GROWTH AND CHOLESTEROL REDUCTION ACTIVITY OF *EUBACTERIUM COPROSTANOLIGENES*

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SUMMARY

Eubacterium coprostanoligenes has been found to be a cholesterol-reducing microorganism. To verify this, the bacteria were grown in Base Cholesterol Medium and its growth was studied by plating growing broth culture on agar solidified medium. It was found that cholesterol was not required for bacterial growth, and the growth was affected by lecithin, $CaCl_2$ and pH of culture medium. In addition, being anaerobic, *E. coprostanoligenes* was found to survive when exposed to ambient air. Morphology of the bacterium was re-affirmed by confocal and transmission electron microscopy to be coccobacilloid.

Cholesterol reduction activity in *E. coprostanoligenes* was studied using gas chromatography because of its practicality and accuracy. With this method, the conversion of cholesterol to coprostanol by *E. coprostanoligenes* was re-affirmed. The cholesterol reduction activity was found to be affected by lecithin, $CaCl_2$ and pH of culture medium. In addition, the reaction could take place under aerobic condition.

Cholesterol reduction activity in *E. coprostanoligenes* was found to increase with increasing cholesterol concentration. A kinetics study of cholesterol reduction activity in these bacteria showed a V_{max} of 14 μM cholesterol reduced/h and K_m of 1 mM cholesterol. The putative cholesterol reducing enzyme(s) appeared to be secreted constitutively and intracellularly. On the other hand, cholesterol reduction in *E. coprostanoligenes* was shown to take place via the indirect pathway. However, attempts to isolate the enzyme(s) by breaking bacterial cells were not successful.

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LIST OF ABBREVIATIONS

ANOVA   analysis of variance

BCM   base cholesterol medium

CHD   coronary heart diseases

GC   gas chromatography

NADH   reduced nicotinamide adenine dinucleotide

R_m relative mobility

R_t relative retention time

TLC   thin layer chromatography

INTRODUCTION

Hypercholesterolemia has been a major health problem particularly in developed countries. Being associated with coronary heart diseases (CHD), it can finally lead to death (Tell *et al.* 1994; Kromhout *et al.*, 1995; Mann *et al.*, 1997; Hegsted and Ausman, 1998). In Singapore, a quarter of the residents was found to have high total cholesterol levels ($\geq$ 6.2 mmol/L) in the National Health Survey conducted in 1998 (Tan, 2000). Nevertheless, some reports have shown that the lowering of cholesterol levels could increase survival rate in CHD patients (Pederson, 1994; Shepherd *et al.*, 1995; Sacks *et al.*, 1996). In view of this, various pharmacological agents (Hunninghake, 1990; März *et al.*, 1997; Staels *et al.*, 1998; Ros, 2000; Istvan, 2003) and dietary supplements (Crouse and Grundy 1979; Benitez *et al.*, 1997; Howard and Kritchevsky, 1997; Danijela *et al.*, 2003) have been developed with the chief aim of lowering plasma cholesterol levels. Statins have been established by far to be the most efficient cholesterol-lowering drug (Istvan, 2003). However, benefits aside, some of these agents (e.g. statins and fibrates) have been reported to incur side effects such as gastrointestinal disturbances and sleep disorders (Christian *et al.*, 1998; Najib, 2002).

Cholesterol-reducing bacteria have the potential to serve as an alternative for cholesterol lowering (Dehal *et al.*, 1991). These bacteria have the ability to convert cholesterol to coprostanol. Cholesterol-lowering ability is achieved as coprostanol is poorly absorbed in human intestines and would be excreted (Bhattacharyya, 1986). Cholesterol-reducing bacteria have been isolated from rat cecal contents (Eyssen *et al.*, 1973), faeces of human (Sadzikowski *et al.*, 1977) and that of baboon (Brinkley *et al.*, 1982). These isolated cholesterol-reducing bacteria have been found to require plasmalogen for growth or for its cholesterol-reduction activity (Eyssen *et al.*, 1973;

Sadzikowski *et al.*, 1977; Brinkley *et al.*, 1982). An exception however is *Eubacterium coprostanoligenes*, one of the isolated cholesterol-reducing bacteria, which has been established to not require plasmalogen for growth or cholesterol reduction activity (Freier *et al.*, 1994). It was therefore a useful experimental microorganism to explore its cholesterol-lowering potential.

The aim of this project is to develop suitable methods to study factors affecting the growth and cholesterol reduction activity of *E. coprostanoligenes*. The information obtained from the study is prospected to be useful for future utilization of *E. coprostanoligenes* in cholesterol lowering in either the food or the pharmaceutical industry.

2   LITERATURE REVIEW

2.1   Cholesterol and health related issues

Cholesterol homeostasis is maintained by balancing intestinal cholesterol absorption and endogenous cholesterol synthesis (Dietschy *et al.* 1993). Intestinal absorption of cholesterol shares complexity to that of triglycerides because both are water-insoluble molecules (Wilson and Rudel, 1994). Its absorption requires steps of emulsification, hydrolysis of ester bonds by specific pancreatic esterase, micellar solubilization, absorption in the proximal jejunum, re-esterification within the intestinal cells, and transport to the lymph in the chylomicrons (Wilson and Rudel, 1994). Only 40 to 60 % of dietary cholesterol is absorbed independent of the amount ingested of up to 600 mg/day (Bosner *et al.*, 1999)

In addition to ingestion, cholesterol is synthesized and secreted from the liver as bile acids (Dietschy *et al.* 1993). A fraction of this biliary cholesterol is absorbed in the intestine due to the efficient re-absorption of bile acids. Dietary absorbed and endogenously synthesized cholesterol are transported as chylomicrons to liver where they are cleared efficiently for further processing (Dietschy *et al.*, 1993). This process has been found to exert regulatory effects on whole-body cholesterol homeostasis (Dietschy *et al.*, 1993). When the delivery of intestinal-absorbed cholesterol to the liver was increased, endogenous cholesterol synthesis is known to be inhibited in a proportional fashion with the increase in bile acids production. In this way, substantial variations of cholesterol intake induced minimal fluctuation in blood cholesterol level on human (Quintao *et al.*, 1971). On the other hand, the response of blood cholesterol to changes in dietary cholesterol was found to vary between individuals (Lin and Cornor, 1980; Maranhao and Quintao, 1983).

Excess cholesterol from diet and bile acids are excreted in faeces (Dietschy *et al.* 1993). This cholesterol mass escaping intestinal absorption will be degraded to coprostanol through reduction of the double bond at C-5 by colonic bacteria before it is excreted (Macdonald *et al.*, 1983). As such, it should be noted that the overall body cholesterol balance is kept mainly by matching cholesterol intake and synthesis with that of faecal loss. The latter is strictly dependent on intestinal cholesterol absorption which in turn is regulated by blood cholesterol levels (Dietschy *et al.* 1993).

Cholesterol absorption appears to be a very specific process (Salen *et al.*, 1970; Connor and Lin, 1981). Phytosterols like β-sitosterol, campesterol, and stigmasterol and marine sterols in shellfish have been found to be absorbed less efficiently (Salen *et al.*, 1970; Connor and Lin, 1981). These sterols are structurally related to cholesterol differing only in the degree of saturation of the sterol nucleus or in the nature of the side chains at C-24. Absorption of β-sitosterol, which differed from cholesterol only by the addition of an ethyl group on C-24, was found to be less than 5 % (Salen *et al.*, 1970).

Gender was found to be unrelated to the efficiency of cholesterol absorption (Bosner *et al.*, 1999). On the other hand, cholesterol absorption has been proposed to be affected by genetics, physiology and dietary factors (Nestel *et al.*, 1973; Vahouny *et al.*, 1980; de Leon *et al.*, 1982; Samuel *et al.*, 1982; Watt and Simmonds, 1984; McMurry *et al.*, 1985; Mahley, 1988; Thurnhofer *et al.*, 1991; Ostlund *et al.*, 1999). For example, studies have shown that polymorphism of apo E, a ubiquitous protein of lipid transport (Mahley, 1988) and mutation in the gene encoding for the putative intestinal cholesterol carrier protein (Thurnhofer *et al.*, 1991) were genetic factors influencing cholesterol absorption. Physiologically, obesity was found to be negatively associated with absorption of cholesterol (Nestel *et al.*, 1973). An increase

in the velocity of intestinal transit was associated with reduced cholesterol absorption and vice versa (de Leon *et al.*, 1982). Detergent capacity of different types of bile acids in the enterohepatic circulation was also reported to influence cholesterol absorption (Watt and Simmonds, 1984). Increased fiber content in a meal would reduce cholesterol absorption due to physical interaction within the intestinal lumen (Vahouny *et al.*, 1980) while the ingestion of cholesterol together with a significant amount of triglycerides in a diet facilitated cholesterol absorption (Samuel *et al.*, 1982).

Hypercholesterolemia is a condition when the plasma cholesterol elevates above 6.2 mmol/L, as defined by the United States Department of Health and Human Services. A survey on cholesterol status among Singaporeans was conducted in 1998 by the Epidemiology and Disease Control Department, Ministry of Health, Singapore. In a random sample of 4723 Singaporeans aged between 18 and 69 years, the survey found that a quarter (25.4 %) of them had high total cholesterol levels (≥ 6.2 mmol/L), 35.3 % with borderline-high levels (5.2-6.2 mmol/L) and 39.3 % at desirable levels (< 5.2 mmol/L) (Tan, 2000). The survey also showed that 94.8 % of Singapore residents had desirable HDL (High Density Lipoprotein)-cholesterol levels (≥ 0.9 mmol/L). On the other hand, 26.5 % of Singapore residents had high LDL (Low Density Lipoprotein)-cholesterol levels (≥ 4.1 mmol/L) and 30.2 % had borderline-high levels (3.3-4.1 mmol/L) (Tan, 2000). More males (27.3 %) than females (23.5 %) had high total cholesterol level. Overall, there was a significant increase in the age-standardized prevalence of high blood cholesterol from 1992 to 1998 (19.4 % and 25.4 %, respectively), mean total cholesterol (1992, 5.3 mmol/L; 1998, 5.5 mmol/L) and crude prevalence of high LDL-cholesterol (1992, 22.9 %; 1998, 26.5 %). There

was no significant difference in the overall age-standardized prevalence low HDL-cholesterol (1992, 6.0 %; 1998, 5.2 %) (Tan, 2000).

CHD have always been related to hypercholesterolemia (McNamara, 2000). Using simple regression analyses, dietary cholesterol has been found to be positively correlated to both plasma total cholesterol level and CHD incidence in many epidemiological studies (Hegsted and Ausman, 1988; Tell *et al.* 1994; Kromhout *et al.*, 1995; Mann *et al.*, 1997).

Hegsted and Ausman (1988) reported that dietary cholesterol was significantly related to CHD incidence. Tell *et al.* (1994) revealed that elevated cholesterol level resulted in a thickened carotid artery wall, which gives rise to CHD. Kromhout *et al.* (1995) measured risk factors for CHD and suggested that dietary cholesterol was an important determinant of the differences in the population rates of CHD death. However, the authors also suggested that cholesterol intake could be a surrogate marker for two other factors which also contributed to increased CHD risk: a) a high intake of saturated fat resulting in elevated plasma cholesterol levels; and b) a dietary pattern low in fruits, grains and vegetables hence resulting in low intakes of B vitamin, antioxidants and dietary fiber. Mann *et al.* (1997) reported that the deleterious effect of dietary cholesterol appeared to be more important in cases of CHD than the protective effect of dietary fiber. In contrast, Esrey *et al.* (1996) and Ascherio *et al.*, (1996) concluded that dietary fat and cholesterol intake were not significantly associated with CHD mortality. Lipid-heart hypothesis which proposes that elevated fat and cholesterol intake increase the risk of developing CHD might be overly simplistic.

