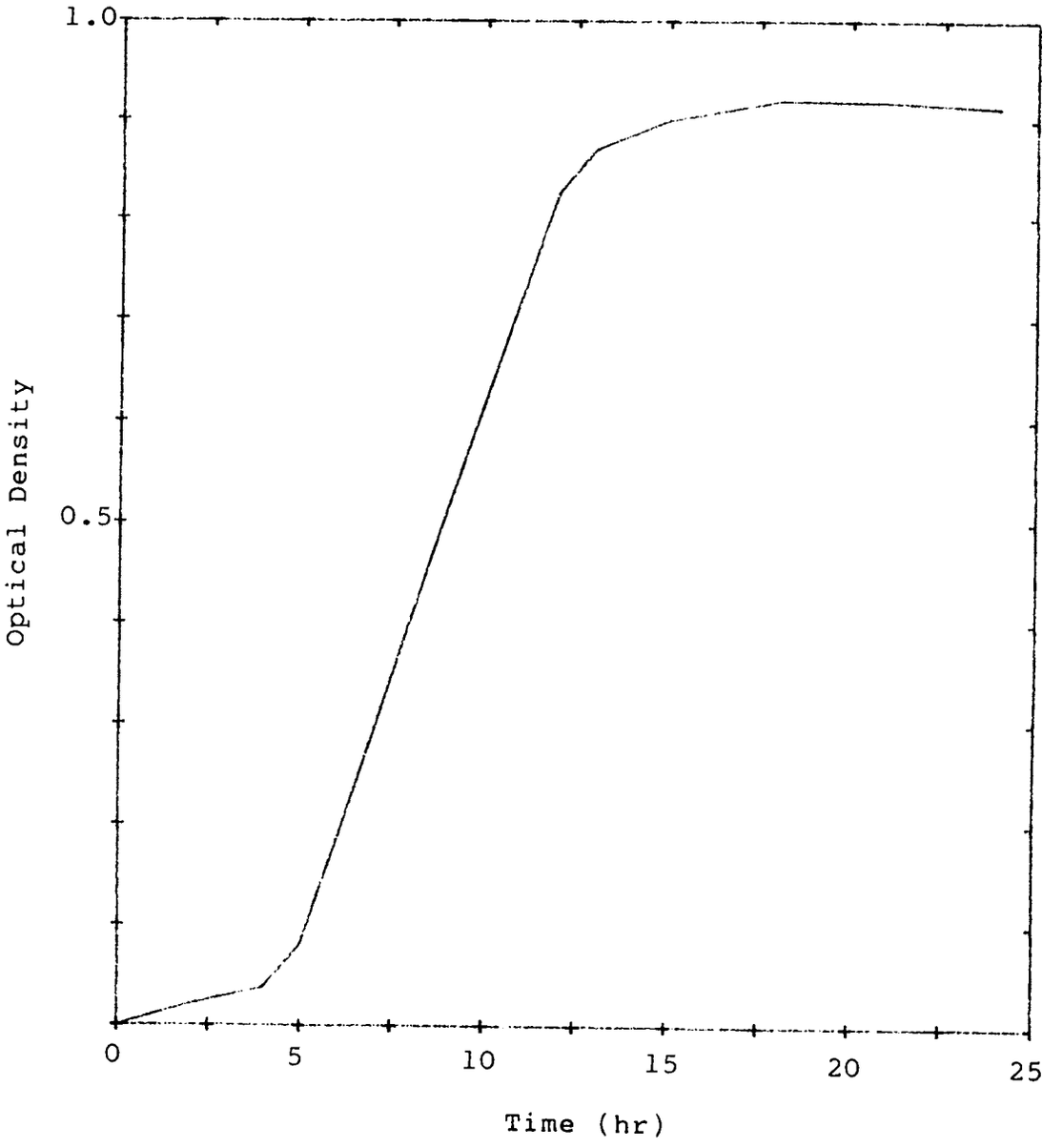


Fig.5.2.1: Growth curve