CHAPTER - 2

REVIEW OF LITERATURE
2.1 Metabolic Engineering

Altering metabolic pathways to improve cell properties and the chances of cell survival is as old as nature itself. Techniques such as genetic modifications via random mutagenesis have yielded, for example, improved strains of *Corynebacterium glutamicum* and its related species *Brevibacterium lactofermentum* and *Brevibacterium flavum* that excrete large amounts of amino acids into the fermentation medium (Kinoshita *et al*., 1958). Random mutagenesis relies heavily on chemical mutagens and creative selection techniques to identify superior strains for achieving a certain objective. Such traditional genetic approaches for strain improvement have been applied extensively in the past also in the areas of antibiotics, solvents, and vitamin production among others.

In this context, metabolic engineering has emerged as the technological and scientific discipline dealing with the introduction of specific modifications to metabolic pathways to improve cellular properties. Metabolic engineering is generally referred to as the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This multidisciplinary field draws principles from chemical engineering, computational sciences, biochemistry, and molecular biology. In essence, metabolic engineering is the application of engineering principles of design and analysis to the metabolic pathways in order to achieve a particular goal. This goal may be to increase process productivity, as in the case in production of antibiotics, biosynthetic precursors or polymers, or to extend metabolic capability by the addition of extrinsic activities for chemical production or degradation. Previous strategies to attain these goals seem more of an art with experimentation by trial-and-error.

In terms of DNA techniques, several approaches have been used for the modification of the host cell to achieve the desired goal. These include: the removal of the enzyme or its inhibition to eliminate a competitive pathway (Green *et al*., 1996; Shimada *et al*., 1998) or a toxic by-product (Aristidou *et al*., 1994), the amplification of a gene or group of genes to improve the synthesis of existing products (Pines *et al*., 1997; Shimada *et al*., 1998), the expression of an heterologous enzyme(s) to extend the substrate range, to produce novel product (Hershberger *et al*., 1996; Ingram *et al*., 1998),
to provide pathways for the degradation of toxic compounds (Xu et al., 1996), or to design a more environmentally resistant plant. Alternatively, the deregulation of existing enzymes might be necessary to overcome the existing control mechanisms of a rigid node. A combination of modifications may be needed to achieve the goal. Additional manipulation of the central metabolic pathway may also be required to generate the precursor, cofactors, and energy needed to sustain the modified pathway (Stephanopoulos et al., 1993). The manipulation of central metabolism has been exemplified in the production of aromatic compounds in *E. coli* growing in glucose.

Metabolic engineering involves manipulation of enzymatic, transport, and regulatory functions of the cell by using recombinant DNA technology (Bouzier et al., 1998; Stephanopoulos et al., 1991). First, various analytical techniques are used to identify and subsequently determine fluxes through critical metabolic pathways in the cell or tissue of interest. This knowledge provides the rational basis for applying, in the second step, molecular biological techniques to enhance metabolic flux through a pathway of interest and minimize metabolic flow to undesired biosynthetically related products. Although a certain sense of direction is inherent in all strain improvement programs, the directionality of effort is a strong focal point of metabolic engineering, compared with random mutagenesis, because this directionality plays a dominant role in enzymatic target selection, experimental design, and data analysis.

Metabolic engineering has found many applications, especially in microbial fermentation. It has been applied to increase the production of chemicals that are already produced by the host organism, to produce desired chemical substances from less expensive feedstock (Brabetz et al., 1991; Kumar et al., 1992), and to generate products that are new to the host organism (Ohta et al., 1991).

Metabolic engineering can also be used to construct cells with desirable properties by altering characteristics such as growth, proliferation, tolerance to exogenous factors, substrate utilization, etc. These characteristics are the result of complex biological functions involving multiple gene products. This complexity may afford greater efficiency or higher quality control or it may exist simply because a single protein cannot provide the required function. Metabolic engineering strategies that co-ordinately modify multigene expression therefore have the potential to achieve previously inaccessible metabolic states.
Escherichia coli, one of the best-characterized prokaryotes, has served as a model organism for countless biochemical, biological, and biotechnological studies. Since the completion of the *E. coli* genome-sequencing project (Blattner *et al*., 1997), this organism has been characterized on the genome-wide scale in terms of its transcriptome, proteome, interactome, metabolome, and physiome by use of DNA microarray, two-dimensional (2-D) gel electrophoresis (2-DE) coupled with mass spectrometry (MS), liquid and gas chromatography coupled with MS, and bioinformatics (Butland *et al*., 2005; Lockhart *et al*., 2000).

Researchers have found engineered *E. coli* to be of enormous value for both scientific and practical applications. To enhance the production of bioproducts and improve the performance of *E. coli* strains in various biotechnological processes, native or foreign genes have been amplified or deleted through recombinant DNA technology. These efforts initially involved trial-and-error approaches, in which various genetic modifications are repeatedly tried until a desired objective is achieved. However, since bioproducts are formed by coordinated enzyme functions acting through the metabolic pathways, it is essential to understand the metabolism and regulation that occur during cell growth and product formation.

From several years, research is going on the capability of microorganisms to degrade aromatic compounds. Microorganisms are not only used for remediation of water or soil, polluted by aromatic hydrocarbons from the chemical industry, but also for their application in compounds. Important flavours are of simple aromatic nature, e.g. benzaldehyde is the principle of bitter almond aroma, or cinnamic aldehyde is the major constituent of cinnamon oil (Rao *et al*., 2000).

Source for biotechnological production of ferulic acid is the phenyl propanoid eugenol. The metabolism of eugenol, which proceeds via ferulic acid and vanillin, has been studied in detail in Pseudomonads (Priefert *et al*., 1997; 1999; Achterholt *et al*., 1998; Narbad *et al*., 1998; Furukawa *et al*., 1998; Overhage *et al*., 1999a,b; 2002; 2003; Brandt *et al*., 2001). Different genes and enzymes which are involved in the corresponding reactions have been characterized recently in *Pseudomonas fluorescens* AN103 (Gasson *et al*., 1998), *Pseudomonas* sp. HR199 (Overhage *et al*., 1999c), *Streptomyces setonii* (Muheim *et al*., 1999), *Amycolatopsis* sp. HR167 (Achterholt *et al*., 2000), *Delftia acidovorans* (Plaggenborg *et al*., 2001) and *Pseudomonas putida* KT2440 (Plaggenborg *et al*., 2003).
2.2 Eugenol

2.2.1 Chemical and Physical Properties

IUPAC name : 4-allyl-2-methoxyphenol

Synonyms : 2-Methoxy-4(2-propenyl)phenol, eugenic, 1-Allyl-3-methoxy-4-hydroxy benzene, Allylguaiacol.

I. Eugenol is a member of the phenylpropanoids class of chemical compounds (Thomas et al., 2007), which is an allyl chain-substituted guaiacol.

II. Eugenol is weakly acidic, slightly soluble in water and soluble in organic solvents.

III. Eugenol is a clear to pale yellow liquid having a characteristic pleasant odour of cloves and a spicy pungent taste. It is responsible for the aroma of cloves (Schiesti et al., 2003).

IV. Specific gravity of eugenol is slightly more than 1.06 at room temperature, due to which it is heavier than water (Oxford University Chemical Safety Data).

V. Boiling point of eugenol is about 252°C. It has about a 2 year half life before its potency begins to seriously degrade, when stored in a cool, dry, and dark place (Oxford University Chemical Safety Data).

Eugenol reacts with alkalis to form water soluble salts. This property is used for its extraction from natural essential oils. Like other phenols, it forms esters when combined with carboxylic acids. These esters are widely used in perfumery. Oxidation of eugenol results in vanillin. Being heated in the presence of catalysts (alkalis or platinum on activated charcoal), eugenol isomerizes to give isoeugenol.

2.2.2 Occurrence of Eugenol

Eugenol, a phytochemical, is a biologically active phenolic component of Syzygium aromaticum (cloves). Aromatic plants like nutmeg, basil, cinnamon and bay leaves also contain eugenol. The name, eugenol which is the main constituent of clove oil, is derived from the species name Eugenia caryophyllata (Lee et al., 2001). It contains a high level of eugenol (45–90%) in addition to acetyleneugenol, chavicol, acetyl salicylate and humulenes. It was first isolated in 1929. Commercial production of
eugenol started in the United States in the 1940s. Synthetic eugenol was produced by the allylation of guaiacol with allyl chloride. However, eugenol is predominantly prepared from natural oil sources. The essential oil is mixed with an excess of aqueous sodium (3%) or potassium hydroxide solution, shaking of solution properly, leads to the formation of a phenolic alkali salt. The insoluble non-phenolic portion is then extracted with a solvent or via steam distillation. The undissolved portion is removed, the alkali solution acidified at low temperatures which liberates eugenol for further purification by fractional distillation (Bedoukian et al., 1986). Essential oils are volatile, ethereal liquids of oily consistency. Eugenol is the most active element in the clove oil. The major clove producers of the world are the West Indies, Madagascar, Tanzania, India, Sri Lanka, Indonesia and Malaysia (Kamatou et al., 2012) (Fig. 2.1).

![Fig. 2.1: The major clove producers of the world (Kamatou et al., 2012).](image)

### 2.2.3 Natural Source of Eugenol

There are different natural source of eugenol (Fig. 2.2) (Usta et al., 2000; Gursale et al., 2005; Stamford et al., 1988). List of aromatic plants which contains eugenol is given below:

1) **Syzygium aromaticum**

Eugenol is present in commercial essential oils isolated from bud, leaf and stem of *Syzygium aromaticum*. The oils were analyzed by GC and ten constituents were
identified from the whole. The major constituent in bud, leaf and stem oils was eugenol, with increasing percentages from bud (72.08 - 82.36%) to leaf (75.04 - 83.58%) and stem (87.52 - 96.65%) (Pathak et al., 2004). In the clove bud essential oil, eugenyl acetate is the second major component (8.6 - 21.3%), while detected in considerably lower amount in the leaf (0 - 1.45%) and stem (0.07 - 2.53%).