The evidence to establish the relationship between dietary cholesterol and CHD incidence has been complicated by the co-linearity of saturated fat with

cholesterol in the diet (Hegsted and Ausman, 1988; Kromhout *et al.*, 1995; Mann *et al.*, 1997). Eggs are high –cholesterol low-saturated fat food. Studies on egg consumption indicated that dietary cholesterol was not associated with risk of CHD (Dawber *et al.*, 1982; Hu *et al.*, 1999). The apparent association between total dietary cholesterol and CHD mortality rates was hence explained by the association between dietary saturated fat calories and dietary cholesterol, and the low fiber intakes in diets high in animal products (Ascherio *et al.*, 1996; Hu *et al.*, 1997; Hu *et al.*, 1999).

Artaud-Wild *et al.* (1993) reported that different populations consuming diets with similar amount of cholesterol and saturated fat could incur different CHD incidence rates. It was shown that maintaining a high intake of cholesterol and saturated fat in the diet, people who consumed more plant foods, including small amount of vegetable oils, and more vegetable (more antioxidants) had lower rates of CHD mortality. Similarly, it has also been shown that patients who died from CHD had a lower vegetable food intake and a higher animal food intake than controls (Kushi *et al.*, 1985).

Even though plasma cholesterol response to dietary cholesterol is highly variable between individuals, the general consensus, as obtained from clinical trials of the effect of dietary cholesterol on plasma cholesterol, is that dietary cholesterol intake does exert a statistically significant, small effect on plasma cholesterol levels (Glatz *et al.*, 1993).

The quantitative importance of dietary fatty acids and cholesterol to blood concentrations of total, LDL-, and HDL-cholesterol was determined by Clarke *et al.*, (1997). The study showed that total blood cholesterol was reduced by about 0.8 mmol/L, with four fifths of this reduction being in LDL-cholesterol, when 60 % of saturated fats were replaced by unsaturated fats in a diet and cutting down 60 % of

dietary cholesterol. However, it should be hereby emphasized that the effects of dietary cholesterol on plasma total cholesterol cannot provide a true estimation of its effects on CHD risk since changes can occur in both the atherogenic LDL-cholesterol as well as in the anti-atherogenic HDL fraction. Numerous cholesterol feeding studies are supporting this notion since they suggest that LDL: HDL cholesterol ratio is unaltered by dietary cholesterol (Ginsberg *et al.*, 1994; Ginsberg *et al.*, 1995; Knopp *et al.*, 1997).

Even though the relationship between dietary cholesterol and incidence of CHD remained elusive, many studies have shown that lowering the cholesterol level could increase survival rate in CHD patients (Pedersen, 1994; Shepherd *et al.*, 1995; Sacks *et al.*, 1996). Pedersen (1994) showed that lowering cholesterol level using simvastatin improved survival in CHD patients by 30 %. This finding was replicated when hypercholesterolemia patients with no history of myocardial infarction were administrated with pravastatin: a reduction in total mortality of 22 % and a reduction in CHD (fatal and non-fatal) of 31 % (Shepherd *et al.*, 1995). The benefit of cholesterol-lowering therapy with pravastatin was also demonstrated in patients with CHD where 24 % reduction in CHD mortality was observed (Sacks *et al.*, 1996).

It was estimated that a long-term reduction in serum cholesterol concentration of 0.6 mmol/L (10 %) could lower the risk of heart disease by 50 % at age of 40, which could then fall to 20 % at age 70 (Law *et al.*, 1994). In view of this, various pharmacological agents (Hunninghake, 1990; März *et al.*, 1997; Staels *et al.*, 1998; Ros, 2000; Istvan, 2003) and dietary supplements (Crouse and Grundy 1979; Benitez *et al.*, 1997; Howard and Kritchevsky, 1997; Danijela *et al.*, 2003) have been developed with the chief aim to lower plasma cholesterol level.

2.2 Pharmacological agents in cholesterol lowering

Pharmacological agents commonly employed in the treatment of hypercholesterolemia include: 1) 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (or statins) (Istvan, 2003); 2) bile acid sequestrants (Packard and Shepherd, 1982; Ast and Frishman, 1990); 3) fibrates (Staels *et al.*, 1998); 4) ursodeoxycholic acid (Ros, 2000) and neomycin (Sedaghat *et al.*, 1975); and 5) lifibrol (März *et al.*, 1997).

The effectiveness of statins is related to the action of HMG-CoA reductase which converts HMG-CoA to mevalonate. This is a control step in the biosynthesis of cholesterol and inhibition of this enzyme will result in a decreased synthesis of cholesterol and other products downstream of mevalonate (Istvan, 2003). Statins are competitive inhibitors of HMG-CoA reductase (Istvan, 2003). They have been therapeutically used to reduce risk of CHD by reducing cholesterol synthesis and upregulating LDL receptors in the liver, consequently giving rise to a decreased level of circulating cholesterol (Istvan, 2003). Other anti-atherogenic effects of statins include: a) reduction of plasma viscosity and decreased platelet aggregation, b) production of a relaxing effect on smooth muscle that could potentially result in a reduction in blood pressure, and c) partially reverse vascular hyper-reactivity associated with hypercholesterolemia (Christian *et al.*, 1998). The most important side effects associated with the use of statins are hepatotoxicity and myopathy. Other common adverse events include gastrointestinal disturbances, dyspepsia, myalgia, headache, sleep disorders and central-nervous-system disturbances (Christian *et al.*, 1998)

Not only is the hepatic synthesis of bile acids from cholesterol a major component of cholesterol homeostasis, it is also a major route of cholesterol excretion.

Bile acids sequestrants basically engaged in hepatic bile acid synthesis and excretion to reduce concentrations of plasma cholesterol (Packard and Shepherd, 1982; Ast and Frishman, 1990). Cholestyramine, a bile acids sequestrant, has been widely prescribed for the treatment of hypercholesterolemia (Hunninghake, 1990) and was reported to cause a 38 % decrease in cholesterol absorption (McNamara *et al.*, 1980).

Fibrates are useful in the treatment of hypercholesterolemia in that it can result in a substantial decrease in plasma triglycerides. It has been found to be able to decrease LDL cholesterol levels while increasing HDL cholesterol concentrations (Staels *et al.*, 1998). Adverse effects of fibrates administration include gastrointestinal symptoms, cholelithiasis, hepatitis, myositis, and rash (Najib, 2002). The combination of fibrate and statin was found to provide complementary cholesterol lowering effects (Farnier *et al.*, 2003).

The fourth pharmacological agent commonly employed is ursodeoxycholic acid, which has the lowest micellar cholesterol-solubilizing ability of all common bile acids (Armstrong and Carey, 1982). Enrichment of endogenous bile acid pool with ursodeoxycholic acid was found to reduce both biliary cholesterol secretion and intestinal absorption as a result of inefficient cholesterol absorption (Fromm, 1984). Neomycin is a non-absorbable aminoglycoside antibiotic with cholesterol-lowering effect by interfering with the micellar solubilization of cholesterol in the digestive tract (Sedaghat *et al.*, 1975).

Last but not least, lifibrol {4-(4'-tert-butylphenyl)-1-(4'-carboxyphenoxy)-2-butanol} has been found to reduce cholesterol absorption from the intestine. It was also shown to moderately decrease hepatic cholesterol biosynthesis and stimulate the expression of LDL receptors (März *et al.*, 1997).

2.3   Dietary supplements in cholesterol lowering

Dietary supplements with cholesterol-lowering property include: 1) plant sterols (Howard and Kritchevsky, 1997); 2) soy lecithin (Boststo *et al.*, 1981; Wilson *et al.*, 1998); 3) sucrose polyester (olestra) (Prince and Welschenbach, 1998); and 4) policosanol (Benitez *et al.*, 1997; Canetti *et al.*, 1997).

Plant sterols (phytosterols), despite being synthesized in plants, are structurally similar to cholesterol. They are however minimally absorbed from the gut (Salen *et al.*, 1970). Ingestion of free phytosterols, especially β-sitosterol, has been shown to reduce plasma cholesterol in both animals and humans (Howard and Kritchevsky, 1997). Saturated plant sterol derivatives (termed plant stanols) are produced by the hydrogenation of sterols (Howard and Kritchevsky, 1997). Addition of plant sterol or stanol to margarine spread reduced serum concentrations of LDL-cholesterol and the risk of heart disease (Low, 2000; Neil and Huxley, 2002). The esterified forms of phytosterols have higher lipid solubility and could be used as cholesterol-lowering agents (Howard and Kritchevsky, 1997). The putative mechanisms by which plant sterols and stanols reduced serum cholesterol were found to include (a) inhibition of cholesterol absorption in the gastrointestinal tract by displacing cholesterol from micelles, (b) limiting the intestinal solubility of cholesterol, and (c) decreasing the hydrolysis of cholesterol esters in the small intestine (Ling and Jones, 1995).

Plasma cholesterol levels were also found to be significantly reduced when rats were fed with soy protein (Boststo *et al.*, 1981). The cholesterol-lowering efficacy of a diet could be enhanced with the addition of soy lecithin (Wilson *et al.*, 1998). It has been found that the inclusion of soybean Leci-Vita, a product rich in polyunsaturated phospholipids (with 7 % lecithin, 17 % soy protein), to a diet

significantly reduced total and LDL-cholesterol in patients with elevated serum cholesterol while causing HDL-cholesterol to significantly increase (Danijela *et al.*, 2003). Jimenez *et al.* (1990) reported that the plasma lecithin-cholesterol-acyltransferase (LCAT) activity increased when lecithin was administrated to hypercholesterolemic rats. Enhanced LCAT activity in turn increased the formation of mature HDL and cholesterol removal.

Olestra is prepared from sucrose and long-chain fatty acids from edible fats and oils such as soybeans, corns and cottonseeds (Prince and Welschenbach, 1998). It has the physical properties of fat but is unabsorbable and hence used exclusively as fat substitute in some commercial snacks (Prince and Welschenbach, 1998). A significant reduction in cholesterol absorption was observed when feeding olestra to human (Crouse and Grundy 1979). No toxicity of olestra was shown when fed to dogs (Miller *et al.*, 1991).

Policosanol comprised of 8 higher aliphatic alcohols obtained from sugar cane (*Saccharum officinarum*) (Canetti *et al.*, 1997). Studies have established the cholesterol lowering effect of policosanol in patients with hypercholesterolemia (Benitez *et al.*, 1997; Canetti *et al.*, 1997). No toxicity was observed even at high dosage of policosanol (Mesa *et al.*, 1994).

2.4   Sterol reductases

Sterol reductases, the enzymes that catalyze the reduction of C=C double bond of sterols have been widely studied (Bottema and Park, 1978; Wiłkomirski and Goad, 1983; Dehal *et al.*, 1991; Taton and Rahier, 1991; Kim *et al.*, 1995; Smith, 1995; Holmer *et al.*, 1998; Silve *et al.*, 1998; Bae *et al.*, 1999; Schrick *et al.*, 2000). Among these, the enzyme catalyzing the reduction reaction of cholesterol was designated as

"cholesterol reductase" irrespective of the reaction mechanism and the biological source (Dehal *et al.*, 1991). This enzyme was reported to convert cholesterol to coprostanol (Dehal *et al.*, 1991). Though coprostanol is structurally similar to cholesterol, the former was found to be poorly absorbed by intestine (Bhattacharyya, 1986). Cholesterol reductase is therefore an efficient way to lower cholesterol concentration.