of CUAC 10 in isoeugenol medium

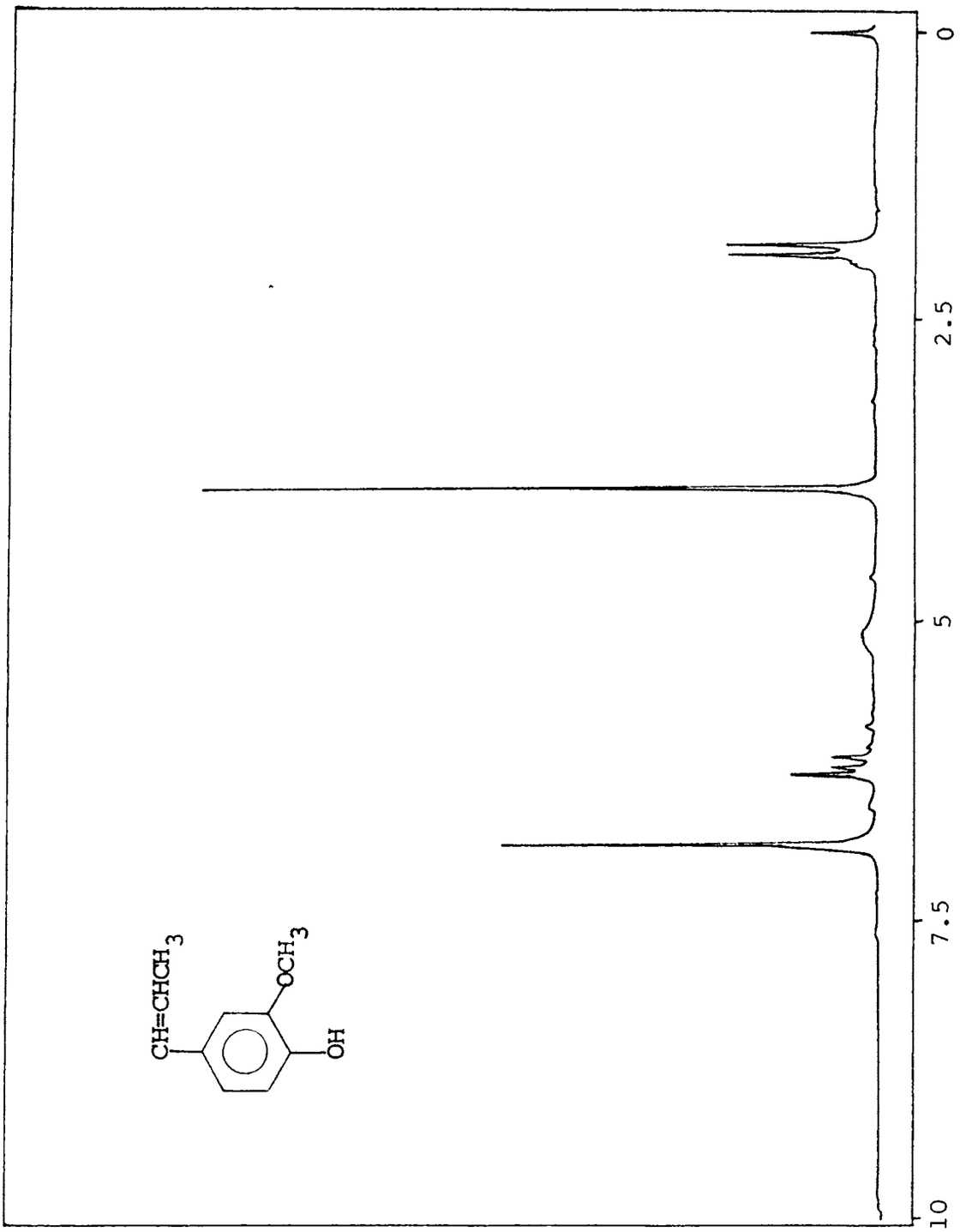