2) *Cinnamomum tamala*

*Cinnamomum tamala*, Indian bay leaf, also known as tejpat, Malabar leaf, Indian bark, Indian cassia, or malabathrum is a tree within the Lauraceae family which is native to India, Nepal, Bhutan, and China. It can grow up to 20 m (66 ft) tall. It has aromatic leaves which are used for culinary and medicinal purposes. It is thought to have been one of the major sources of the medicinal plant leaves known in classic and medieval times as malabathrum (Dighe et al., 2005). A simple, rapid and precise reverse-phase high performance liquid chromatographic method has been developed for the quantitative determination of eugenol from the extract of dried powder of *Cinnamomum tamala* leaves.

3) *Myristica fragrans*

Nutmeg oil is a volatile oil obtained by steam distillation of seed of *Myristica fragrans* (Bennet et al., 1988). Nutmeg oil has capacity to inhibit platelets aggregation in vitro. It has been found that eugenol and isoeugenol play the major role in the detected activity of nutmeg. Medicinally, nutmeg oil and nutmeg powder can be replaced by eugenol and/or isoeugenol.

4) *Melissa officinalis*

Lemon balm (Melissa officanils) also known as balm or balm mint is a perennial herb in mint family Lamiaceae, native to south-central Europe and the Mediterranean region. It grows to 70-150 cm tall. Leaves have gentle lemon scent related to mint, its flavour comes from citronella (24%), linalyl acetate (12%) and caryophyllene (12%). Eugenol is present in leaves (Bullerman et al., 1977).

5) *Ocimum basilicum, Ocimum tenuiflorum*

Eugenol is obtained from various parts of the *Ocimum basilicum* (Johnsan et al., 1999), *Ocimum tenuiflorum* or Tulsi (Lee et al., 2001) such as leaves, flowers and stems.
Basil contains important phytochemicals that have been reported to afford protection against several chronic diseases due to their anti-inflammatory and antioxidant activities. Basil is a rich source of essential oils and has been used in confectioneries, condiments, sausages and meats, salad dressings, nonalcoholic beverages and ice cream.

![Fig. 2.2: Structure and source of Eugenol (Usta et al., 2000).](image)

6) *Ocimum gratissimum*

*Ocimum gratissimum* also known as clove basil, African Basil is a species of Ocimum. The essential oil of *Ocimum gratissicum* contains eugenol and shows some evidences of antibacterial activity (Oboh et al., 2009). Oil is obtained from leaf extract of *O. gratissimum*.

7) *Illicium anisatum*

It is an evergreen shrub or small tree growing up to 8 meters high. Fruit contain contains a volatile oil and fixed oil. Eugenol is present in volatile oil (Kim et al., 2009).
8) *Cinnamomum verum*

Cinnamon also known as true cinnamon, Ceylon Cinnamon, is a small evergreen tree belonging to the family Lauraceae native to Sri Lanka. Its inner bark is used to make cinnamon. The essential oil obtained from the bark of Cinnamomum verum Blume (Lauraceae) contain eugenol as one of components (*Encyclopaedia Britannica*. 2008). Eugenol is present in oil.

**2.2.4. Applications**

For centuries, natural products have been used to treat microbial infections and numerous essential oil molecules have demonstrated the ability to inhibit the growth of various pathogens (Singh *et al.*, 2007). In detergents and soaps, large quantities of eugenol are used because of their spicy aroma, but due to their phenol structure, they cause discolouration. The ester form of eugenol is non-discolouring and stable, but it is rarely used in applications such as perfumes, because they do not possess the powerful spicy notes of the free phenolic compound (Bedoukian *et al.*, 1986; Barceloux *et al.*, 2008).

Eugenol is used in perfumeries, flavorings, essential oils and in medicine as a local antiseptic and anesthetic (Jadhav *et al.*, 2004). It is a key ingredient in Indonesian kretek (clove) cigarettes. It is used to make zinc-oxide eugenol paste for temporary fillings of teeth (Bohnert *et al.*, 2008). It also makes a good local anaesthetic for temporary relief from toothache. They are used in formulating insect attractants, analgesics, biocides and antiseptics (Obeng *et al.*, 1997). They are also used in manufacturing stabilizers and antioxidants for plastics and rubbers.

Clove oil is growing in popularity as an anaesthetic for use on aquarium fish as well as on wild fish when sampled for research and management purposes where, readily available over-the-counter from pharmacies, it may be a humane method to euthanise sick and diseased fish either by direct over-dose or to induce sleep before an overdose of ethanol. It is also used in some mousetraps.

Anecdotal evidence suggests that it may have healing properties for sores and help to relieve rashes though it should not be used on open wounds. It may be beneficial when ingested in moderate amounts (keeping down the free radicals). Various eugenol
derivatives has been developed for intravenous injection such as propanlidid and G.29.505. The latter produced unacceptable side effects around the site of injection in many patients (Right et al., 1962). Hence patients should be discouraged from using it in its pure form, either to alleviate toothache or dentine hypersensitivity.

In recent years, eugenol has attracted the attention of many researchers because of its anti-inflammatory and chemopreventive effects, as well as its superior anti-oxidant activity due the presence of its phenolic group. As a result of its broad range of pharmacological and biological activities, studies on eugenol and clove products still remains a research priority. It was used in the production of isoeugenol, for the manufacture of vanillin. It is therefore of significant value to coherently unite some of the most noteworthy research findings related to eugenol to highlight its importance in phytotherapy and industry as well as to elucidate its mechanisms of action where possible.

2.2.5 Toxicity and Allergenicity of Eugenol

Eugenol has been classified as ‘generally recognised as safe (GRAS)’ by the U.S. Food and Drug Administration (WHO report, 1982). However, the toxicity of eugenol should be determined as higher concentrations are used for other applications. The cytotoxicity and internucleosomal DNA fragmentation of eugenol and related compound was examined by Okada (Okada et al., 2005). The reactive metabolites of eugenol and recombinogenic compounds are responsible for the genotoxicity of eugenol (Monerato et al., 2005). It is impossible to consume too much eugenol. In fact the National Library of Medicine defines eugenol overdose as "poisoning from swallowing a large amount of eugenol oil (clove oil)." Some of the symptoms are shallow and rapid breathing, coughing up blood, blood in urine, burns in mouth and throat, abdominal pain, nausea, rapid heartbeat, dizziness, seizures, and even coma. Obviously, this is not a substance to be consumed in mass quantities. Persons experiencing an overdose, should seek immediate medical attention. Vomiting should not be induced unless it is recommended by an official. A good step is to contact the Poison Control Center. A 2-year old boy nearly died after taking between 5 and 10 ml, according to a report published in 1993 (Hartnoll et al., 1993). Eugenol is generally used without incident but in a sensitised individual, it can cause a range of tissue effects, from low-grade local reactions to the rare, but serious, anaphylactic reaction.
2.3 Ferulic acid

2.3.1 Structure and Chemical properties

**IUPAC name**: 3-(4-hydroxyl-3-methoxy-phenyl)prop-2-enoic acid.

**Synonyms**: 3-methoxy-4-hydroxycinnamic acid, 4-hydroxy-3-methoxy cinnamic acid, Ferulate, coniferic acid.

1. Ferulic acid is aromatic homomonocyclic compound which is hydroxycinnamic derivative.
2. It exists in crystalline powdered form which is white to yellow in colour and have characteristics odour.
3. Melting point of ferulic acid is 168-171°C.
4. It is slightly soluble in water and soluble in organic solvent.

2.3.2 Occurrence of Ferulic Acid

Ferulic acid (C_{10}H_{10}O_{4}), a phytochemical is an organic compound which is found in plant cell walls, leaves, seeds. Pure ferulic acid is a yellowish powder. A good amount of ferulic acid is found in oats, brown rice, whole wheat, peanuts, apples, and pineapples (Sakai *et al.*, 1999). It is a derivative of trans-cinnamic acid. Ferulic acid is a precursor in the manufacture of other aromatic compounds.

Ferulic acid can be made from the metabolism of phenylalanine and tyrosine. Ferulic acid can be extracted from different parts of plants or it may be produced from eugenol, isoeugenol etc. It is one of the most abundant phenolic acids in plants. Amount of ferulic acid varies from 5kg^{-1} in wheat bran to 9gkg^{-1} in sugar-beet pulp and 50gkg^{-1} in corn kernel (Rosazza *et al.*, 1995; Karoon *et al.*, 1997). Ferulic acid is rarely found in the free form in plants, but it is is found in bounded form. It is usually found as ester cross-links with polysaccharides in the cell wall, such as pectin in spinach, arabinofurans in grasses, sugar beet and xyloglucans in bamboo (Iiyama *et al.*, 1994). Ferulic acid can also cross-link with proteins (Figueroa Espinoza *et al.*, 1999). In wheat, Ferulic acid is ester linked to cell wall carbohydrates and occurs in higher concentration in the alcurone, pericarp and embryo cell wall. The trans isomer predominant and account for 90% of total phenolic acid in common flour (Fulcher *et al.*, 1983). It is a major constituent of
some fruit such as oranges and some vegetable such as tomato, carrot etc. It is also found in sweet corn (Balasubhashine et al., 2003).

It is bound to the primary cell walls of all gymnosperm families. Unlignified primary cell walls which contain ester-linked ferulic acid fluoresce blue in UV rays which further changes to green with increased intensity on treatment with ammonium hydroxide.

In angiosperms, ferulic acid has been found ester-linked to primary cell walls, in the monocotyledons and in the dicotyledon order Caryophyllales. It has been found at concentrations >3.5mgg⁻¹ cell walls, and has been detected in primary cell walls by its autofluorescence using ultraviolet fluorescence microscopy (Harris et al., 2010).

### 2.3.3 Application of Ferulic Acid

Ferulic acid can be used to preserve foods, because of its antioxidant and antimicrobial activities. Ferulic acid is an effective antioxidant, in some food-related system, such as lecithin-liposomes and aqueous emulsions (Frankel et al., 1994; Huang et al., 1997). Ferulic acid may be used as a precursor in the manufacturing of vanillin, which is a synthetic flavoring agent and can be used in place of natural vanilla extract. Studies have shown that biotechnological processes are the most efficient method to use ferulic acid as a precursor and research is still ongoing. Kraft Foods has patented the use of sodium ferulate to mask the bitter aftertaste of the artificial sweetener acesulfame potassium.