Other than cholesterol reductase, 7-dehydrocholesterol reductase that catalyzes the reduction of C-7 double bond of 7-dehydrocholesterol to cholesterol was identified in microsomes of *Zea mays* (Taton and Rahier, 1991). Two genes, assigned as TM7SF2 and DHCR7, with strong sequence similarity to carboxyl-terminal domain of human lamin B receptor and 7-dehydrocholesterol reductase were described (Holmer *et al.*, 1998). They were reported as human gene family encoding proteins that functioned in nuclear organization and/or sterol metabolism. The cDNA encoding rat 7-dehydrocholesterol reductase had since been cloned and sequenced (Bae *et al.*, 1999). It appears to share a closed amino acid identity with mouse and human 7-dehydrocholesterol reductase and highly hydrophobic. Mutations in the 7-dehydrocholesterol reductase gene have been known to give rise to Smith-Lemli-Opitz Syndrome characterized by facial dysmorphisms, mental retardation and multiple congenital anomalies (Wassif *et al.*, 1998; Waterham *et al.*, 1998).

C14-sterol reductase catalyzes the reduction of C8=C14 or C7=C14 double bond of sterols (Kim *et al.*, 1995). It was identified in *Saccharomyces cerevisiae* (Bottema and Parks, 1978). Following that, it has been purified from rat microsomes and was found to be induced by cholesterol (Kim *et al.*, 1995). *Schizosaccharomyces pombe erg24* cDNA which encodes a C14-sterol reductase has been cloned and sequenced (Smith, 1995). It was found to bear significant homology with that of

Saccharomyces cerevisiae. Human lamin B receptor was suggested as a C14-sterol reductase because it restored the C14 reduction step when transformed in mutated *Saccharomyces cerevisiae* lacking C14-sterol reductase (Silve *et al.*, 1998). FACKEL, a gene that required for organized cell division and expansion in *Arabidopsis* embryogenesis was found to encode a C14-sterol reductase (Schrick *et al.*, 2000). The C14-sterol reductase activity was found to be inhibited by 15-azasterol (Bottema and Park, 1978), 7-aminocholesterol (Elkihel *et al.*, 1994), fenpropimorph and tridemorph (Silve *et al.*, 1998).

C25-sterol reductase, an enzyme that catalyzes the conversion of (24S)-24-ethylcholesta-5,22,25-trien-3β-ol to (24S)-24-ethylcholesta-5,22-dien-3β-ol was identified in alga *Trebouxia sp.* (Wiłkomirski and Goad, 1983). Mutation in the C24-sterol reductase gene was found to cause desmosterolosis, which is characterized by multiple congenital anomalies (Waterham *et al.*, 2001). 23-Azacholesterol was found to inhibit C24-sterol reductase in *Saccharomyces cerevisiae* (Pierce Jr. *et al.*, 1978). Genetic defects of sterol metabolism in humans and mice that involved impairment of sterol reductases has been discussed (Moebius *et al.*, 1998).

2.5   Cholesterol reductase in plants

Cholesterol functions in plants as hormone and hormone precursors, architectural components of membrane and have also been postulated to play a role in seed germination and plant growth (Grunwald, 1975). Generally speaking, the amount of cholesterol present in a given plant source is of no indication to its relative importance because the turnover rate of cholesterol is very high (Hefmann, 1984).

Examination of the structures of the various steroids formed from cholesterol by plants indicated that cholesterol must have undergone a series of oxidation and

reduction reactions in the process (Hefmann, 1984). The oxidation of cholesterol to 4-cholesten-3-one was demonstrated *in vitro* with *Solanum tuberosum* and *Cheiranthus cheiri* leaves as well as with suspension cultures of *Brassica napus* and *Glycine max* (Hefmann, 1984). 4-Cholesten-3-one has been found to undergo reduction to 5α-cholestan-3β-one in the presence of *Strophanthus kombé*, and *Cheiranthus cheiri* leaf homogenates. It is converted to 5α-cholestan-3β-ol in the suspension cultures of rape and soya cell (Hefmann, 1984). 5α-Cholestan-3β-ol (isomer of coprostanol) was found to be absorbed only half as efficiently as cholesterol by intestine (Bhattacharyya, 1986).

Various steroid transformations have been found to occur in plants (Hefmann *et al.*, 1967; Lin *et al.*, 1983). For example, in *Lycopersicon pimpinellifolium*, the C5=C6 double bond of cholesterol is reduced to form tomatidine (Hefmann *et al.*, 1967). Lin *et al.* (1983) observed that androst-4-en-3,17-dione was metabolized into a variety of steroids in cucumber plants (*Cucumis sativum*). Dehal *et al.* (1988, 1990a, 1990b) studied the conversion of cholesterol to coprostanol in plants. The homogenate from young cucumber leaves was found to catalyze the reduction of 7 % of cholesterol to coprostanol (Dehal *et al.*, 1988). Last but not least, partial purification of cholesterol reductase from alfalfa (*Medicago sativa*) leaves and identification of cholesterol reductase activity in pea (*Pisum sativum*) were also reported (Dehal *et al.*, 1990a, 1990b; Yang and Beitz, 1992).

2.6   Cholesterol reductase in bacteria

In view of the fact that coprostanol is found in faeces, many attempts have been made to isolate bacteria capable of reducing cholesterol to coprostanol from human and animal faeces (Snog-kjaer *et al.*, 1956; Crowther *et al.*, 1973). Certain

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anaerobic bacteria from human faeces are known to hydrogenate cholesterol *in vitro* (Snog-kjaer *et al.*, 1956). On the other hand, microbial degradation of cholesterol and plant sterols have been found to occur in *Mycobacterium sp.* NRRL B-3683 and *Mycobacterium sp.* NRRL B-3805 producing androsta-1,4-diene-3,17-dione and androst-4-ene-3,17-dione (Marsheck *et al.*, 1972). Cholesterol reduction by common intestinal bacteria such as *Bifidobacterium*, *Clostridium*, and *Bacteriodes* has also been reported (Crowther *et al.*, 1973). Goddard and Hill (1974) found that bacterial flora in the guinea pig gut can degrade cholesterol. The *in vivo* reaction was abolished by pretreatment of the animals with antibiotics which suppressed the gut bacterial flora. On the other hand, degradation of cholesterol from liquid media was reported in fast-growing non-pathogenic mycobacteria (Av-Gay and Sobouti, 2000).

Wiggers *et al.* (1973) showed that despite the high cholesterol level (250 mg/kg body weight daily) fed to calves, their plasma cholesterol was not higher than in grain-fed calves which had received no cholesterol in their diet. It was thus postulated that the cholesterol ingested had undergone microbial degradation in the ruminoreticulum. The postulation was confirmed by Ashes *et al.* (1978) who showed that cholesterol was hydrogenated by anaerobic incubation with sheep rumen fluid. The principal product of cholesterol hydrogenation was later identified to be coprostanol.

Microorganisms that have the ability to hydrogenate cholesterol to coprostanol have been isolated from rat cecal contents (Eyssen *et al.*, 1973), the faeces of human (Sadzikowski *et al.*, 1977) and that of baboon (Brinkley *et al.*, 1982). The cholesterol-reducing microorganism isolated from rat cecal contents, *Eubacterium* ATCC 21408, is an obligate anaerobe, measuring 0.3 to 0.5 µm by 1 µm in size, and is gram positive in very young culture. This strain is different from the previously described

Eubacterium in its requirement of cholesterol for growth (Eyssen *et al.*, 1973). The bacteria are able to reduce C5=C6 double bond of cholesterol, campesterol, β-sitosterol and stigmasterol to yield the corresponding 5β-saturated derivatives. No reduction reaction has been known to occur when 3-hydroxyl functional group was absent or altered (Eyssen *et al.*, 1973).

An anaerobic, gram-positive diplobacillus that reduced cholesterol to coprostanol was also isolated from human faeces (Sadzikowski *et al.*, 1977) and it was found to display similar characteristics to the cholesterol-reducing bacterium isolated from rat cecal contents by Eyssen *et al.* (1973). These anaerobes would not form colonies and were isolated and cultivated in an anaerobic medium containing homogenized pork brain (naturally high in cholesterol). They also required free or esterified cholesterol and alkenyl ether lipid (plasmalogen) for growth (Sadzikowski *et al.*, 1977).

Nine strains of cholesterol-reducing bacteria have been isolated and characterized from faeces and intestinal contents of baboons (Brinkley *et al.*, 1982). Unlike previously reported strains, these nine strains did not require cholesterol and plasmalogen for growth (Brinkley *et al.*, 1982). However, only two strains reduced cholesterol in the absence of plasmalogen. These two strains also produce succinate as end product (Brinkley *et al.*, 1982).

The role of cholesterol in growth of these organisms has not been reported. Eyssen *et al.* (1973) suggested that cholesterol could be the terminal electron receptor. However, all strains isolated from faeces and intestinal contents of baboons had not required cholesterol for growth (Brinkley *et al.*, 1982). Therefore, an alternative electron would have to be used by these strains when cholesterol was not available (Brinkley *et al.*, 1982). That aside, it has been reported that *Eubacterium* ATCC

21408 is able to grow well in standard brain medium (Eyssen *et al.*, 1973). However, colonies of the bacteria did not develop on the media solidified with agar (Brinkley *et al.*, 1980). Colonies of the bacteria were found to develop when cholesterol concentration was increased to 5 % (Brinkley *et al.*, 1982) which suggested the importance of cholesterol in bacterial growth.

The usual end product of microbial cholesterol reduction in soil and sediments was found to be 5α-cholestan-3β-ol while that in the intestine was coprostanol (5β-cholestan-3β-ol) (Gaskell and Eglinton, 1975). Coprostanol, cholesterol, stigmasterol and β-sitosterol have been detected in natural water and sediments (Hassett and Lee, 1977). Coprostanol, a ubiquitous organic residue in the soil, has been selected to be a biomarker of a variety of human activities such as settlement organization and manuring practices in archaeological study as it provides an indication of prior human settlement (Bethell *et al.*, 1994). On the other hand, the faecal stanol/sterol ratio has been established to be a suitable parameter for the comparison of sewage contamination in sediments (Chan *et al.*, 1998). The amount of coprostanol in urine collection tank can also be used as an indicator of faecal cross-contamination (Sundin *et al.*, 1999).

The mechanism of cholesterol reduction to coprostanol has been studied (Schoenheimer, 1935; Rosenfeld *et al.*, 1956; Björkhem and Gustafsson, 1971). According to Schoenheimer (1935), bacterial conversion of cholesterol to coprostanol involved the initial oxidation of cholesterol to 4-cholesten-3-one, followed by the successive reduction to coprostanone and finally to coprostanol. In contrast, Rosenfeld *et al.* (1956) eliminated the ketones from the pathway for coprostanol formation. This direct stereospecific reduction of the C5=C6 double bond was later invalidated by Björkhem and Gustafsson (1971) who demonstrated that conversion of

cholesterol into coprostanol by cecal contents of rat proceeded to at least 50 % by means of the formation of the intermediate 4-cholesten-3-one.

2.7   *Eubacterium coprostanoligenes*

The cholesterol-reducing bacteria discussed in this literature review thus far require plasmalogen for growth or cholesterol-reduction activity (Eyssen *et al.*, 1973; Sadzikowski *et al.*, 1977; Brinkley *et al.*, 1982). Plasmalogen was provided to the bacteria by the inclusion of brain extract in the growth medium (Mott and Brinkley, 1979) which consequently made the culture medium viscous. This in turn made the separation of the bacteria from growth medium very difficult.

Freier *et al.* (1994) reported a new bacteria species, *Eubacterium coprostanoligenes*, which was isolated from hog sewage lagoon in Iowa, U.S.A. The coccobacilloid cells are small and occurred singly or in pair. They are nonmotile, gram positive and non-spore forming. Optimal growth and coprostanol production were reported to be at pH 7.0 and at 35 °C (Freier *et al.*, 1994). These bacteria could metabolize lecithin, a substrate necessary for growth. Cholesterol was found to be reduced to coprostanol by the bacteria, but was not required for growth (Freier *et al.*, 1994). Unlike previously described cholesterol-reducing bacteria, plasmalogen was neither required for growth nor for cholesterol-reduction activity in this case. In addition, while the bacteria required anaerobic conditions to grow, they could survive long exposure to atmospheric oxygen for up to 48 hours (Freier *et al.*, 1994). Li *et al.* (1995b) considered *E. coprostanoligenes* to be more amenable than previously studied cholesterol-reducing bacteria for application in the food and pharmaceutical industries.