Fig.5.2.2: NMR spectrum of isoeugenol

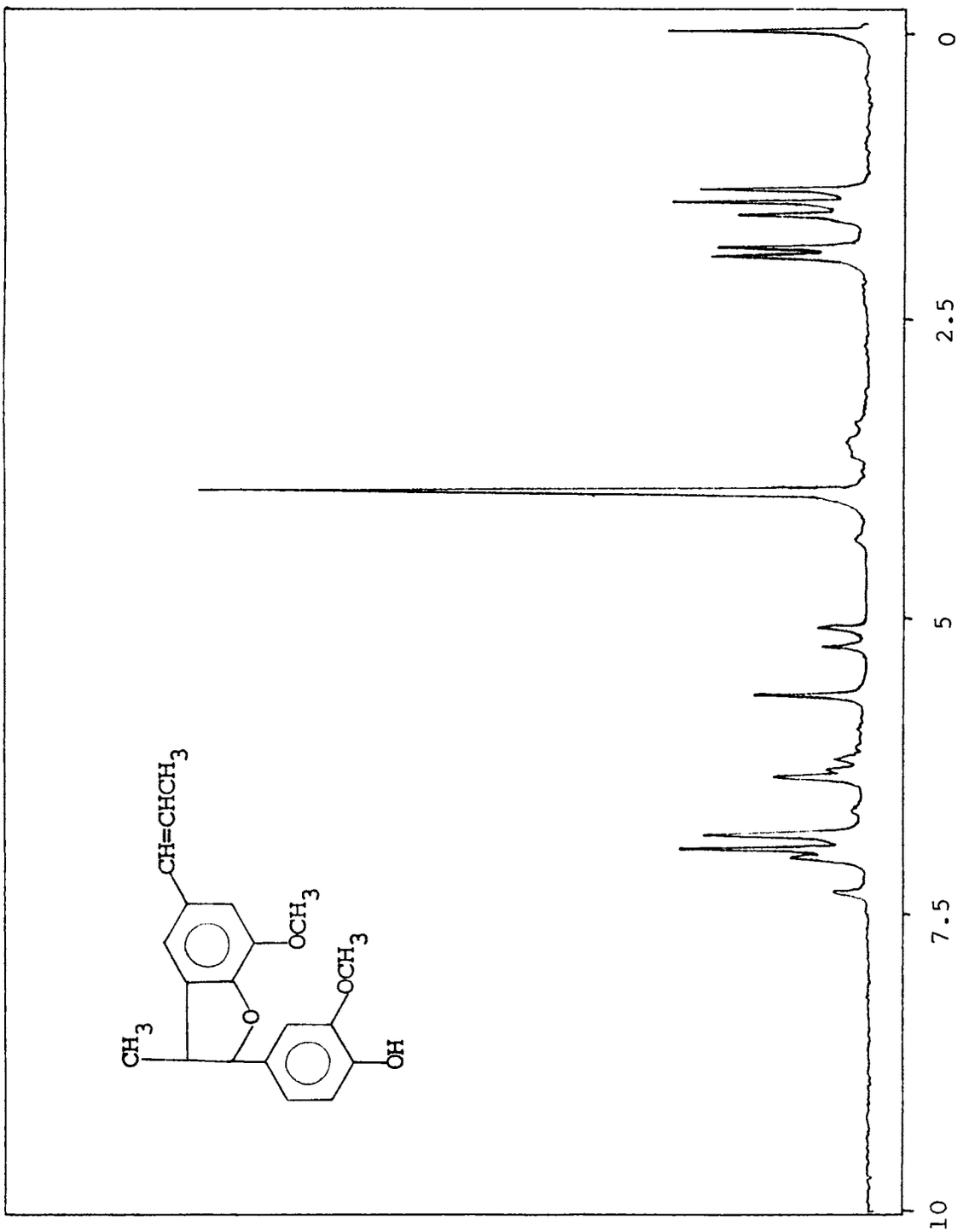
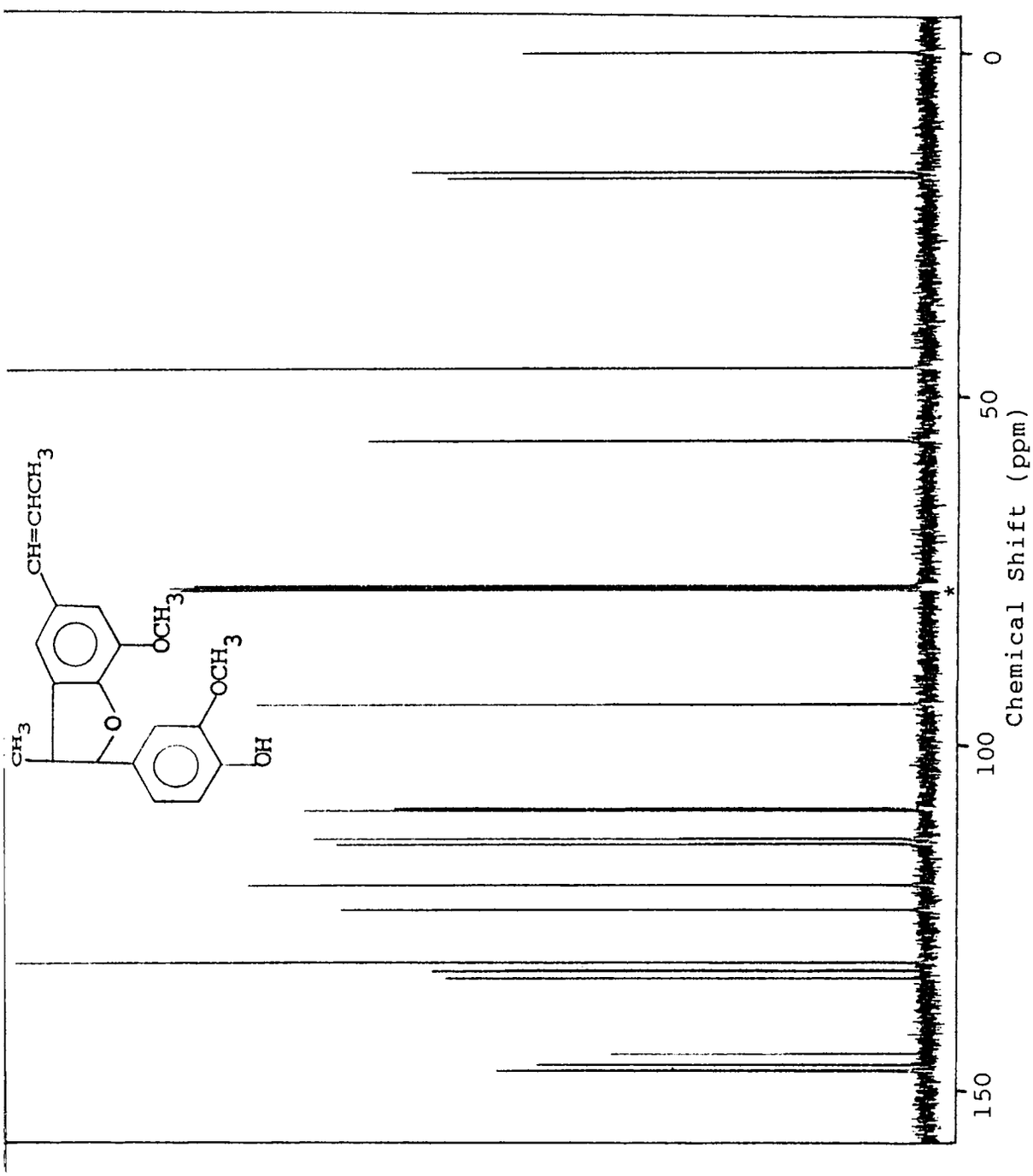