Addition of ferulic acid to a topical preparation of ascorbic acid and vitaminE reduces oxidative stress and formation of thymine dimers in skin. Research has shown that oral supplements of ferulic acid can inhibit melanin production in the process of skin whitening. It can be used as a matrix for proteins in MALDI mass spectrometry analysis (Meetani et al., 2005).

### 2.3.3.1 Medicinal application

#### 1. Antioxidant activity

Ferulic acid is an antioxidant, which neutralizes those free radicals (superoxide, nitric oxide and hydroxyl radical) which could cause oxidative damage of cell
membranes and DNA. Ferulic acid helps to prevent damage to our cells caused by ultraviolet light. Exposure to ultraviolet light actually increases the antioxidant potency of ferulic acid.

In human body, free radicals and reactive oxygen species are the underlying cause for different diseases, such as atherosclerosis, cancer, cataracts, macula lutea etc. (Young et al., 2001). Free radicals in the human body are generated by different endogenous and exogenous processes. In human body, various endogenous processes cause production of superoxide and hydroxyl radicals, which are associated with many diseases. Superoxide are produced by oxidation of the molecules of adrenaline flavine nucleotides, thiol compounds and sugars in the presence of oxygen (Becker et al., 1999). Exogenous factors such as ultraviolet light, air pollutants, cigarette, smoke cause the production of free radicals which may also cause damage to our body. The metabolism of nitrates and nitrites produces the nitrogen dioxide radical which is known to be toxic agent in human body. As free radicals are constantly produced in the human body, so a defensive system that can prevent the damage caused by free radicals, is essential to human health. In human body, though endogenous antioxidant systems already exist, but still the intake of antioxidants is necessary for strengthening the body defence systems.

Now a days, an increasing awareness of the bioactivity and potential health benefits of hydroxycinnamates (e.g. ferulic, caffeic, p-coumaric and sinapic acid) and their conjugates has been found. Similar to many natural phenols, ferulic acid is an antioxidant since it forms a resonance stabilized phenol radical (Young et al., 2001). In vitro, ferulic acid is reactive toward different free radicals such as reactive oxygen species (ROS). These ROS and free radicals are responsible for DNA damage, cancer, accelerated cell aging. Ferulic acid showed high scavenging activity for hydrogen peroxide, superoxide, hydroxyl radical and nitrogen dioxide free radicals (Ou et al., 1999). By using the pulse radiolysis technique, it has been shown that hydroxycinnamic acid derivatives, including ferulic acid, sinapic acid and caffeic acid, scavenged nitrogen dioxide radicals (Zhang et al., 1998). Ferulic acid can also prevent peroxynitrite-mediated nitration of protein-bound and free tyrosine and peroxynitrite-mediated oxidation of dihydorhodamine 1,2,3 and DNA (Ketsawas et al., 2000). Ferulic acid, not only scavenges free radicals, but also increases the activity of enzymes which are responsible for scavenging free radicals. Ferulic acid also inhibits enzymes that catalyze the production of free radicals. Ferulic acid, as well as its amino acid derivatives,
showed strong tyrosinase inhibitory activity and superoxide dismutase-like activity (Kayahara et al., 1999).

2. **Prevention against thrombosis and atherosclerosis**

To repair bloodletting injury, platelet aggregation is a natural mechanism and this is related to thrombosis. In China, to cure thrombosis, ferulic acid-rich herbs have long been used. The compound is capable of inhibiting platelet aggregation providing a therapeutic means to cure thrombosis. The mechanism underlying ferulic acid inhibition of platelet aggregation in the circulatory system involves inhibition of phosphodiesterase and increase of adenosine-3’,5-cyclic monophosphate (cAMP) and guanosine-3’,5-cyclic monophosphate (cGMP) (Gao et al., 1988; Zhang et al., 1993). cAMP and cGMP are two important cyclic nucleotides in the organism (Kayahara et al., 1999). Ferulic Acid can inhibit malondialdehyde (MDA) production from platelets (Yin et al., 1986). It can also inhibit erythrocyte lysis induced by MDA and hydroxyl radical (OH- free radical) and inhibit lipid peroxidation induced by H₂O₂ and O₂ (Pan et al., 1985). The antioxidative activity of ferulic acid is due to direct capturing of free radicals by phenolic hydroxyl group (Ju et al., 1990; Meng et al., 1994).

3. **Antimicrobial and anti-inflammatory activity**

Ferulic acid shows inhibitory activity against growth and reproduction of viruses, such as influenza virus, respiratory syncytial virus and AIDS. Ferulic acid and isoferulic acid show anti-inflammatory activity. Both are the active components of the rhizoma of Cimicifuga species and this species is used frequently as anti-inflammatory drugs in Japanese Oriental medicines. The effect of ferulic acid and isoferulic acid on murine interleukin-8 (IL-8) production in response to influenza virus infections in vitro and in vivo by antibody-sandwich enzyme-linked immunosorbent assay has been proven (Hirabayashi et al., 1995). Both ferulic acid and isoferulic acid influence the production of macrophage inflammatory protein-2 (MIP-2) in a murine macrophage cell line, RAW264.7, in response to respiratory syncytial virus (RSV) infection (Sakai et al., 1999).

Ferulic acid has been found to possess a wide spectrum of antimicrobial activity. It exhibited antimicrobial activity towards Gram-positive bacteria, Gram-negative
bacteria and yeasts (Jeong et al., 2000). Ferulic acid also showed strong inhibitory effects on the growth of various bacteria associated with human gastrointestinal microflora, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter koseri*, *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Shigella sonnei*. (Nilsson et al., 1999; Lo et al., 1999; Tsou et al., 2000) The antimicrobial mechanism of ferulic acid was found to be related to its inhibition of arylamine N-acetyltransferase in the bacteria (Lo et al., 1999). Ferulic acid, at a concentration of 500mgL⁻¹, can also appreciably inhibit growth of yeasts such as *Pichia anomala*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae*, but it is less effective than potassium sorbate (Stead et al., 1995).

Exposure of skin to ultraviolet light cause skin damage, which causes both precancerous and cancerous skin lesions and acceleration of skin ageing (Carbonare et al., 1992). Reactive oxygen species are responsible for some of the deleterious effects of UV light upon skin. Prolonged skin exposure to UV light results in a severe decrease of antioxidant content of skin (Podda et al., 1998). Ferulic acid oxide from keratinocytes seems to play a major role in the integrated response leading to erythema production and inflammation processes following UV radiation exposure (Romero et al., 1997). Due to the high degree of conjugated unsaturation (as a strong UV absorber) and its permeability to the skin (Saija et al., 2000), ferulic acid is an active ingredient in many skin lotions, sunscreens and hair creams designed for photoprotection and for skin tumor inhibition (Murakami et al., 2002).

### 4. Anti-cancer effect

From different animal studies and in vitro studies, it is clear that ferulic acid may show direct antitumor activity against breast cancer and liver cancer. Ferulic acid may also show pro-apoptotic effects on cancer cells, which helps in their destruction. Cancer induced by exposure to the carcinogenic compounds, such as benzopyrene and 4-nitro quinoline-1-oxide may be treated by ferulic acid. Ferulic acid shows the chemopreventive effects on oral and large bowel carcinogenesis. In rats, the effects of ferulic acid on oral cancer was studied by feeding ferulic acid in the diet at a dose of 0.5gkg⁻¹ after exposure to 4-nitroquinoline-1-oxide for 5 weeks in drinking water at a dose of 0.02gkg⁻¹ (Mori et al, 1999). It was found that the incidences of tongue carcinomas and preneoplastic lesions (severe dysplasia) were significantly lower on termination of the experiment (32 weeks) than the group with the carcinogen alone. The
results suggest that ferulic acid has chemopreventive activity on oral cancer. Recently many researchers have focused their attention on the anti-cancer activity of ferulic acid on colon and rectal cancer (Mori et al., 1999; Kawabata et al., 2000; Wargovich et al., 2000).

One of main cause of the development of cancer is production of free radicals, the anti-cancer effects of some antioxidants is related to their ability for scavenging free radicals. Tropical application of polyphenols such as ellagic acid, tannic acid, caffeic acid and ferulic acid simultaneously with phorbol-12-myristate-13-acetate or mezerein resulted in significant protection against 7,12-dimethylbenz[a]anthracene-induced skin tumors (Kaul et al., 1999).

Since free ferulic acid does not enter enterohepatic circulation, via oral or intravenous route so it cannot easily reach the colon. It has been found that dietary fiber-bound ferulic acid can reach the colon and is partly released by colon microorganism, but the concentration of the released ferulic acid is too low to act as a chemopreventive agent. So to solve this problem, attempts are being made to study the synthesis of enzyme-resistant derivatives of ferulic acid which can be delivered to the colon safely. Maize starch and ferulic acid can be used to synthesize starch ferulate and it was found that the starch ferulate was only partly hydrolyzed (less than 10%) by diastase and the bound ferulic acid was largely released by colon microorganisms (Ou et al., 2001).

Some derivatives of ferulic acid showed even higher biological activities than the free form. For these reasons, synthesis of enzyme-resistant and high-biological-activity derivatives of ferulic acid will be a focused area of future studies. Extensive toxicological and biological experiments are needed to test this kind of product. Ferulic acid seems to protect against cancer, bone degeneration, menopausal symptoms (hot flushes) (Ou et al., 2001).