E. coprostanoligenes possesses phospholipase activity. It was suggested that the metabolites of phospholipase activity alter the bacterial membrane, thus increasing the accessibility of the cholesterol to cholesterol reductase (Freier *et al.*, 1994). It was also suggested that calcium chloride in the growth medium provided the net positive charge required for phospholipase activity. The subsequent hydrolysis of phosphatidylcholine by phospholipase is either a cofactor or is directly involved in coprostanol formation (Freier *et al.*, 1994). A resting-cell assay was established to evaluate the cholesterol reductase activity of *E. coprostanoligenes* (Li *et al.*, 1995b).

The reduction mechanism of cholesterol to coprostanol by *E. coprostanoligenes* was studied by incubating the bacterium with a mixture of α- and β-isomers of [4-^{3}H, 4-^{14}C] cholesterol (Ren *et al.*, 1996). The results suggested that the major pathway for cholesterol reduction in *E. coprostanoligenes* involved the intermediate formation of 4-cholesten-3-one and coprostan-3-one followed by the reduction of latter to coprostanol.

The hypocholesterolemic effect of *E. coprostanoligenes* has been studied in rabbits (Li *et al.*, 1995a), laying hens (Li *et al.*, 1996a) and germ-free mice (Li. *et al.*, 1998). Oral administration of the bacteria caused a significant hypocholesterolemic effect in rabbits (Li *et. al*, 1995a). The effect was explained by the conversion of cholesterol to coprostanol in the intestine. In laying hens, plasma cholesterol concentrations were not affected by the bacterial treatment despite an increase in the coprostanol-to-cholesterol ratio in faeces (Li *et al.*, 1996a). The hypocholesterolemic effect of *E. coprostanoligenes* was found to be transient in germ-free mice as the bacteria did not colonize the intestine of the mice (Li. *et al.*, 1998).

GROWTH OF *EUBACTERIUM COPROSTANOLIGENES*

3.1   Introduction

E. coprostanoligenes was isolated by Freier *et al.* (1994). It was reported as a small, anaeorobic and gram-positive coccobacillus that was able to convert cholesterol to coprostanol. It showed optimal growth at pH 7 and at temperature of 35 °C. Growth was not evident at pH 5.5 or 8 and at temperatures of 25 or 45 °C (Freier *et al.*, 1994). Other than *E. coprostanoligenes*, cholesterol-reducing bacteria have also been isolated from rat cecal contents (Eyssen *et al.*, 1973), faeces of human (Sadzikowski *et al.*, 1977) and baboon (Brinkley *et al.*, 1982). The requirement of a strict anaerobic condition posed an obstacle to the investigation of growth of these organisms (Eyssen *et al.*, 1973; Sadzikowski *et al.*, 1977; Brinkley *et al.*, 1982). *E. coprostanoligenes* should be more easily studied since it was reported to survive exposure to air for up to 48 hours and not required plasmalogen for growth (Freier *et al.*, 1994).

The objectives of this chapter are to study the growth of *E. coprostanoligenes* as well as various factors affecting its growth. The study would provide useful information on the growth behavior of these special bacteria and how its growth could be enhanced.

3.2   Materials and Methods

3.2.1   *E. coprostanoligenes* and Base Cholesterol Medium (BCM)

E. coprostanoligenes was purchased from American Type Culture Collection (ATCC Number: 51222, isolated from hog waste lagoon, Iowa). BCM was prepared by mixing cholesterol (2 g) and lecithin (1 g) with stirring in 200 ml of milli-Q water under nitrogen gas for 10 min, and subsequently combined with 800 ml of milli-Q

water dissolved with casitone (10 g), yeast extract (10 g), sodium thioglycollate (0.5 g), $CaCl_2$ (1 g) and resazurin (1 mg). The medium was adjusted to pH 7.5 with 5 M KOH and boiled under N_2 until resazurin turned colorless before autoclaving at 121 °C for 20 min. BCM was mixed well after autoclaving and placed in anaerobic chamber (Sheldon Manufacturing Inc., U.S.A.) before being inoculated with the bacteria. Cholesterol-free BCM was prepared with the same procedure without adding cholesterol. Bacterial cultures were maintained by weekly transfers of 20 ml bacterial culture to 200 ml fresh BCM.

3.2.2 Plating of bacteria on agar solidified medium

Agar solidified medium was prepared as BCM with the addition of 1.5 % (w/v) agar before autoclaving. About 25 ml medium was dispensed into each 90 mm diameter Petri dish. Solidified medium were placed in anaerobic chamber for 2 hours to ensure a fully reduced (deoxygenation) state of medium. Bacterial culture (100 μl) was spread evenly on agar solidified medium with glass beads, and sealed with parafilm to avoid dehydration. The bacterial culture could be diluted to avoid overcrowding of colonies on surface of solidified medium. Inoculated plates were inverted and incubated overnight under anaerobic conditions at 37 °C. Colonies formed on surface of solidified medium were counted with naked eyes.

To investigate the suitability of plate counting as a method to study growth of *E. coprostanoligenes,* bacterial culture was diluted at 10^3 to 10^8 times and inoculated on agar solidified medium in triplicate. To study the effect of cholesterol on growth of *E. coprostanoligenes*, agar solidified medium were prepared and inoculated with growing broth culture from BCM and cholesterol-free BCM. Inoculation and counting of colonies were conducted daily in triplicate until the growth of bacteria reached

death phase (as reflected by a decrease in the number of colonies on agar solidified medium).

3.2.3 Microscopy study

3.2.3.1 Confocal microscopy

Fresh culture was grown in liquid medium, pelleted by centrifuging at 10,000 g, washed twice with 1 % (w/v) NaCl and suspended in the same solution. A drop of the suspended culture was transferred onto a slide with an inoculation loop and covered with a cover-slip. Images of *E. coprostanoligenes* observed in the transmission mode after excitation at 543 nm were captured with Zeiss LSM 510.

3.2.3.2 Gram staining

Fresh culture was grown in liquid medium, pelleted by centrifuging at 10,000 g, washed twice with 1 % (w/v) NaCl and suspended in the same solution. A drop of the suspended culture was transferred onto a slide with an inoculation loop and smeared into a very thin layer using a wooden stick. The culture was then air dried. A drop of crystal violet stain (2 g of crystal violet was dissolved in 20 ml of 95 % ethanol as solution A; 0.8 g of ammonium oxalate was dissolved in 80 ml of milli-Q water as solution B; solutions A and B were mixed and stored for 24 hours before use) was added over the dried culture for 10 seconds. Excess stain was then poured off. The culture was then further rinsed gently with a stream of water from a plastic water bottle.

Iodine solution (1 g of iodine crystal and 2 g of KI were dissolved in 300 ml of milli-Q water) was added just enough to cover the culture and allowed to stand for 10 seconds. After that, the iodine solution was poured off and the slide was rinsed with

water. A few drops of decolorizer (acetone/ethanol, 50:50 v/v) were added and allowed to trickle down the slide. The decolorizer was rinsed off with water after 5 seconds. Rinsing was continued until the decolorizer was no longer colored as it flowed over the slide. The smear was counterstained with saffranin solution (2.5 g of saffranin O was dissolved in 100 ml of 95 % ethanol as stock solution; 10 ml of stock solution was diluted with 90 ml of milli-Q water as working solution) for 60 seconds. The saffranin solution was washed off with water and the slide was blotted dry. The specimen was examined under Olympus BH-2 light microscope. Images of stained cells were captured with Olympus CAMEDIA C-5050 Zoom digital camera.

3.2.3.3 Transmission electron microscopy

Fresh culture was grown in liquid medium, pelleted by centrifuging at 10,000 g, washed twice with 1 % (w/v) NaCl and suspended in the same solution. A drop of suspended culture was placed onto the Formvar-coated copper grid. One drop of 2 % (v/v) phospho-tungstate acid was added onto the copper grid and allowed to stand for 1 minute. Excess stain was blotted dry and the copper grid was dried under table lamp for 3 min. The specimen was examined under Philips CM10 electron microscope.

3.2.4   Factors affecting growth of bacteria

BCM containing 1 mM cholesterol with a) lecithin concentrations varying from 0 to 10 g/l; b) $CaCl_2$ (calcium chloride) concentrations varying from 0 to 10 g/l; and c) pH adjusted to 4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10, were prepared and autoclaved, respectively. The media were then reduced in anaerobic chamber for 2 hours. Ten ml each of these media was inoculated with 1 ml of 24-hour-old culture (containing approximately 10^6 cells) and incubated at 37 °C in anaerobic chamber. Plate counting

was performed after 24 hours of incubation to study the growth of bacteria at different lecithin and $CaCl_2$ concentrations and pH. Each test was carried out in triplicate.

3.2.5 Aerotolerance of *E. coprostanoligenes*

BCM containing 1 mM cholesterol with sodium thioglycollate concentrations varying from 0 to 5 g/l were prepared and autoclaved. The media were then reduced in anaerobic chamber for 2 hours. Ten ml of media containing different sodium thioglycollate concentrations was inoculated with 1 ml of 24-hour-old culture (containing approximately 10^6 cells) and incubated at 37 °C in anaerobic chamber. Plate counting was performed after 24 hours. Each test was carried out in triplicate

BCM with (0.5 g/l) and without sodium thioglycollate were prepared and autoclaved. Ten ml of each media was inoculated with 1 ml of 24-hour-old culture (containing approximately 10^6 cells) and incubated in anaerobic chamber at 37 °C. On the other hand, 10 ml of each media was exposed to ambient air (aerobic condition) by shaking in a shaker incubator for two hours. They were then inoculated with the same bacterial culture and incubated in the same shaker incubator at 37 °C. Plate counting was carried out every 12 hours for 60 hours. Each test was carried out in triplicate.

3.2.6 Statistical analysis

Where necessary, statistical tests were conducted using one-way ANOVA (Tukey's Test) to determine if the treatments in each experiment were significantly different from one another at 95 % confidence level.

3.3   Results and Discussion

3.3.1   Culture medium for *E. coprostanoligenes*

E. coprostanoligenes was cultured in BCM which is a cloudy lipid suspension. Lecithin in BCM is required for growth of *E. coprostanoligenes* (Freier *et al.*, 1994). Boiling the medium before autoclaving is an important step in the preparation of BCM as cholesterol and lecithin are not readily dissolved in the mixture. Boiling will enable a finer lipid suspension to be formed which might facilitate bacterial growth as lecithin would be then more accessible to the bacteria. Autoclaved medium was placed in the anaerobic chamber for at least two hours to ensure that the medium fully achieved a reduced state before inoculation with bacterial culture. In addition to anaerobic chamber, anaerobic jar can be used to generate anaerobic environment for culture of *E. coprostanoligenes*.

The yeast extract in BCM could provide a variety of organic nitrogenous constituents which would fulfill the general nitrogen requirement; plus, it also contains most of the organic growth factors likely to be required by *E. coprostanoligenes*. Sodium thioglycollate, a reducing agent, is necessary as it maintains the medium in reduced (deoxygenated) state to facilitate growth of anaerobic *E. coprostanoligenes*. Some other common reducing agents used in anaerobic culture include ascorbic acid, cysteine and dithiothreitol (Holland *et al.*, 1987). On the other hand, resazurin acts as indicator of deoxygenation of growth media (Holland *et al.*, 1987). It will change from blue to pink (oxidized) to colorless (reduced) as an indication that deoxygenation has occurred.