Fig.5.2.3: NMR spectrum of product IE_{20}



* Signals due to the solvent, $CDCl_3$

Fig.5.2.4: ^{13}C NMR spectrum of product IE_{20}

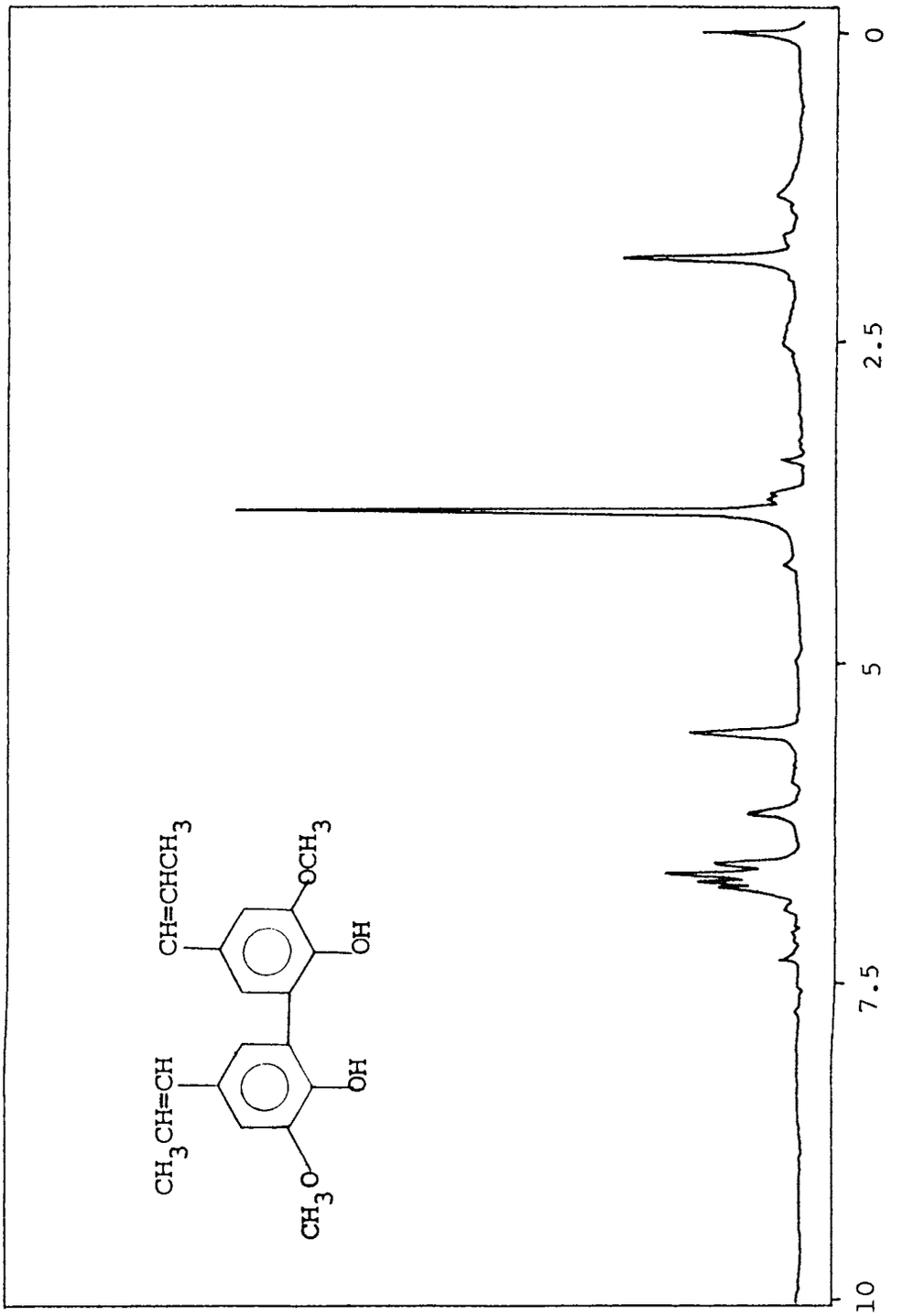


Fig.5.2.5: NMR spectrum of product IE_{30}

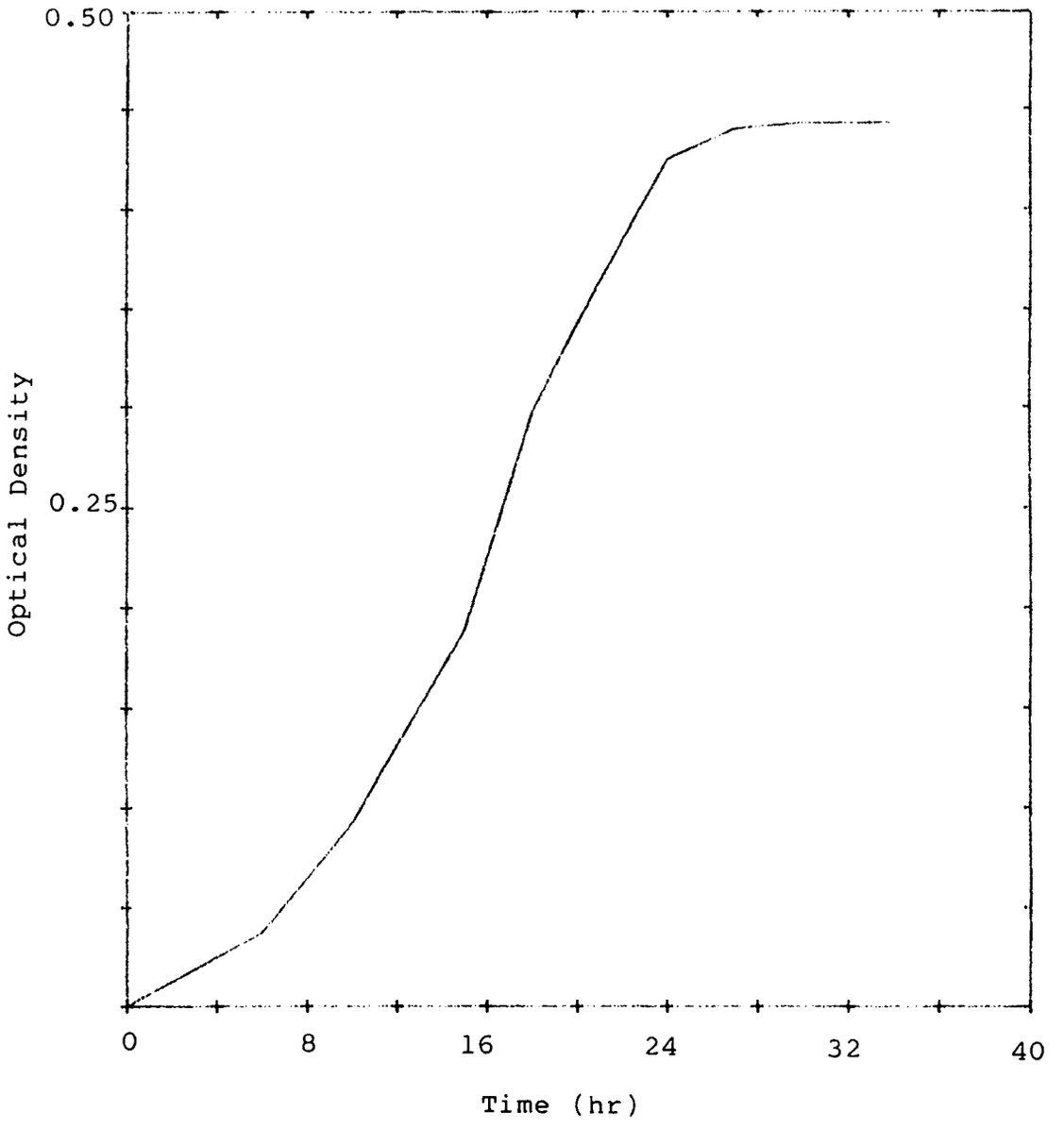


Fig.5.3.1: Growth curve of CUAC 30

CHAPTER 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Microbial transformations of organic compounds have been one of the most significant developments in the field of synthetic organic chemistry in recent years. They display far greater specificities than conventional organic reactions and have great potential. The production of commodity chemicals is currently one of the fastest developing fields where microbial transformations are applied. In this work, microbial transformations of eugenol and isoeugenol are undertaken with a view to converting them into more useful products like vanillin. Eugenol and isoeugenol, both terpene-related aromatic compounds, are the main constituents of many essential oils like clove oil, cinnamon oil and ylang-ylang oil. Vanillin, an important flavouring agent, is manufactured from eugenol or isoeugenol using chemical methods.

Since the stock cultures available in our laboratory were found to be inactive on both eugenol and isoeugenol, organisms that transform these substrates were isolated from the aerial and soil flora. These organisms were purified and identified upto the generic level based

on their morphological and biochemical characters. Of the three organisms isolated, one belonged to the Bacillus sp. and the other two to the Pseudomonas sp. They were kept on nutrient agar and mineral agar slants, respectively. The effect of various physico-chemical factors like substrate concentration, sodium chloride concentration, presence of different nitrogen sources, pH and temperature and the influence of an additional carbon source like glucose on the growth of organism and the transformation of the substrates were extensively studied to obtain the optimal conditions. The growth of the organism was monitored periodically to obtain the growth curve and a correlation was obtained between the growth rate of the organism and the transformation rate of the substrate.

After incorporating the optimal conditions the substrate was fermented in a mini jar fermentor. The progress of fermentation was monitored by withdrawing samples from the broth periodically and subjecting them to analysis by chromatography. After fermentation the broth was made acidic by adding dilute sulphuric acid and extracted with solvent ether. The ether extract, after concentrating under reduced pressure, was subjected to

column chromatography to separate the products. The products were purified by recrystallisation from appropriate solvents. The pure products were identified by analysis of their spectral data such as UV, IR, NMR and mass spectra and also by comparison of their physical constants like melting points and Rf values (TLC) with authentic samples. Suitable derivatives like acetates or methyl esters of these products were also prepared and characterised.