5. Antifertility Activity

The capacity of human sperm fertilization mainly depends on sperm motility and membrane integrity. Different reactive oxygen species, such as superoxide anion and hydrogen peroxide impair sperm motility and membrane integrity by inducing membrane lipid peroxidation. Ferulic acid, which is an effective constituent of different medicinal herbs, scavenge oxygen free radicals and increase the intracellular cAMP and cGMP. In
both fertile and infertile spermatozoa, the viability, trans-membrane migration ratio and the levels of intracellular cAMP and cGMP in ferulic acid-treated spermatozoa were significantly higher than those of spermatozoa in control groups. The effect of ferulic acid was found to be concentration dependent. These showed that ferulic acid is beneficial to sperm viability and motility in both fertile and infertile individuals. Reduction of lipid peroxidative damage to sperm membranes and increase of intracellular cAMP and cGMP may be due to ferulic acid. It is possible that ferulic acid may be used for cure of asthenozoospermic infertility. cAMP has been found to play a central role in sperm motility (Hoskin et al., 1973; Morton et al., 1974; Schoenifed et al., 1975; Garber et al., 1978; Miller et al., 1985). A high cGMP level has also been shown to increase sperm metabolism and motility in the sea urchin and other mammalian species (Garber et al., 1978; Miller et al., 1985) thus; it may affect mammalian sperm motility as well.

Ferulic acid also inhibits the effect of androgens on the rat prostate. It antagonized the effect of exogenous androgens on the ventral prostate in castrated rats as well as the effect of endogenous androgens in intact rats. However, ferulic acid showed no effect on the seminal vesicles and levatorani muscle, nor oestrogenic effect in female rats and mice (Saito et al., 1995).

Ferulic acid and other hydroxycinnamic acids are found in plant tissues mainly as low-molecular-weight, water soluble conjugates which are present in the cytosol and in bound forms esterified or etherified to cell wall polymers. These water-soluble conjugates are more readily absorbed as ester or the glycone in the upper digestive tract of non-ruminants and bound acids are primarily released by microbial action in the hindgut of non-ruminants species, including humans (Chesson et al., 1999).

Ferulic acid is often added as ingredient of anti-aging supplements to other chemicals. Ferulic acid can decrease blood glucose levels and can be of help to diabetes patients. Like many other antioxidants, it also reduces the level of cholesterol and triglyceride, thus help in reducing the risk of heart diseases.

2.3.3.2 Applications in Food

Ferulic acid is of low toxicity with LD$_{50}$ of 2445mgkg$^{-1}$ body weight in male and 2113mgkg$^{-1}$ body weight in female rat (Tada et al., 1999). In Japan, ferulic acid has been
approved as a food additive and used as a natural antioxidant in foods, beverages and cosmetics. Also, in the USA and most European countries, numerous medical essences and natural extracts of herbs, coffee, vanilla beans, spices and other botanicals are selected for their high content of ferulic acid and added to foods as an FDA-approved antioxidant concoction (Graf et al., 1992).

i. Ferulic acid as a Biopreservative

Ferulic acid can be used to preserve foods because of its antioxidant and antimicrobial activities. It was first used in Japan in 1975 to preserve oranges and to inhibit the autooxidation of linseed oil, lard and soybean oil (Graf et al., 1992). Ferulic acid has two main advantages. First, ferulic acid has strong antioxidant activity. Second, it is much less affected by pH changes than other phenolic compounds, such as chlorogenic acid, caffeic acid and gallic acid (Friedman et al., 2000).

Preservative function of ferulic acid is also due to its antimicrobial activity. Ferulic acid can inhibit the growth of bacteria, fungi and yeasts. Ferulic acid is found to be an active ingredient of extracts of some plants showing antimicrobial activity which is useful as processed food preservation.

ii. Packaging for food industry

As ferulic acid cross-links with polysaccharides, so it is used to increase the viscosity and thus ferulic acid form gels from some polysaccharides such as pectin and arabinoxylans (Micard et al., 1999; Oosterveld et al., 2000; 2001). Feruloylated arabinoxylans and proteins using cross-linking agents such as ammonium sulfate, hydrogen peroxide (Oosterveld et al., 2000) and laccase (Micard et al., 1999) affect gel formation. This property of ferulic acid is very important, because ferulic acid make it possible to use polysaccharides of lower molecular weight having low viscosity and poor gel formation capacity, to make new gels for use in food processing. Ferulic acid and its oxide, quinoid ferulic acid, can react with some amino acids in proteins such as tyrosine, lysine, cysteine (Hurrell et al., 1984; Oudgenoeg et al., 2001) and cross-link protein molecules. Thus, it can be used as a cross-linking agent to improve the properties of protein-based edible films.

Ferulic acid at concentration of 5.5 mmoll−1 enhanced the heat stability of milk at 140°C. The addition of caffeic acid resulted in a reduction of the reactive lysine and
sulphhydryl content and inhibited the dissociation of $\kappa$-casein-rich protein from the casein micelles in milk on heating. On heating in milk, caffeic acid was thermally oxidized to quinones which then interacted with nucleophilic amino acid residues to inhibit $\kappa$-casein dissociation from the casein micelle. Caffeic acid and its related phenolic compounds, such as ferulic acid find their prospective applications in the processing of products (O’Connell et al., 1999).

iii. Ingredient in sport foods

Since ferulic acid is found to be an antioxidant and stimulate hormone secretion in humans, so it is widely used as an ergogenic substance in sport foods (Berning et al., 1998; Headley et al., 1999).

iv. Production of vanillin

Vanillin is an important aromatic flavor compound used in foods, beverages, perfume and pharmaceuticals. It is produced on a large scale in the industry through chemical synthesis. However, the vanillin obtained by chemical synthesis cannot be considered as natural. Ferulic acid can be used to produce vanillin through biotransformation in microorganisms by three major pathways. The first is decarboxylation of ferulic acid by decarboxylase to produce 4-vinylguaiacol and then vanillin. The second is the reduction of ferulic acid to dihydroferulic acid, from which vanillic acid and vanillin are formed. The third is to produce vanillic acid and vanillin by formation of coniferyl alcohol from ferulic acid (Priefert et al., 2001).

2.3.4 Production of Ferulic Acid

There are two ways for preparation of ferulic acid. Ferulic acid can be prepared by chemical synthesis and through biological transformation. The first way is chemical synthesis involving the condensation reaction of vanillin with malonic acid catalyzed by piperidine (Adams et al., 1952). Though this method produces a mixture of trans- and cis-isomers of ferulic acid with a high yield of ferulic acid, it takes as long as three weeks to complete the reaction. This method was improved by using benzylamine as the catalytic agent, methylbenzene as the solvent and a reaction temperature of 85–95°C. The improved method increased the yield and reduced the reaction time to 2h.
The other way is extraction from natural sources where ferulic acid is one of the most abundant phenolic acids in plants. The extraction of ferulic acid using various methods such as enzymatic (Mathew et al., 2005), alkaline (Saulnier et al., 1995; Kim et al., 2006), and acidic extraction (Xu et al., 2005) have been performed. Different techniques are employed for extraction, which help in releasing various constitutive monomers from the cell wall polysaccharides and can be used for further specific application. The extraction method may affect the yield and profile of phenolic acids released because these exist in esterified forms in plant cell walls (Saadi et al., 1998). Alkaline treatment essentially involves hydrolytic cleavage of ester linkages between lignin and plant polysaccharides thereby releasing phenolic acids (Lozovaya et al., 1990).

Ferulic acid can be produced from waste oil, an alkaline oil cake, and a crude fatty acid which are discarded as waste materials or obtained as a by-product in the manufacture of rice salad oil. They are subjected to hydrolysis in the presence of an alkali so as to efficiently manufacture ferulic acid (Taniguchi et al., 1996). The bran oil waste material was hydrolyzed with sodium hydroxide or potassium hydroxide at 90–100°C for 8 h under atmospheric pressure, producing crude ferulic acid with purity of 70–90%. The solution containing alkaline salt of ferulic acid was acidified with dilute sulfuric acid to precipitate ferulic acid.

As polysaccharide ferulate is a natural and abundant source of ferulic acid, preparation of ferulic acid from plant cell wall materials will be a prospective pathway. Feruloyl esterases, a subclass of the carboxylesterases can release ferulic acid from a range of esterified substances including methyl ferulate, feruloylated oligosaccharides and polysaccharides (Kroon et al., 1999). These enzymes are secreted by fungal, bacterial and yeast microbes, such as Aspergillus niger, Pycnoporus innabarinus, Streptomyces avermitilis, Clostridium thermocellum, Bacillus sp., Lactobacilli, Pseudomonas fluorescens and Brettanomyces anomalus.

The conventional technique for extracting ferulic acid is the percolation method, but this method has some disadvantages. For example, it is time-consuming and has some undesirable reactions such as hydrolysis, oxidation, degradation and rearrangement, therefore alternative extraction techniques are preferable. Surface Fluid Extraction (SFE) method has been used for producing ferulic acid (Tilay et al., 2008).
SFE possesses several advantages including improved selectivity, expeditiousness, automation and environmental safety. It has been extensively studied for separation of many active compounds from natural products matrices, such as roots, seeds, flowers and leaves.

Ferulic acid can be biologically synthesized. It is obtained from eugenol through a simple enzymatic oxidative conversion process. Eugenol is natural substrate that can be isolated from essential oil of the clove tree *Syzygium aromaticum* on industrial scale (Rabenhorst *et al.*, 1996). Chemically, it can be prepared from sugarcane bagasse (Shiyi *et al.*, 2006) by alkaline hydroxylase and by resin exchange chromatography. It can be extracted from clove oil along with isoeugenol.

### 2.3.4.1 Extraction of ferulic acid from Angelica sinensis

Ferulic acid may be extracted from the root of Angelica sinensis by Surface fluid extraction method (Sun *et al.*, 2006). The extract yields were 0.87–4.06% at temperatures from 45 to 65°C and pressures from 30 to 50MPa, and the maximum content of ferulic acid in the extracts was about 0.35–0.37%, which is lower than that of 0.61–0.85% by conventional percolation methods. As ferulic acid is a polar compound, its solubility in supercritical carbon dioxide is very low, which is between $0.94 \times 10^{-5}$ and $5.22 \times 10^{-5}$ (mass fraction solubility) at the temperatures from 301 to 333K and pressure from 12 to 28MPa. The most used fluid for SFE is CO$_2$ because of its low critical properties, low toxicity and chemical inertness. In order to extract ferulic acid efficiently, the addition of polar co-solvent may be needed. Ethanol was used as co-solvent in different ratios to raw materials in order to increase the content of ferulic acid in the extracts. Extract yields and the content of ferulic acid in the extracts were increased as compared with pure CO$_2$ extraction. When the ratio of ethanol to the raw material was 1.6, the content of ferulic acid in the extracts was 0.91–1.27%, indicating that supercritical fluid extraction (SFE) with CO$_2$ in the presence of suitable co-solvent is superior to percolation in extracting polar ferulic acid from Angelica sinensis.