3.3.2   Growth of bacteria

3.3.2.1 Evaluation of solid plate counting

As shown in Figure 3.1, as the bacterial culture was diluted, the number of colonies formed on solid agar plate was reduced accordingly. This method thus can be used to monitor growth of *E. coprostanoligenes*. Dilution of culture was necessary to avoid over-crowding of the colonies on the surface of solidified medium. It was found that only plates that contained 30 to 300 colonies should be considered for counting from a practical point of view. Colonies usually formed after 24 hours of incubation under anaerobic conditions. Surface colonies of *E. coprostanoligenes* on anaerobic plates were fine, round, white and powdery in texture with approximately 0.2 mm in diameter (Figure 3.2a to 3.2e).

Plate count will measure only the living cells in a population, that is, those capable of reproduction (Ingraham and Ingraham, 1995). The indirect techniques that measure a property of the mass of cells in a population (e.g. turbidity, dry weight or metabolic activity) are not applicable for the present study of growth as BCM is a cloudy suspension.

3.3.2.2 Growth patterns of *E. coprostanoligenes*

There was no significant difference in growth for the bacterial cultured in medium with or without cholesterol (Figure 3.3). This indicated that cholesterol was not necessary for growth of *E. coprostanoligenes*. Our observation agreed with that of Freier *et al.* (1994).

E. coprostanoligenes culture grew through three distinct and sequential phases: the log, stationary and death phases (Figure 3.3). The lag phase characterized by slow microbial growth was not observed when the growth was monitored at a 24-hour
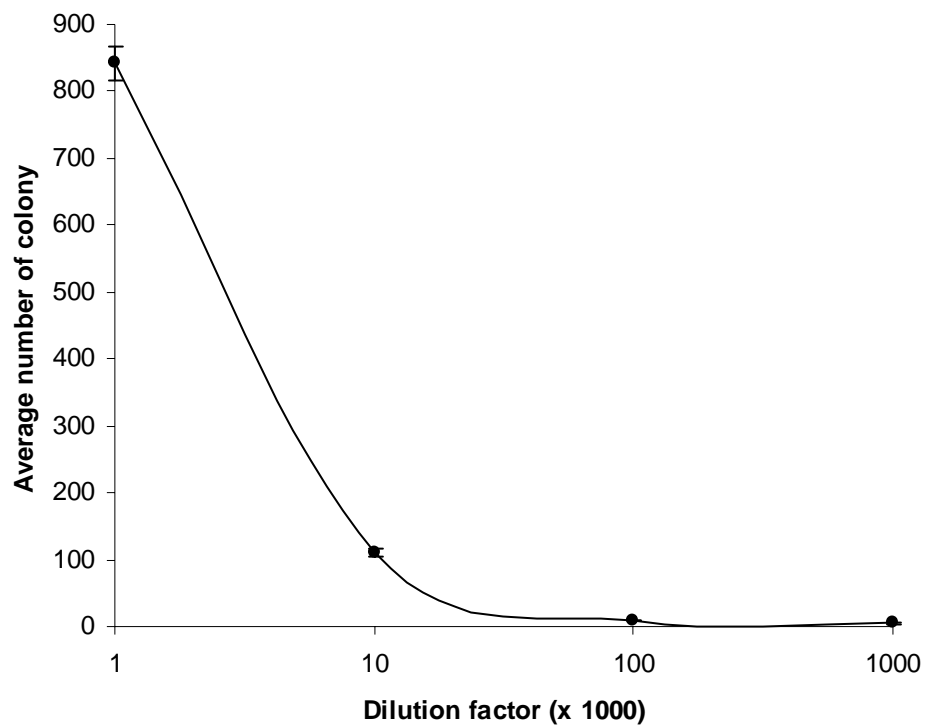
Fig 3.1: Solid plate counting as a method to monitor bacterial growth. Number of colony was plotted against dilution factor. Vertical bars denote SE (n=3). Growing broth culture was spread evenly on agar solidified medium, sealed, inverted and incubated at 37 °C overnight under anaerobic condition. Colonies formed were counted with naked eyes.
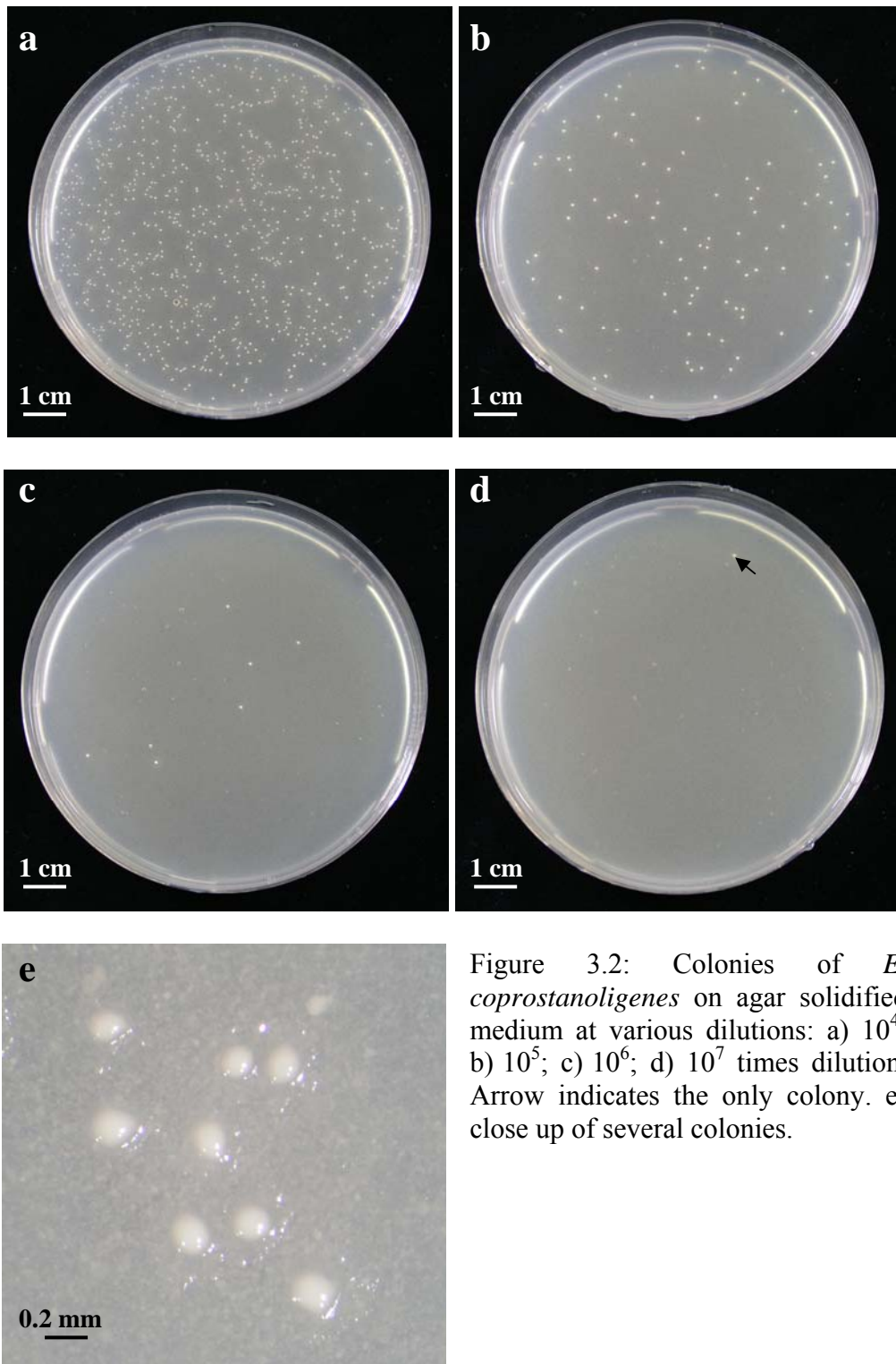
Figure 3.2: Colonies of *E. coprostanoligenes* on agar solidified medium at various dilutions: a) 10^4; b) 10^5; c) 10^6; d) 10^7 times dilution. Arrow indicates the only colony. e) close up of several colonies.
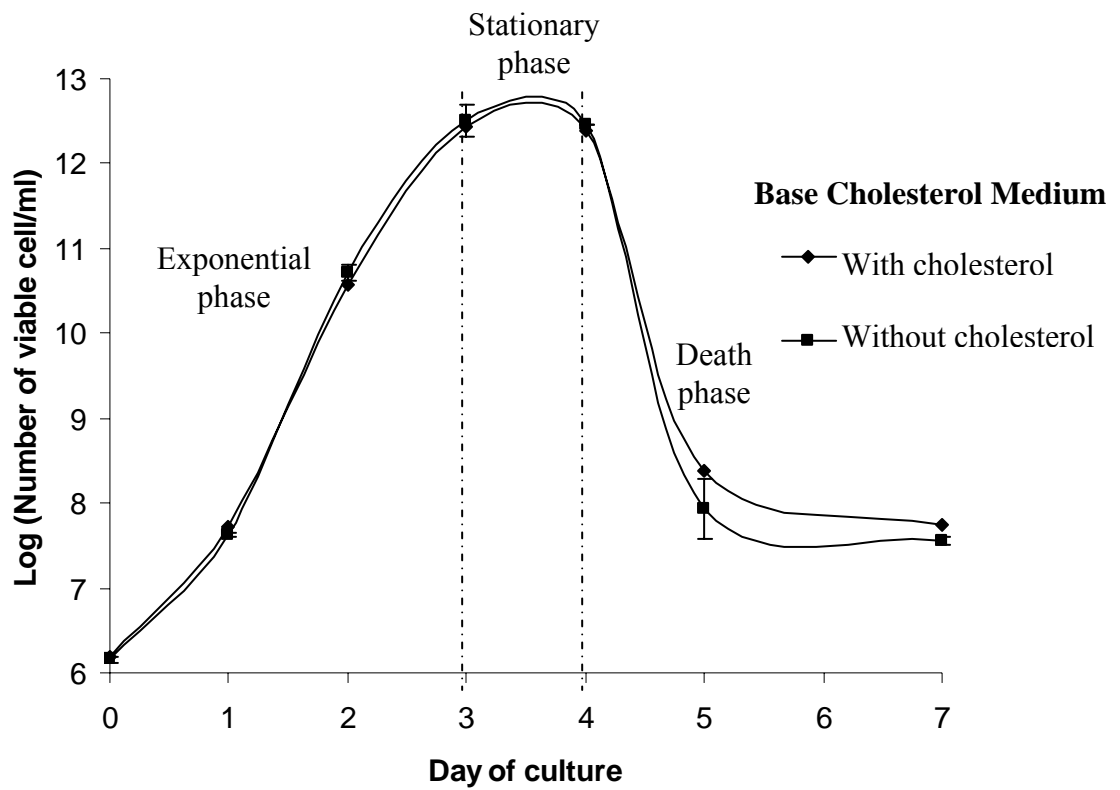
Fig. 3.3: Growth curve of *E. coprostanoligenes* cultured in BCM with and without cholesterol. Plate counting for viable cells was carried out daily for a period of 7 days. Vertical bars denote SE (n=3).

interval. The bacterial culture might have undergone the lag phase within the first 24 hours. The log phase persisted for three days after which came the stationary phase. The bacteria underwent exponential growth during the log phase and achieved a population number of approximately 3×10^{12}/ml culture. The stationary phase lasted for a day before the death phase characterized by a drastic decrease in number of viable cells. Doubling time is the period required for cells in a microbial population to grow, divide and to produce two new cells for each one that existed before (Ingraham and Ingraham, 1995). During the 72-hour log phase, *E. coprostanoligenes* culture has doubled 21 times which was equivalent to a doubling time of approximately 3 hours and 25 min, or 0.3 doubling per hour.