The Bacillus sp. CUAC 10 was not able to grow in a mineral medium containing eugenol or isoeugenol as the sole carbon and energy source. However, the organisms did grow when an easily assimilable carbon source like glucose or another nutrient was also added to it. This strain converted eugenol to bisdehydroeugenol (BDHE) in 45.4% yield. The yield of BDHE increased to 47.5% when eugenol acetate was used as the substrate. The bacteria transformed isoeugenol to dehydrodiisoeugenol (DHDI) and bisdehydroisoeugenol (BDHI) with yields of 46.5% and 28.7% respectively. As in the case of eugenol acetate, the yields of these products increased to 49% and 30.4% respectively when isoeugenol acetate was used as the substrate. Fifteen hours old culture cells were immobilised

in calcium alginate beads and transformations of eugenol and isoeugenol were performed using these immobilised whole cells. Optimum pH and temperature were found out for the maximum conversion of eugenol and isoeugenol with the immobilised cells. Though the extent of transformation with the immobilised cells was less than that with the intact cells, the retention time was less and the immobilised cells could be used continuously for more than one week without considerable depreciation in the activity of the cells.

The Pseudomonas sp. CUAC 20 was able to grow in a mineral medium utilising eugenol as the sole carbon and energy source. But it was not able to metabolise isoeugenol The products of the metabolism of eugenol were isolated by column chromatography and identified as ferulic acid, vanillin, vanillic acid and protocatechuic acid. The protocatechuic acid was subsequently metabolised via ortho-cleavage of the aromatic ring by the enzyme protocatechuic acid 3,4-oxygenase to β-carboxy-cis, cis-muconic acid semialdehyde which was then further degraded to β-ketoadipic acid. The maximum yield of vanillin was only 4.62% which was noted at 17 hours after inoculation. The maximum

yields of ferulic acid and vanillic acid were 6.25% and 8.9%, noted at 19 and 23 hours respectively. After that time the concentration of all these products decreased due to further oxidation. Several experiments including fermentation of eugenol acetate and addition of glucose as an additional carbon source, were tried for the improved production of vanillin. But the results were not promising. Culture cells, 18 hours old, were immobilised in calcium alginate beads and eugenol was transformed with the immobilised cells. The pH and temperature were optimised for the maximum conversion of eugenol. The cell free extracts of the culture were prepared and the transformation of eugenol with the CFE was also studied. From these data the degradation pathway of eugenol with the present strain was proposed. Eugenol was then fermented with Pseudomonas aeruginosa reported earlier which gave only 1.47% vanillin and the results were compared with that of present strain.

The Pseudomonas sp. CUAC 30 was able to assimilate isoeugenol as the sole carbon and energy source in a mineral medium. Isoeugenol was fermented in the mini jar fermentor, incorporating the results obtained from the optimisation studies. The products of fermentation were

isolated by column chromatography and identified as ferulic acid, vanillin, vanillic acid and protocatechuic acid. Protocatechuic acid was subsequently metabolised via meta-cleavage of the aromatic ring by the enzyme protocatechuic acid-4,5-oxygenase to γ-carboxy-α-hydroxy-cis,cis-muconic acid semialdehyde. The maximum yield of vanillin (6.3%) was observed at 24 hours after inoculation of the culture. The yields of ferulic acid (8.2%) and vanillic acid (9.0%) were maximum at 25 and 28 hours respectively. The concentration of all these products subsequently decreased due to further oxidation, as in the case of eugenol. Twentyfour hours old culture cells were immobilised in calcium alginate beads and transformation studies were carried out on isoeugenol with the immobilised cells. The cell free extract of the strain was prepared and isoeugenol was fermented with the CFE. The strain also assimilated isoeugenol acetate, and the products and their yields were almost the same as that with isoeugenol. From these results, the degradation pathway of isoeugenol with the present strain was proposed.

Thus the present work led to the isolation and identification of three new strains of bacteria which could transform eugenol, isoeugenol and their acetates.

The products of transformation of these compounds were isolated, purified and identified. The physico-chemical parameters were optimised for the maximum conversion of these substrates. The pathway proposed earlier for the degradation of eugenol was confirmed and a pathway was proposed for the degradation of isoeugenol. Though the yields of the products formed by the oxidative coupling of the substrates were substantial, that of the products formed by the oxidative degradation of the substrates especially vanillin were not satisfactory.

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