### 2.3.4.2 Ferulic Acid from Agricultural Wastes

Ferulic acid is found in various agricultural wastes such as maize bran, rice bran, wheat bran, wheat straw, sugarcane bagasse, pineapple peels, orange peels and
pomegranate peels. Maize bran is found to contain the highest amount of Esterified Ferulic Acid (EFA) (Tilay et al., 2008). Alkaline extraction of EFA from maize bran was carried out using 2M NaOH. Response surface methodology (RSM) was used for optimization of EFA extraction, which resulted in a 1.3-fold increase as compared to the unoptimized conventional extraction technique. The extraction method may affect the yield and profile of phenolic acids released because these exist in esterified forms in plant cell walls. Alkaline treatment essentially involves hydrolytic cleavage of ester linkages between lignin and plant polysaccharides, thereby releasing phenolic acids (Mathew et al., 2004). EFA was released by alkaline treatment and the etherified FA leftovers as it is in residue. By changing various extraction parameters, such as NaOH, molarity, temperature, and time, extraction of FA from maize bran were optimized. The maximum FA extraction was observed with optimized conditions such as NaOH 4M, temperature 21.6°C and time 24 h. Increasing demands for antioxidants such as FA require efficient extraction and purification techniques. Ferulic Acid was analyzed by means of high-performance liquid chromatography (HPLC). Purification was carried out by adsorption chromatography using Amberlite XAD-16 followed by preparative high-performance thin-layer chromatography (HPTLC). The recovery of Amberlite XAD-16 purified FA was up to 57.97% with HPLC purity 50.89%. The fold purity achieved was 1.35. After preparative HPTLC, the maximum HPLC purity obtained was 95.35% along with an increase in fold purity up to 2.53.

### 2.3.4.3 Ferulic Acid from Clove Oil

The useful antioxidant ferulic acid may be produced from clove oil containing abundant eugenol (Furukawa et al., 2003). The growth of eugenol-degrading microorganism in the presence of clove oil was examined. *Pseudomonas fluorescens* E118, a clove-oil-tolerant strain, accumulated 6.1 g/l ferulic acid under optimized culture conditions with the intermittent addition of eugenol. Since clove oil is much cheaper than eugenol, ferulic acid production from clove oil by the bacterium is promising for the industrial production of ferulic acid. Clove oil is an essential oil isolated from buds and leaves of Syzygium aromaticum by steam distillation. Eugenol accounts for approximately 80% (w/w) of clove oil as the main component and has been used as an aromatherapy oil, mouth sterilizer or painkiller.
2.3.4.4 Ferulic Acid from Sugar Beet Pulp

A microorganism, *Penicillium chrysogenum* 31B, that has high ability to release ferulic acid from sugar beet pulp was recognized (Sakamoto *et al*., 2005). Approximately 85% of alkaline-extractable ferulic acid in sugar beet pulp could be released using the culture supernatant of *P. chrysogenum* 31B. However, the culture supernatant did not efficiently extract ferulic acid from wheat bran, peel of corn seed, or sugar-cane bagasse. A ferulic acid esterase (FAE-1) was purified from the culture filtrate of *P. chrysogenum* 31B. The molecular mass of the enzyme was determined to be 62 kDa by SDS-PAGE. Optimum conditions for enzyme activity were 50°C and pH 6-7. The substrate specificity of FAE-1 seemed to be similar to that of ferulic acid esterase (CinnAE) of *Aspergillus niger*. However, there was a difference between FAE-1 and CinnAE in respect to activity towards methyl vanillate. It is remarkable that FAE-1 hydrolyzed methyl vanillate.

The agro-industries generate thousands of tons of by-products, such as bran or pulps each year. They are at best used for cattle feeding. Through biocracking, this biomass may constitute a renewable source for various molecules of interest for the industry. For instance, ferulic acid can be recovered from agriculture residue like corn bran by the use of enzyme ferulic acid estrase (FAE) (Mathew *et al*., 2004). Hydrolysis of cell wall of green coconut agriculture husk provides a sufficient natural source of ferulic acid (Soccal *et al*., 2000; 2003). But this large amount of production generates a large amount of residue that cause serious environmental problems (Pandav *et al*., 1998; Soccal *et al*., 2003).

2.4 Advantages of microbial catalysis

There are three possible ways in which flavour production can be carried out, biocatalysed by plant tissues, by isolated enzymes, or by microorganisms. Chemical synthesis currently dominates flavour production. It often results in environmentally unfriendly production processes and lacks substrate selectivity, which might cause the formation of undesirable reaction mixtures, thus reducing process efficiency and increasing downstream cost. Moreover, according to European legislation, the use of these flavours is restricted in food, beverages and cosmetics. Therefore, natural flavours are sought-after in the worldwide market despite their considerably higher prices. However, natural flavour production by direct extraction from botanic materials is
subjected to many problems. Ferulic acid production from plants is expensive and not ideal for tissue culture techniques because plants are slow growing and pathway is not actively expressed (Prince et al., 1994; Lomascolo et al., 1999). Concentration of ferulic acid in plant is low, making the extraction process expensive. The harvest is also influenced by weather conditions and plant diseases (Longo et al., 2006).

Ferulic acid, an extremely abundant cinnamic acid derivative, is found in the cell walls of woods, grasses, and corn hulls. It is not easily accessible from these natural sources, since it is covalently linked to the various carbohydrates as a glycosidic conjugate, or it occurs as an ester or amide. Thus, it can be released from these natural products only by alkaline hydrolysis. However, ferulic acid gained from this chemical process cannot be considered as “natural.” Attempts to release, ferulic acid enzymatically from lignin employing ferulic acid esterases or cinnamoyl ester hydrolases are still far from being an implementable process due to unacceptable low yields. Therefore natural flavours cannot satisfy the large market, and alternative sources and processes are needed (Dignum et al., 2001). One of the emerging fields for the production of natural flavours is biotechnology.

In general, the advantages of biotechnological approaches are as follows:

(i) relatively mild reaction conditions.
(ii) high substrate or product specificity leading to only one product isomer.
(iii) fewer environmental problems (Schmid et al., 2001).

Owing to these important advantages, research into biotechnological production of flavours has increased in recent years. Enzymatic and microbial catalysis has the advantages of high substrate-selectivity and few by-products, resulting in easy product isolation procedures, and it is therefore considered the most promising strategy. Among the catalytic reactions, biocatalysis using whole microbial cells has emerged as an important tool for the large-scale synthesis of flavours, bulk chemicals and food ingredients.

2.5 Tolerance of E. coli XL1-Blue towards eugenol

Bacteria cannot survive in presence of eugenol. The structure of antibiotic substance in flavoring agents was studied by Katayama. They found that the benzene
ring compound containing hydroxyl group has antibacterial efficacy even when diluted to more than 2000 times (Katayama et al., 1960). Eugenol also contains this structure. Sara et al. studied the antibiotic mechanism of eugenol and other flavoring agents, in which the cell wall becomes degraded, which damages the plasma membrane and membrane protein and exposes the cell contents. The cytoplasm coagulates, causing the proton-motive force to move far away (Sara et al., 2004).

The biological activity of essential oils is generally investigated without emphasis on the mechanism of action. The mechanism of action of eugenol was studied to evaluate the effect on the bacterial membranes of *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *E. coli* by observing changes in membrane composition and monitoring the leakage of protein and lipid. The study revealed that eugenol induced cell lysis through leakage of protein and lipid contents. In addition both the cell wall and membrane of the treated Gram-negative and Gram-positive bacteria were significantly damaged and eugenol caused high protein content leakage after 120min of exposure (Oyedemi et al., 2006).

The effect of eugenol on the growth of Gram-positive (*Bacillus cereus*, *B. Subtilis*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Salmonella typhi*; *Pseudomonas aeruginosa*) bacteria using the agar well diffusion method was investigated by Singh (Singh et al., 2007). At 1,000ppm, eugenol inhibited the growth of the bacteria and complete inhibition was obtained against *P. aeruginosa* at a high concentration of 2,000ppm. Several other studies have confirmed the antibacterial activity of eugenol against various pathogens such as *E. coli*, *B. cereus*, *Helicobacter pylori*, *S. aureus*, *S. epidermdis*, *Streptococcus pneumoniae* and *S. pyogenes* amongst numerous others (Laekeman et al., 1990; Ali et al., 2005; van Zyl et al., 2006; Leite et al., 2007). The effect of eugenol on adherence and biofilms of two pathogenic *P. aeruginosa* isolates was studied. The treatment of *P. aeruginosa* with eugenol (0.5%) resulted in 60% adherence inhibition for *P. aeruginosa*. Inhibition of more than 90% was obtained when eugenol was tested against *P. aeruginosa* (ATCC 27853) biofilms (El-Abed et al., 2011). Several studies have investigated the combination of an essential oil molecule with a conventional antibiotic to determine possible synergistic interaction. For instance, the combination of eugenol with vancomycin and β-lactam antibiotics resulted in an increase of activity by a factor of 5–1,000 with respect to their individual MIC values. This synergistic effect could be explained by the fact that eugenol is able to
damage the membrane of Gram-negative bacteria. It was found that a concentration of 1mM damaged nearly 50% of the bacterial membrane allowing increased penetration of vancomycin and β-lactam antibiotics and therefore a greater antimicrobial effect (Hemaiswarya et al., 2009).