The bacteria in this study belong to the genus *Eubacterium*. It is a common genus in the intestinal flora (up to 10^{11} cells/ g of faeces). It has previously been found that *E. ruminantium* and *E. aerofaciens* constituted up to 7 % of bovine rumen flora and 10 % of human faeces, respectively (Holland *et al.*, 1987).

3.3.3 Microscopy study

Figure 3.4a and 3.4c show the confocal and transmission electron microscopy images of *E. coprostanoligenes*. The coccobacilloid cells were 0.5 to 0.7 µm in diameter and 1 to 1.2 µm in length. They occurred either singly or in pairs. These observations agreed well with that reported by Freier *et al.* (1994). *E. coprostanoligenes* were Gram positive (Figure 3.4b).
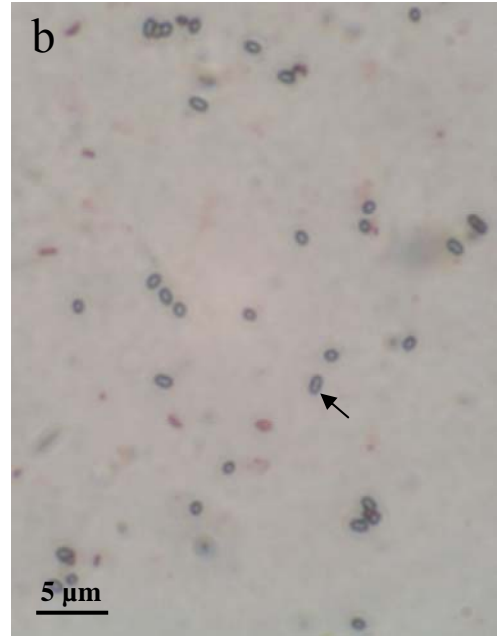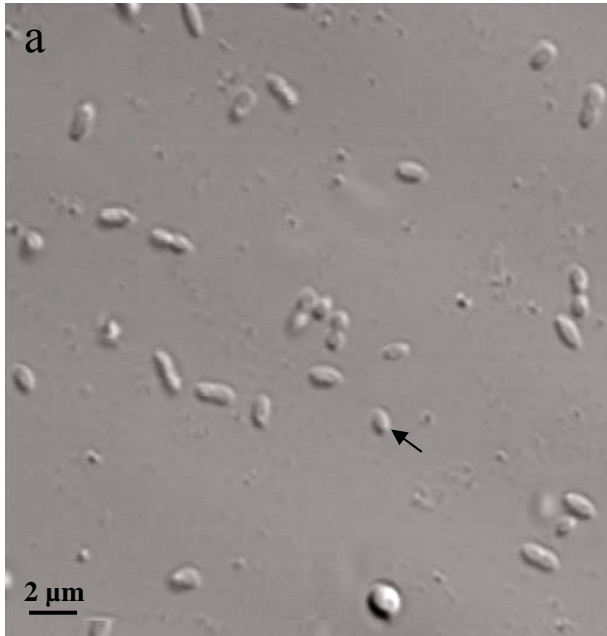
Fig. 3.4: Microscopy study of *E. coprostanoligenes*. a) Confocal microscopy. The arrow indicates a coccobacilloid cell. b): Gram stains. The arrow indicates a single cell. c): Transmission electron microscopy showing a bacterium.

3.3.4 Factors affecting growth of *E. coprostanoligenes*

3.3.4.1 Effect of lecithin

The number of bacterial cells increased with increasing lecithin concentration and achieved optimal growth at 1 g/l with approximately 5.4×10^8 cells/ml culture (Figure 3.5). This was a 50-fold increase in number of cells compared to growth in BCM without lecithin. When lecithin concentration was increased to 5 g/l and greater, the culture media became very viscous which consequently resulted in a reduction of viable bacteria. Freier *et al.* (1994) had reported that lecithin was metabolized in *E. coprostanoligenes* and was required for growth. However, bacterial growth was observed in our experiment even when lecithin was not supplied in culture medium. This could be due to the presence of any residual lecithin from inoculating culture.

The ability of *E. coprostanoligenes* to utilize lecithin could be conferred by lecithinase, which was probably a mixture of phospholipases (Ratledge, 1994). Freier *et al.* (1994) speculated that the product of phospholipase action might alter the bacterial membrane thereby increasing the accessibility of cholesterol to cholesterol reductase. These metabolites could also affect the micelle structure in which cholesterol was imbedded, which in turn increased the availability of cholesterol (Freier *et al.*, 1994).

3.3.4.2 Effect of CaCl$_2$

Growth of *E. coprostanoligenes* was not significantly decreased at CaCl$_2$ below 2.5 g/l and was found to be in the range of 1.6 to 2.4×10^7 cells/ml culture (Figure 3.6). CaCl$_2$ above 2.5 g/l severely reduced bacterial growth. The number of viable cells at 10 g/l CaCl$_2$ was only one-sixth of that at 2.5 g/l. Freier *et al.* (1994) suggested that calcium ions were necessary as they supplied a net positive charge to

Fig 3.5: Effect of lecithin on growth of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Different letters (above each bar chart) indicate significant difference between treatments (one-way ANOVA Tukey's Test, 95 % confidence level).
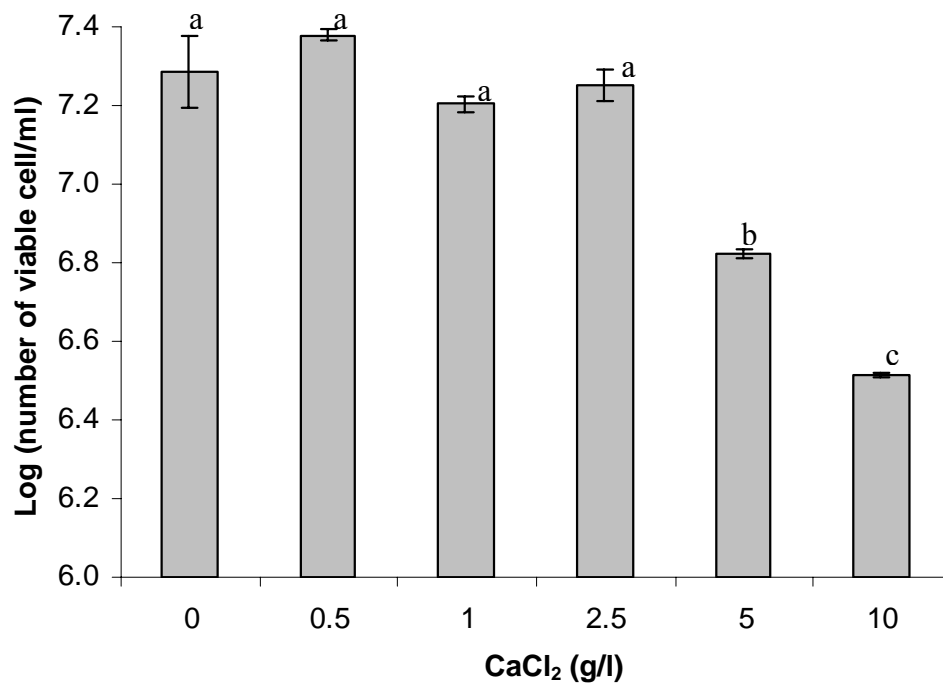
Fig 3.6: Effect of $CaCl_2$ on growth of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Different letters (above each bar chart) indicate significant difference between treatments (one-way ANOVA Tukey's Test, 95 % confidence level).

lecithin which could then function as an activator of phospholipase enabling it to undertake lecithin hydrolysis. Flores-Díaz *et al.* (2004) had also reported that calcium ions played a key role in phospholipase in interaction with substrates in *Clostridium perfringens.*

3.3.4.3 Effect of pH

There was no significant difference in growth of *E. coprostanoligenes* from pH 6 to 9 which was found to be in the range of 1.7 to 6.7×10^8 cells/ml culture (Figure 3.7a). Cell multiplication was not observed at pH 4 and 10 after 24 hours of incubation. On the other hand, Freier *et al.* (1994) reported optimal growth of *E. coprostanoligenes* at pH 7 to 7.5 and no growth at pH 5.5 or 8.

For those media showing growth of bacteria (media of pH 5 to 9), it was interesting to find out that the pH were shifted to the range of 6.4 to 7.1 after 24 hours of incubation regardless of the starting pH of culture media (Figure 3.7b). The pH shifted because *E. coprostanoligenes* might be releasing acid or alkali during its growth.