E. coli is able, neither to use the aromatic substrates eugenol or ferulic acid as the carbon source for growth, nor to convert or degrade ferulic acid. Thus, it is an eligible host for establishing a biochemical pathway enabling biotransformation of eugenol to ferulic acid. Eugenol tolerance of E. coli XL1-Blue was studied by Overhage. They studied different concentrations of eugenol ranging from 0.01% (vol/vol) up to 0.1% (vol/vol) in E. coli XL1-Blue culture for 48 hours (Overhage et al., 2003). Optical densities of the cultures at 600 nm were determined. They found that growth of E. coli XL1-Blue was only slightly affected by eugenol concentrations of up to 0.025% (vol/vol). There was a notable inhibition of E. coli XL1-Blue at a eugenol concentration of 0.05% (vol/vol), and the strain was no longer able to grow at concentrations higher than 0.075% (vol/vol).

2.6 Biotransformation of Eugenol to Ferulic Acid

Vanillin metabolic pathway proceeds from eugenol, via ferulic acid to vanillin. It has been studied in detail in Pseudomonads (Brandt et al., 2001). One of the most important aromatic flavor compounds vanillin is used in the food and perfume-producing industries. “Artificial” or “nature-identical” vanillin is currently produced from petrochemicals and from lignin, but there is a growing interest in producing "natural" vanillin by biotransformations (Hagedorn et al., 1994; Krings et al., 1998; Priefert et al., 2001). Phenolic stilbenes, eugenol (4-allyl-2-methoxyphenol), ferulic acid (4-hydroxy-3-methoxycinnamate), and lignin were found to be potential substrates for these biotransformation processes. A biotransformation with similar efficiency based on the same substrate using S. setonii as a biocatalyst was developed (Muller et al., patent application). However, these biotransformations depend on the use of the expensive substrate ferulic acid. A different source for a natural ferulic acid feedstock is eugenol, which is a commercially available natural raw material extracted from clove tree leaves. Although, eugenol is highly toxic for microorganism even in low concentrations (Friedman et al., 2002), but still eugenol can be used as precursor substrate for the
production of aromatics. Eugenol and isoegenol have attracted attention as natural renewable sources for the production of different useful chemicals (Rabenhorst et al., 1996).

Eugenol has great potential as a starting material for the synthesis of aromatic flavorings and aromas. *Pseudomonas* and *Corynebacterium* strains degrade eugenol to vanillin via coniferyl alcohol, coniferyl aldehyde, and ferulic acid (Takada et al., 1977; 1983). The initial conversion of eugenol to coniferyl alcohol is catalyzed by eugenol hydroxylase, which is encoded by *ehyAB* (Furukawa et al., 1998; Priefert et al., 1999; Brandt et al., 2001). Eugenol degradation involves two oxidation reactions, where first step involves oxidation of double bond of side chain to coniferyl alcohol, and in the second oxidation step, it is converted into ferulic acid via coniferyl aldehyde (Priefert et al., 2000) (Fig. 2.3).

![Fig. 2.3: Biochemical Pathway for Conversion of Eugenol to Ferulic acid.](image)

The initial step of eugenol degradation was confirmed to be the double-bond-transferring hydroxylation catalyzed by eugenol dehydrogenase (Furukawa et al., 1998; Wieser et al., 1999). The gene loci *ehyA* and *ehyB* of *Pseudomonas* sp. HR199 were identified as the structural genes of eugenol hydroxylase (dehydrogenase). Genes *ehyAB*, *calA* and *calB*, encoding eugenol hydroxylase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase were studied in different organisms.

These genes were expressed in *E. coli* to establish conversion of eugenol to ferulic acid. No biotransformation was achieved with this strain, although the
corresponding enzyme activities were detectable in protein extracts of the recombinant cells in vitro. Thus, the initial reaction leading from eugenol to coniferyl alcohol could not be established by expression of the eugenol hydroxylase genes in *E. coli*. *Pseudomonas* sp. strain HR199 has been used to produce natural ferulic acid; however, the yield of 6.7 g of ferulic acid liter\(^{-1}\) from 13.9 g of eugenol liter\(^{-1}\) within 78 h was too low for a commercial process.

To achieve higher yields, the said genes of *Pseudomonas* sp. Strain HR199 were expressed in *R. eutropha* H16, which is not able to degrade ferulic acid (Overhage *et al*., 2003). With this recombinant strain, 3.5 g of ferulic acid liter\(^{-1}\) from 3.2 g liter\(^{-1}\) eugenol was obtained within only 20 h, which corresponded to a molar yield of 93.8%. The major difficulty of this biotransformation process was the sensitivity of *R. eutropha* H16 to eugenol, demanding a stringent eugenol addition mode to keep the final concentration of eugenol in the medium below 3.25 mM (corresponding to 0.05% [vol/vol]).

The same reaction is also catalyzed by vanillyl alcohol oxidase of *Penicillium simplicissimum* (Fraaije *et al*., 1995; 1997), encoded by *vaoA* (Benen *et al*., 1998). This enzyme shares extensive regions of homology with the flavoprotein subunit of the eugenol hydroxylases (Benen *et al*., 1998; Priefert *et al*., 1999; Brandt *et al*., 2001). However, it consists of only a single subunit type and lacks a cytochrome *c* subunit. The gene *vaoA* was chosen to establish the initial eugenol biotransformation step in *E. coli*. Expression of *vaoA* enabled *E. coli* XL1-Blue to convert eugenol to coniferyl alcohol, coexpression of *vaoA*, *calA*, and *calB* was expected to enable *E. coli* to convert eugenol to ferulic acid efficiently. Resting cells of the corresponding recombinant strain *E. coli* XL1-Blue (pSKvaom Pcal4mcalB) converted eugenol to ferulic acid with a molar yield of 91% within 15 h on a 50-ml scale, reaching a ferulic acid concentration of 8.6 g liter\(^{-1}\). The maximum production rate for ferulic acid at that scale was 14.4 mmol per h per liter of culture. The maximum concentration of ferulic acid obtained was 14.7 g liter\(^{-1}\) after a total fermentation time of 30 h, which corresponded to a molar yield of 93.3% with respect to the added amount of eugenol. In different organisms, different genes are used for biotransformation of eugenol to ferulic acid (Fig. 2.4).

VAO has been used in metabolic engineering experiments with the aim of creating a bacterial whole cell biocatalyst that is able to form vanillin from eugenol. Since VAO is poorly expressed in bacteria, resulting in a relatively low intracellular
VAO activity and low yields of purified VAO when *Escherichia coli* is used as the expression host. A gene *eugo* encoding a eugenol oxidase has been identified in the genome from *Rhodococcus sp.* strain RHA1 (Jin et al., 2007).

### 2.7 Genes of Eugenol to Ferulic acid Metabolic Pathway

In metabolic pathway of eugenol to ferulic acid, translated product of eugenol oxidase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase genes catalysed various reactions of metabolic pathway.

#### 2.7.1 Eugenol Oxidase (*EUGO*)

In the genome of *Rhodococcus sp.* strain RHA1, a gene encoding a eugenol oxidase was identified. Fosmid clone of genome of *Rhodococcus* are prepared in genomic library. Different genes are located in fosmid which can be used for amplification of desired genes. Fosmids are similar to cosmids but are based on the bacterial F-plasmid. The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule. Fosmids can hold DNA inserts of up to 40kb in size; often the source of the insert is random genomic DNA. A fosmid library is prepared by extracting the genomic DNA from the target organism and cloning it into the fosmid vector.

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Fig. 2.4: Genetic Organization of Eugenol Biotransformation Cassettes in various studies amongst different organisms.
The ligation mixture is then packaged into phage particles and the DNA is transfected into the bacterial host. Bacterial clones propagate the fosmid library. The low copy number offers higher stability than vectors with relatively higher copy numbers, including cosmids.

Fig. 2.5: (A) Crystal structure of VAO in which the histidyl-bound FAD cofactor is shown in sticks. The dimer–dimer interacting loop, missing in EUGO, is indicated. (B) Superposition of the VAO structure (black) and the modeled apo-EUGO structure (gray). His422 of VAO, linking the FAD cofactor, aligns with His390 of EUGO (Jin et al., 2007).
Fosmids may be useful for constructing stable libraries from complex genomes. Fosmids have high structural stability and have been found to maintain human DNA effectively even after 100 generations of bacterial growth. Eugo shares 45% amino acid sequence identity with vanillyl alcohol oxidase from the fungus *Penicillium simplicissimum*. The bacterial oxidase efficiently oxidizes eugenol into coniferyl alcohol ($K_M \approx 1.0\text{m}$, $k_{cat} \approx 3.1\text{s}$). Vanillyl alcohol and 5-indanol are also readily accepted as substrates, whereas other phenolic compounds (vanillylamine, 4-ethyl guaiacol) are converted with relatively poor catalytic efficiencies. The catalytic efficiencies with the identified substrates are strikingly different when compared with vanillyl alcohol oxidase. The ability to efficiently convert eugenol may facilitate biotechnological valorization of this natural aromatic compound. Vanillyl alcohol oxidase from *Penicillium simplicissimum* is active on a range of phenolic compounds. As well as oxidizing alcohols, the fungal enzyme is also able to perform amine oxidations, enantioselective hydroxylations, and oxidative ether-cleavage reactions. The bacterial oxidase was found to be most active with eugenol, and hence has been named eugenol oxidase. EUGO represents a true oxidase, as it can efficiently use molecular oxygen as electron acceptor. It shares this property with VAO.

A number of VAO homologs can be found such as 25 bacterial and fungal homologs with sequence identity of $>30\%$. A putative VAO from *Rhodococcus sp.* strain RHA1 was found to display sequence identity with VAO (45%) (40% with PCMH). Sequence alignment with its characterized homologs revealed that it contains a histidine residue (His390) at the equivalent position of the FAD-binding histidine in VAO. This suggested that this enzyme might represent a bacterial VAO (Jin *et al*., 2007).

Eugenol oxidase could be expressed at high levels in *Escherichia coli*, which allowed purification of 160 mg of eugenol oxidase from 1L of culture (Jin *et al*., 2007). Gel permeation experiments and macromolecular MS revealed that the enzyme forms homodimers. Eugenol oxidase is partly expressed in the apo form, but can be fully flavinylated by the addition of FAD. Cofactor incorporation involves the formation of a covalent protein–FAD linkage, which is formed autocatalytically. Modeling using the vanillyl alcohol oxidase structure indicates that the FAD cofactor is tethered to His390 in eugenol oxidase. The model also provides a structural explanation for the observation that eugenol oxidase is dimeric whereas vanillyl alcohol oxidase is octameric (Jin *et al*., 2007).
The flavoenzyme vanillyl alcohol oxidase (VAO, EC 1.1.3.38) from *Penicillium simplicissimum* is active on a range of phenolic compounds (Jong *et al.*, 1992; Fraijee *et al.*, 1995). It contains a covalently linked FAD cofactor, and the holoprotein forms stable octamers. Recently, VAO has been used in metabolic engineering experiments with the aim of creating a bacterial whole cell biocatalyst that is able to form vanillin from eugenol (Overhage *et al.*, 2003; 2006). However, VAO is poorly expressed in bacteria, resulting in a relatively low intracellular VAO activity (Overhage *et al.*, 2006) and low yields of purified VAO when *Escherichia coli* is used as the expression host (Benen *et al.*, 1998). A putative VAO from *Rhodococcus* sp. strain RHA1 was found to display sequence identity with VAO (45%).

Sequence alignment with its characterized homologs revealed that it contains a histidine residue (His390) at the equivalent position of the FAD-binding histidine in VAO (Fig. 2.5). This suggested that this enzyme might represent a bacterial VAO.

### 2.7.2 Coniferyl Alcohol Dehydrogenase (*calA*)

Sequence of coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase are available in genome of *P. nitroreducens* Jin1. *P. nitroreducens* Jin1 is an aerobic, gram-negative soil bacterium, first isolated from oil brine in Japan. It was used to isolate bacteria capable of growing on eugenol and isoeugenol (Izuka *et al.*, 1964). Gram negative rod is of size 0.4 to 0.6 by 1.4 to 1.8, occur singly or in pair. Colonies are circular, smooth or slightly rough entire raised glistening. It is able to synthesise polyhydroxybutyrate homopolymer (a polyester) from medium chain length fatty acids. A soil bacterium isolate was found to be capable of utilizing eugenol and isoeugenol as a sole source of carbon and energy and was named Jin1 (Ryu *et al.*, 2010). Based on 16S rRNA analysis, *P. nitroreducens* Jin1 has been placed in the *P. aeruginosa* group (Anzai *et al.*, 2000). *P. nitroreducens* Jin1 is having genomic DNA of size 1,37,693 bp. It has different genes like *lolC, lolD, lolE, gpd, calB, calA* etc. (Ryu *et al.*, 2010).

A group of isozymes that catalyses the oxidation of primary and secondary alcohol to aldehyde and ketones respectively are known as alcohol dehydrogenase (ADH). ADH can also catalyse the reverse reaction (Fig. 2.6). These are found in both prokaryotic and eukaryotic organisms. Reduction/oxidation (Redox) reaction involves
the coenzyme nicotinamide adenine dinucleotide. ADH also catalyzes the reverse reaction which is a part of fermentation reaction to ensure a constant supply of NAD$^+$ in yeast, plants and many bacteria. Alcohol dehydrogenase (ADH) was first isolated and purified by Negelein and Wuluff from *Saccharomyces cerevisiae* (baker's yeast) (Negelein *et al.*, 1937).

**Fig. 2.6: Bioconversion of Coniferyl Alcohol into Coniferyl Aldehyde.**

Animal and bacterial ADH showed different properties such as the yeast enzyme is double the size of animal ADH and its activity is 100 times more. The yeast enzyme is more specific than mammal ADH.

Different ADH have been divided between short-chain dehydrogenase, medium-chain dehydrogenases and long-chain dehydrogenases/ reductases on the basis of distinct sequence motifs, protein chain length, mechanistic features and structural comparisons (Krook *et al.*, 1992; Anderson *et al.*, 1996; Oppermann *et al.*, 1997). From study of different genomes it has been found that about 1/4 of all dehydrogenases found are Short Dehydrogenase Reductase (SDRs).

SDR superfamily was found to be the largest, having more than 60,000 non-redundant sequences (over 30,000) of the ‘classical’ type and close to 30,000 of the ‘extended’ type (Venter *et al.*, 2001). Currently more than 47,000 primary structures are found in this superfamily. These sequences are available in sequence databases and over 300 crystal structures are now deposited in the Protein Data Bank. All the members of SDR show early divergence, most of family members show only low pair-wise sequence identity (typically 20–30%), but have several properties in common.

SDR superfamily is present in all domains of life. Of the large number of completely sequenced bacterial genomes (close to 400 in January 2007), about 3/4 of all known SDR forms are of bacterial origin. The members of SDR family belong to domain cd05328 of NCBI Conserved Domain Database (www.ncbi.nlm.nih.gov). The
short name of this domain is alpha_HSD__SDR_c. Pair-wise sequence identity amongst different SDR enzymes is typically 15-30%. All available 3D structures (27 deposited in PDB at present) show a high similarity of α/β folding pattern with the Rossmann-fold being present in all. The Rossmann fold is characterised by presence of a typical central β-sheet which is flanked by α-helices. The fold is also common to other subdivisions of oxidoreductases. The active site is constituted from a triad of catalytically important Ser, Tyr, Lys residues. Out of these three, Tyr is found to be the most conserved residue within the whole family (Jornvall et al., 1995). NNAG around position 86-89, singular Asp around position 60, another Asn around position 111 and an IRVN sequence preceding a P-G motif around position 180, followed by a conserved Thr around position 188 (Jornvall et al., 1995; Filling et al., 2001) are other conserved sequences found in members of SDR. The functions of all these residues have been found by combinations of chemical modifications, sequence comparisons, their structure analyses and site-directed mutagenesis (Krook et al., 1992; Prozorovski et al., 1992; Anderson et al., 1996; Zhou et al., 1999; Filling et al., 2001). In the 3D SDR structure, all these sequence motifs are assigned to three different regions. Namely (i) the coenzyme binding region with the central β-sheet, (typical of a Rossmann-fold) (ii) the active site and (iii) sections proximal to the substrate binding region. A core structure in most of SDR enzymes is 250-350 residues (Jornvall et al., 1995) in length, which have N- or C-terminal trans-membrane domains or signal peptides, or form parts of multi-enzyme complexes (Kalberg et al., 2002).

There are three major classes of microbial alcohol dehydrogenases depending on categorised specificities (Reid et al., 1994).

1. NAD(P) dependent enzymes which are further subdivided into three classes;
   
   Zn-dependent alcohol dehydrogenase
   
   Zn-independent alcohol dehydrogenase
   
   Iron activated alcohol dehydrogenase
   
2. NAD(P) independent alcohol dehydrogenase which are pyrroloquinoline quinine, heme or cofactor F420.
   
3. FAD dependent ADH.
Coniferyl alcohol dehydrogenase (CADH) are mostly NAD dependent. They occur in many prokaryotic as well as eukaryotic species. In eukaryotes, it is mainly found in the plants such as sorghum, sweet potato, oryza, populous etc. Cinnamyl alcohol dehydrogenase (CAD) occurs in Sorghum, Sweet potato, Eucalyptus gunnii, Oryza, Populus, Aspergillus nidulans etc. (Barakat et al., 2009). CAD participates in phenylpropanoid biosynthesis and catalyses the reversible conversion of p-hydroxycinnamaldehyde to its corresponding alcohol, which further leads to the biosynthesis of lignin in plants (Barakat et al., 2009; MaQH et al., 2010). In lignin biosynthesis, CAD is a key enzyme which catalyzes the last step in the synthesis of monolignols. Their role is not very well defined beyond plants. Molecular mass of plants CADH is yet to be established. The CAD gene family has been studied in Arabidopsis thaliana, Oryza sativa and partially in Populus (Barakat et al., 2009). Cinnamyl alcohol dehydrogenase plays a role in synthesis of coniferyl alcohol in tobacco xylem of Eucalyptus gunnii (Daimini et al., 2005). In Aspergillus nidulans (Belaish et al., 2007), it is involved in conidial germination and cell wall morphogenesis.

Sequential degradation pathway of eugenol has been found in different species of bacteria such as Acinetobacter (Dalneri et al., 1995), Corynbacterium (Marnens et al., 2005), Streptomyces (Sutherland et al., 1987; Nishimura et al., 1987) and Helicobacter pylori (Mee et al., 2005). Coniferyl alcohol dehydrogenase (CADH) has been studied in many bacteria such as Rhodococcus erythropolis, Pseudomonas sp. HR199 (Achterholt et al., 1998) and plants (Mansell et al., 1976). In Helicobacter pylori, the gene for cinnamyl alcohol dehydrogenase (HpCAD) has been cloned in Escherichia coli. The recombinant enzyme was characterized for substrate specificity (HpCAD) (Mee et al., 2005). It was found to be a monomer of 42.5 kDa located predominantly in the cytosol of the bacterium. This enzyme uses NADP(H) specifically as cofactor and has a broad substrate specificity for alcohol and aldehyde substrates. In Streptomyces NL15-2K, two isoforms of CADH have been found which are CADH1 and CADHII. CADH1 has high substrate affinity for cinnamyl alcohol than coniferyl alcohol. It also favours aromatic alcohols without 4-hydroxy-3-methoxy groups. CADHII acts on only coniferyl alcohol, cinnamyl alcohol and 3-(4-hydroxyl-3-methoxy phenyl)-1-propanol. Molecular mass of native CADH from Streptomyces (CADHIII), Rhodococcus and Pseudomonas was found to be 151kDa, 200kDa and 54.9kDa respectively. CADH from Pseudomonas is a dimer composed of two identical subunits with identical molecular mass of 27 kDa. CADHII is
tetrameric. This shows molecular mass and quaternary structure vary among bacterial enzymes. In *Rhodococcus* spp., subunits are not identified. Coniferyl alcohol is the best substrate for enzymes from both *Streptomyces* and *Rhodococcus*.