3.3.5 Aerotolerance of *E. coprostanoligenes*

Growth of *E. coprostanoligenes* was not significantly affected when the bacteria were cultured in media with and without sodium thioglycollate, and under aerobic or anaerobic conditions (Figure 3.8). In all cases, the number of bacterial cells increased to 1.4 to 2.1×10^8 cells/ml culture after 24 hours of incubation. *E. coprostanoligenes* remained viable after 60 hours of exposure to ambient air. However, BCM incubated under anaerobic condition tended to have approximately 20 % more viable cells than that under aerobic condition; and BCM without sodium
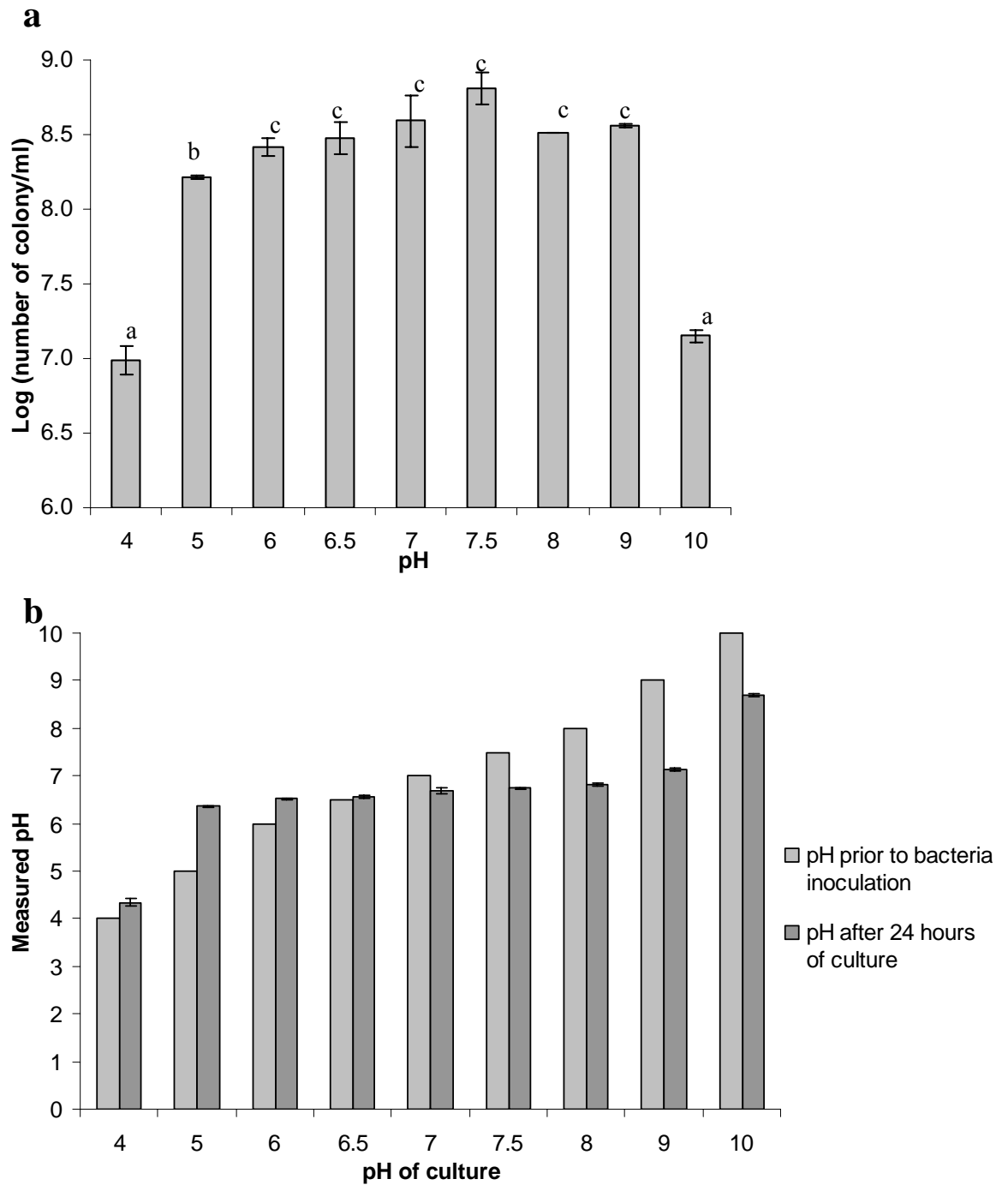
Fig 3.7: Effect of pH on growth of *E. coprostanoligenes*. a) Growth of bacteria in BCM of various pH. Vertical bars denote SE (n=3). Different letters (above each bar chart) indicate significant difference between treatments (one-way ANOVA Tukey's Test, 95 % confidence level). b) pH of BCM before and 24 hours after inoculation of *E. coprostanoligenes*. Vertical bars denote SE (n=3).
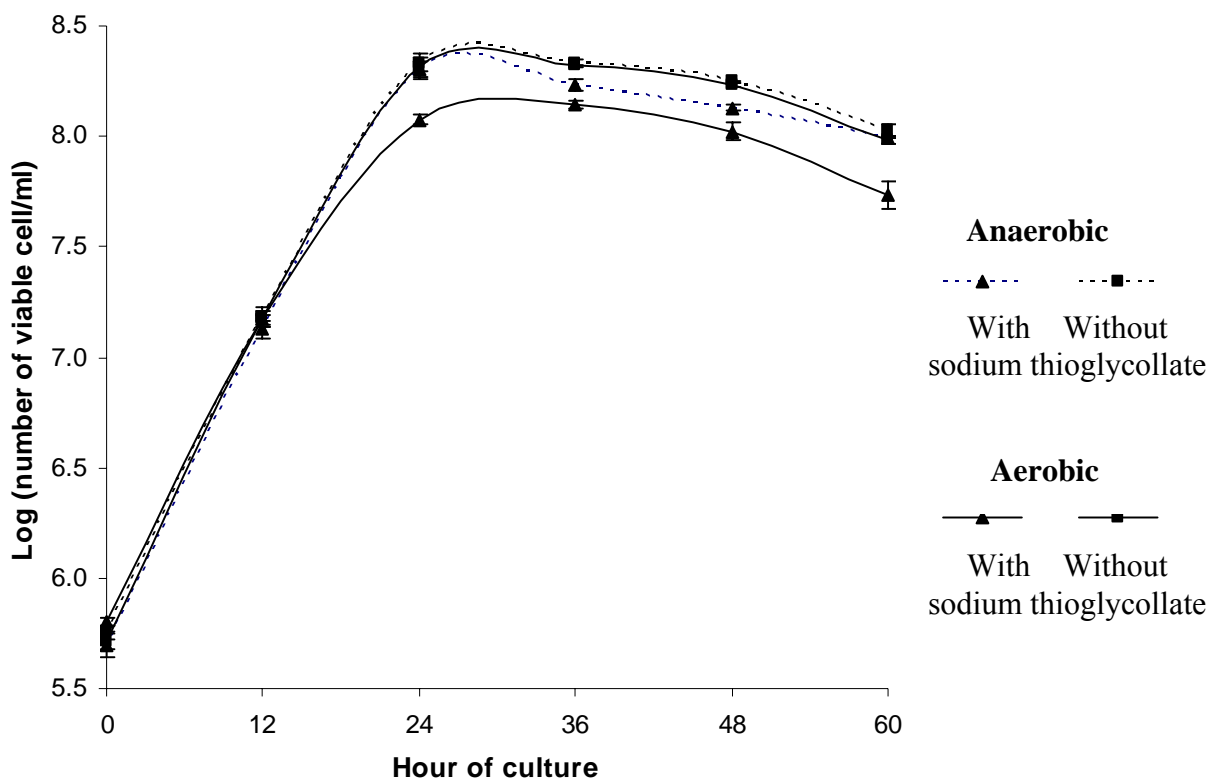
Fig 3.8: Aerotolerance of *E. coprostanoligenes* cultured in BCM with and without sodium thioglycollate, under aerobic or anaerobic conditions. Vertical bars denote SE (n=3). No significant difference between treatments was found (one-way ANOVA. Tukey's Test, 95 % confidence level).

thioglycollate would have approximately 25 % more viable cells. Sodium thioglycollate above 1 g/l in BCM severely reduced the number of viable *E. coprostanoligenes* by approximately 30 times (Figure 3.9). *E. coprostanoligenes* was reported to survive exposure to air for at least 48 hours (Freier *et al.*, 1994).

Sodium thioglycollate is a common reducing agent used in anaerobic culture. However, preparation of thioglycollate-containing media in the presence of oxygen might result in the formation of oxidized products, which may be toxic to some anaerobic bacteria (Holland *et al.*, 1987). Hence, it was recommended that the reducing agent should be added only after the medium has been deoxygenated. Low-toxicity cysteine was reported to be an alternative reducing agent in anaerobic culture and its slow reducing capability could be enhanced using illumination (Fukushima *et al.*, 2002).

Aerotolerance of some anaerobic bacteria has been studied (de Macêdo Farias *et al.*, 1999; Beerens *et al.*, 2000; Farias *et al.*, 2001). The atmospheric oxygen sensitivity of bacterial strains of genus *Fusobacterium* was heterogeneous (de Macêdo Farias *et al.*, 1999). This heterogeneity in oxygen sensitivity could be due to difference in the origin of the bacteria (Beerens *et al.*, 2000). It was also reported that varying aerotolerance capability was influenced by the isolation site, laboratory handling and growth stage. This capability could be important for the adaptation of bacteria to the environment (Farias *et al.*, 2001). Hence, the aerotolerance capability of *E. coprostanoligenes* might be conferred by the nature of its isolation site which is not strictly anaerobic.

Mechanisms of aerotolerance in *Brachyspira hyodysenteriae* (Stanton and Sellwood, 1999), *Clostridium perfringens* (Trinh *et al.*, 2000) and *Bacteroides fragilis* (Rocha *et al.*, 2003) have been studied. Anaerobic *Brachyspira*
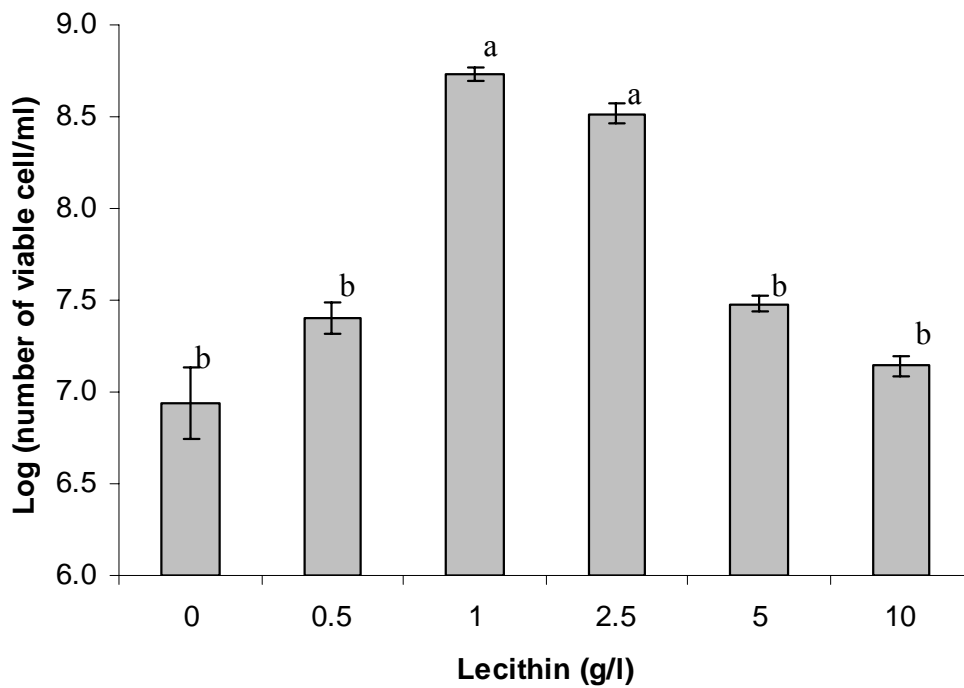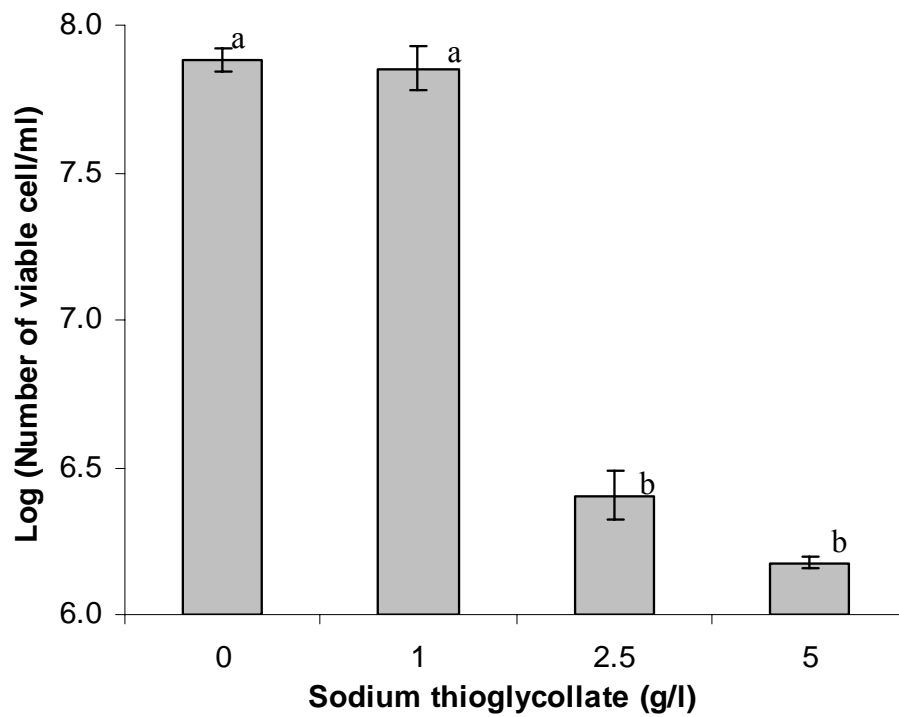
Fig 3.9: Effect of sodium thioglycollate on growth of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Different letters (above each bar chart) indicate significant difference between treatments (one-way ANOVA. Tukey's Test, 95 % confidence level).

hyodysenteriae has been reported to metabolize oxygen through NADH oxidase. The NADH oxidase gene has been cloned and characterized (Stanton and Sellwood, 1999). An adaptive response to oxidative stress was suggested in *Clostridium perfringens* in which cells at stationary phase exhibited more resistance than cells in mid-exponential growth (Trinh *et al.*, 2000). In addition, *Bacteroides fragilis* was shown to induce an array of genes including genes for catalase and superoxide dismutase producing more than 28 proteins when subjected to oxidative stress (Rocha *et al.*, 2003). A regulator, OxyR, was identified to respond quickly to oxidative stress inducing the oxidative-stress-response genes. This phenomenon was considered as a protective mechanism and metabolic adaptation (Rocha *et al.*, 2003). The ability of *E. coprostanoligenes* to survive when exposed to oxygen might indicate the presence of such mechanisms.