This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD\(^+\) or NADP\(^+\) as acceptor. The systematic name of this enzyme class is coniferyl alcohol: NADP\(^+\) oxidoreductase. Coniferyl alcohol is the main product in the biotransformation of eugenol to ferulic acid. Eugenol can be used as a source for the production of ferulic acid and *calA* gene product oxidises coniferyl alcohol to coniferyl aldehyde, which is then oxidized to ferulic acid (Theorell *et al.*, 1961).

Coniferyl alcohol dehydrogenase (*calA*) gene has been isolated and characterized from many prokaryotes such as *Escherichia coli*, *Pseudomonas* sp. HR199 (Overhage *et al.*, 1999) and *Ralstonia eutropha* (Priefert *et al.*, 1999). While in some others it is known but not cloned as from *Pseudomonas nitroreducens* Jin1.

### 2.7.3 Coniferyl aldehyde dehydrogenase (*calB*)

Coniferyl aldehyde dehydrogenase enzyme is member of the family oxidoreductase, which acts on the aldehyde or oxo group of donor with NAD\(^+\) or NADP\(^+\) as acceptor. The systematic name is aldehyde : NAD\(^+\) aldehyde oxidoreductase. Other common names are: CoA-independent aldehyde dehydrogenase, m-methyl benzaldehyde dehydrogenase, NAD-aldehyde dehydrogenase, NAD-dependent-4-hydroxynonenal dehydrogenase, NAD-dependentaldehyde dehydrogenase, NAD-linked aldehyde dehydrogenase, propionaldehyde dehydrogenase and aldehyde dehydrogenase (NAD) (Boyer *et al.*, 1963).

Aldehyde dehydrogenase belongs to class of NADPH dependent enzymes which catalyze reduction of various phenylpropenyl aldehyde derivatives. These enzymes mainly catalyse the oxidation (dehydrogenation) of aldehydes to carboxylic acid (Marchitti *et al.*, 2008). Aldehyde dehydrogenase gene is found in many organisms from prokaryotes such as bacteria, fungus to eukaryotes such as mammals. They belong to aldehyde dehydrogenase domain (IPR015590). Aldehyde dehydrogenase family consists of enzymes which are involved in conversion of a broad variety of aliphatic and aromatic aldehydes to their respective carboxylic acids (Marchitti *et al.*, 2008;
Perozich et al., 1999; Wang et al., 2009). Dehydrogenase enzymes may be dimers or tetramers of identical subunits (Perozich et al., 1999). It is clear from crystal structures of enzymes that subunits of different ALDHs contain very similar domains like each has catalytic, nucleotide binding and oligomerization domains (Liu et al., 1997; Steinmetz et al., 1997; Lamb et al., 1999; Moore et al., 1998; Johansson et al., 1998; Cobessi et al., 1999; Tsybovsky et al., 2007).

Aldehyde dehydrogenases participate in 17 different metabolic pathways (Racker et al., 1949) such as glycolysis/gluconeogenesis (Kim et al., 2011), ascorbate and aldarate metabolism (Bánhegyi et al., 1997), bile acid synthesis (Staels et al., 2010), fatty acid metabolism, valine, leucine and isoleucine degradation, urea cycle, metabolism of amino acids, histidine metabolism, lysine degradation, tryptophan metabolism, glycerolipid metabolism, pyruvate metabolism, 1,2_dichloroethane degradation, propanoate metabolism butanoate metabolism, 3-chloroacrylic acid degradation, limonene and pinene degradation, butanoate metabolism.

**Aldehyde + NAD+ + H2O ⇄ an acid + NADH + H+**

It is an enzyme that catalyses the oxidation of aldehyde to ferulic acid. Coniferyl aldehyde, NAD+ and H2O are the 3 substrates of this reaction giving rise to 3 products ferulic acid, NADH and H+. Structurally the enzyme consists of three domains which are: a NAD(P)+ cofactor-binding domain, a catalytic domain and a bridging domain. The catalytic mechanism of enzyme involves cofactor (NAD(P)+) binding, which results in a conformational change and activation of an invariant catalytic cysteine nucleophile. It has been found that ALDH reaction is completed in two steps, acetylation and deacetylation (Steinmetz et al., 1997; Moore et al., 1998; Feldman et al., 1972; Marchal et al., 2000).

In first step, invariant active site cysteine makes a nucleophillic attack on the carbonyl carbon of the aldehyde molecule (Fig. 2.7) and thus thio-hemiacetal intermediate is formed. From this intermediate, hydride ion is transferred from aldehyde to the C4 atom of the nicotinamide ring of the NAD(P)+. This causes a collapse of the thio-hemiacetal to thioester intermediately. In second step, an activated water hydrolyses the thioester intermediate which releases the product. Deprotonation of catalytic cysteine before nucleophillic attack on the substrate, is an essential step in this mechanism. It may be caused by either glutamate 268 or 399 (Steinmetz et al., 1997; Moore et al., 1998;
Sheikh et al., 1997; Mann et al., 1999; Wymore et al., 2007). The resulting thiolate ion is likely stabilized by the positively charged nicotinamide ring of the coenzyme and/or adjacent main chain amide groups. The proton abstracted by Glu-268 may then go to bulk water (Tsybovsky et al., 2007; González-Segura et al., 2009). In addition, Glu-268 may be most likely residue which activates water molecule in the second step of the reaction (Steinmetz et al., 1997; Moore et al., 1998; Tsybovsky et al., 2007; Marchal et al., 2000; Wang et al., 1995).

Beside cysteine and glutamate many other residues interact with the NAD(P)$^+$ to hold it in place. Magnesium may help in the function of enzyme but the amount of magnesium which helps the enzyme can vary between different classes of aldehydes. In reaction, the aldehyde enters the active site through a channel located on the outside of the enzyme. The active site contains a Rossman fold. Interactions between the cofactor and the fold allow the isomerization of the enzyme while keeping the active site functional (Liu et al., 1997). Rossman fold containing NADP-binding protein, is a large family of proteins which share Rossman fold NAD(P)H/NADP(+) binding NADB domain. In metabolic pathway of numerous dehydrogenases such as glycolysis and many other redox enzymes the NADB domain is found. Numerous H–bond and vanderwall’s contacts are involved in the NAD binding. In aldehyde dehydrogenase enzyme, active site is largely conserved throughout the different classes of the enzyme and although the number of amino acids present in a subunit can change, but there is little change in overall function of the site (Marchal et al., 2000; Wang et al., 1995).

Different organisms have several distinct ALDH genes. In E. coli, in one species the largest number of sequences of ALDH present are 13. In human, eleven sequences of ALDH are present, excluding the ALDH8 protein (Hsu et al., 1997). In Bacillus subtilis, 10 sequences and 14 from various species of Pseudomonas are known. Most of these
sequences are involved in metabolising aromatic aldehydes. In Clostridium butylicum ATCC 824, a gene *aad* has been found whose N terminal shows homology to aldehyde dehydrogenase and C-terminal shows homology to alcohol dehydrogenase (Nair *et al*., 1994).

In bacteria, acylating acetaldehyde dehydrogenase forms a bifunctional heterodimer with metal-dependent 4-hydroxy-2-ketovalerate aldolase. Enzyme is used in the bacteria for degradation of toxic aromatic compounds. During degradation, the enzyme’s crystal structure shows that intermediates are shuttled directly between the aldolase active site and the acetaldehyde dehydrogenase active site. Such communication between proteins allows for the efficient transfer of substrates from one active site to the next (Manjasetty *et al*., 2003).

Coniferyl aldehyde dehydrogenase (*calB*) is also a member of aldehyde dehydrogenase family with conserved domain cd07133 (NCBI). *calB* occurs in many bacteria such as Pseudomonas fluorescens AN103 (Gasson *et al*., 1998), Pseudomonas sp. HR199 (Overhage *et al*., 1999), Streptomyces setonii (Muheim *et al*., 1999), Amycolatopsis sp. HR167 (Achterholt *et al*., 2000), Delftia acidovorans (Plaggenborg *et al*., 2001) and Pseudomonas putida KT2440 (Plaggenborg *et al*., 2003). *CalB* converts coniferyl aldehyde to ferulic acid. Ferulic acid may be further converted into vanillin. Thus *calB* also plays role in the production of vanillin. Ferulic acid, vanillic acid, coniferyl alcohol, coniferyl aldehyde are the main products in the biotransformation of eugenol to vanillic acid catalysed by *Pseudomonas* sp. HR199 and *Rhodococcus opacus* PD630 (Overhage *et al*., 2002). In the experiment to improve the yield of vanillin, metabolic engineering was carried out by introducing valinyl alcohol oxidase gene (*vaoA*) from *P. simplicissimum* CBS170.90 in *R. opacus* PD630 and *Amycolatopsis* sp. HR167, together with the coniferyl alcohol dehydrogenase (*calA*) and coniferyl aldehyde dehydrogenase (*calB*) genes from *Pseudomonas* sp. HR199. The recombinant strains converted eugenol to ferulic acid, which could be transformed to vanillin. Therefore, a vanillin production process was constructed (Overhage *et al*., 2006). The process may be further improved upon.

It has been found that coniferyl aldehyde dehydrogenase enzyme from *Pseudomonas* sp. HR199 is homodimer of two identical subunits. Each subunit is 49.5kD in size. Optimum temp (Achterholt *et al*., 1998) for its activity is 26°C. In *Ralstonia*
eutropha H16, by using ehyAB, calA, and calB genes encoding eugenol hydroxylase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase respectively the biotransformation of eugenol to ferulic acid was studied (Priefert et al., 1999). Later the biotransformation in industrially approved bacterium Ralstonia eutropha H16 was carried out using vao gene instead of ehyAB gene (Overhage et al., 2002), which quantitatively improved production of ferulic acid in R. eutropha H16 to $<0.01$U/mg of protein. However, further two Gram-positive microorganisms, Amycolatopsis sp. HR167 and S. setonii, which exhibited a very high tolerance towards vanillin, were identified for transformation with vao, calA, and calB genes (Achterholt et al., 2000).