3.4   Concluding Remarks

E. coprostanoligenes was successfully cultured and maintained in BCM. Solid plate counting, which indicates the number of viable cells, was found to be a reliable method to monitor the growth of these bacteria. *E. coprostanoligenes* was found to undergo three days of exponential growth before it reached stationary and death phases. In addition, cholesterol was found to have no effect on its growth.

Colonies of *E. coprostanoligenes* on agar were fine, round, white and powdery in texture. Confocal and transmission electron microscopy revealed that the bacteria were coccobacilloid cells of 0.5 to 0.7 μm in diameter and 1 to 1.2 μm in length. The cells were gram positive. These features of *E. coprostanoligenes* agreed with the observation of Freier *et al.* (1994).

Growth of *E. coprostanoligenes* was affected by lecithin, $CaCl_2$ and pH of culture medium. The number of bacterial cells increased with increasing lecithin

concentration and achieved optimum at 1 g/l with approximately 5.4×10^8 cells/ml culture. No significant difference in growth was found for bacteria cultured in medium of CaCl$_2$ below 2.5 g/l, and in medium of pH 6 to 9. *E. coprostanoligenes* was also found to survive when exposed to ambient air for at least 60 hours.

The observations have provided useful information on the growth patterns and characteristics of *E. coprostanoligenes* and will enable us to manipulate the bacteria better. Further studies, however, are essential in order to comprehend lecithin metabolism and roles of various factors in growing *E. coprostanoligenes*. Experiments focusing on the cholesterol reduction activity of the bacteria are also important.

4 CHOLESTEROL REDUCTION ACTIVITY OF *E. COPROSTANOLIGENES*

4.1 Introduction

E. coprostanoligenes was found to be able to convert cholesterol to coprostanol (Freier *et al.*, 1994). This reaction involves the saturation of C5=C6 double bond of cholesterol to form coprostanol. As the latter is poorly absorbed by human intestinal system (Bhattacharyya, 1986), *E. coprostanoligenes* holds promise for use in treating hypercholesterolemia. Knowledge of cholesterol reduction activity in *E. coprostanoligenes* is necessary for its future application. To date, cholesterol reduction activity in these bacteria was investigated using radiolabeled cholesterol incorporated with thin layer chromatography (Freier *et al.*, 1994; Li *et al.*, 1995b). The method is laborious and poses certain harm as radioisotope is involved.

The objective of this chapter is to develop a simple, accurate and reliable method to study cholesterol reduction activity. In doing so, the factors affecting the cholesterol reduction activity can be investigated.

4.2 Materials and Methods

4.2.1 Cholesterol measurement using Infinity® Cholesterol Reagent

(a) cuvette method

Cholesterol sample (10 μl) was added to 1 ml of Infinity® Cholesterol Reagent (Sigma Diagnostics®), mixed well and incubated at 37 °C in water bath for 5 min. Absorbance was then measured at 500 nm (DU® 640B, Beckman, U.S.A.). Calibration was performed using Cholesterol Calibrators (Sigma®) at 1, 2 and 4 g cholesterol/l.

(b) microtiter plate method

Cholesterol samples (10 μl) were added to 200 μl of Infinity® Cholesterol Reagent in a microtiter-plate well, mixed well and incubated at 37 °C in ELISA (SPECTRAMAX 340, Molecular Devices) reader for 5 min. Absorbance was then measured at 500 nm using ELISA reader. Calibration was performed using Cholesterol Calibrators at 1, 2 and 4 g cholesterol/l.

4.2.2   Analysis of cholesterol reduction using thin layer chromatography (TLC)

Cholesterol, coprostanol, 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one of concentrations 0.1 to 5 mg/ml were prepared using chloroform: methanol (2:1, v/v) as solvent. Silica gel TLC plates were used without any pretreatment. Sterol of each concentration was then applied on TLC plates 2 cm from the bottom of the plates. Elution was carried out in glass tanks filled with approximately 50 ml of hexane: ethyl acetate (80:20, v/v). TLC was stopped after the solvent front had traveled 16 cm, which required about 70 min. The plates were sprayed with 10 % (v/v) sulfuric acid in 50 % (v/v) methanol followed by heating at 100 °C for 8 min to detect the sterols. Relative mobility, R_m, was calculated based on distance traveled by sterol divided by distance traveled by solvent front.

4.2.3   Analysis of cholesterol reduction using gas chromatography (GC)

HP-5 column (Agilent J&W, 30m × 0.32 mm i.d. × 0.25 μm) with polysiloxane stationary phase was installed to GC machine (HP 5890 series II) equipped with flame ionization detector. Injector and detector temperatures were set at 280 and 300 °C, respectively. Oven temperature was maintained isothermally at 230 °C. Helium carrier gas was maintained at 1.7 ml/min. Cholesterol, coprostanol, 5-

cholesten-3-one, 4-cholesten-3-one and coprostan-3-one at 1 mM in chloroform: methanol (2:1, v/v) were prepared separately. These sterol standards (0.5 µl) were then injected consecutively for GC analysis. Analysis was carried out in triplicates. Sterols were identified as peaks on chromatograms. Retention time is the time taken for sterol to appear as peak on chromatogram. Relative retention time, R_t, was calculated based on the retention time of each sterol divided by the retention time of cholesterol. Hence, retention time of cholesterol was taken as 1. As for the calibration of cholesterol and coprostanol, these sterols were dissolved separately in chloroform: methanol (2:1, v/v) at concentrations ranging from 0 to 2.5 mM and 0 to 1 mM, respectively. Sterol solutions (0.5 µl) were then injected consecutively for GC analysis. Calibration curves were plotted with area under peak against amount of sterol.

4.2.4 Cholesterol reduction activity of *E. coprostanoligenes*

BCM containing 1 mM cholesterol was prepared and autoclaved. Ten ml of medium was dispensed in a tube and inoculated with 1 ml of 24-hour-old culture (approximately 10^6 cells). The culture was incubated at 37 °C under anaerobic conditions. Bacterial culture (1 ml) was withdrawn from the tube and extracted twice with two volumes of chloroform: methanol (2:1, v/v). The combined organic extracts were concentrated to 500 µl for analysis using GC according to the procedure outlined in section 4.2.3. The test was carried out for a period of 5 days in triplicate.

Cholesterol and coprostanol in a sample were identified by comparing their respective retention times with that of standards obtained in section 4.2.3. For subsequent analysis, the interpretation of a chromatogram was based on "internal normalization" in which the areas under "cholesterol peak" and "coprostanol peak" in

a chromatogram were summed up and "normalized" to 100 %. Cholesterol and coprostanol were then reported as a percentage of the total.

$$\text{Cholesterol (\%)} = \frac{\text{Area under cholesterol peak}}{\text{Area under cholesterol peak} + \text{Area under coprostanol peak}} \times 100\ \%$$

It was assumed that response factors for cholesterol and coprostanol were identical; the area of each peak divided by the sum of the areas of all peaks in the chromatogram represented the concentrations of the compounds directly.

4.2.5   Effects of lecithin, CaCl$_2$ and pH on cholesterol reduction activity

BCM containing 1 mM cholesterol with a) lecithin concentrations varying from 0 to 10 g/l; b) calcium chloride concentrations varying from 0 to 10 g/l; and c) pH adjusted to 4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10, were prepared and autoclaved, respectively. The media were then reduced in the anaerobic chamber for 2 hours. Ten ml each of these media was dispensed in a tube, respectively, and inoculated with 1 ml of 24-hour-old culture (approximately 10^6 cells) and incubated at 37 °C in anaerobic chamber. One ml of bacterial culture was withdrawn from the tube after 24 hours of incubation for sterol extraction and analysis using GC. Each test was carried out in triplicate.

4.2.6   Cholesterol reduction activity of *E. coprostanoligenes* under aerobic condition

BCM of 1 mM cholesterol with and without sodium thioglycollate were prepared and autoclaved. For each medium, one set was reduced in anaerobic chamber (anaerobic condition) with the other set exposed to ambient air (aerobic

condition) by shaking in a shaker incubator for two hours before being inoculated with *E. coprostanoligenes*. Ten ml of media in the anaerobic chamber and shaker incubator was dispensed into tube, respectively, and inoculated with 1 ml of 24-hour-old culture (approximately 10^6 cells) and incubated at 37 °C. One ml of the bacterial culture was withdrawn from each tube at 12 hours interval for 60 hours followed by sterol extraction and analysis using GC. Each test was carried out in triplicate.

4.3   Results and Discussion

4.3.1   Development and optimization of analytical method for cholesterol reduction activity

4.3.1.1 Cholesterol measurement using Infinity® Cholesterol Reagent

Cholesterol measurements using Infinity® Cholesterol Reagent in cuvette and microtiter plate were compared. Cholesterol concentrations as low as 10 μg could be measured, with every 0.1 change in absorbance corresponded to approximately 0.6 μg of cholesterol (Figure 4.1a). On the other hand, analysis carried out in microtiter plate offered a ten times higher sensitivity (Figure 4.1b), and it used up only one-fifth of the amount of reagent required in cuvette assays for the same measurement. In addition, measurement using microtiter plate had a higher throughput than cuvette because 96 samples could be measured simultaneously. Therefore, cholesterol measurement using Infinity® Cholesterol Reagent in microtiter plate is recommended.

Cholesterol analysis was generally accomplished using a three-enzyme assay and indicator method devised by Richmond (1973). The first enzyme, cholesterol esterase, freed the esterified cholesterol present in a sample. The free cholesterol was then subjected to oxidation by the second enzyme, cholesterol oxidase, releasing hydrogen peroxide at the same time. A peroxidase enzyme subsequently reduced the
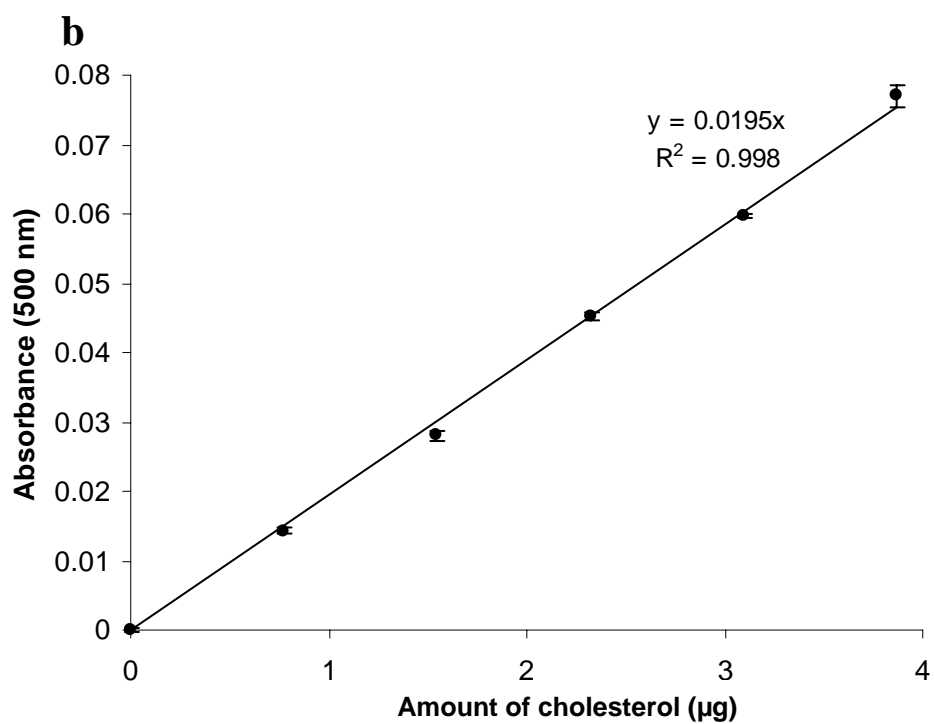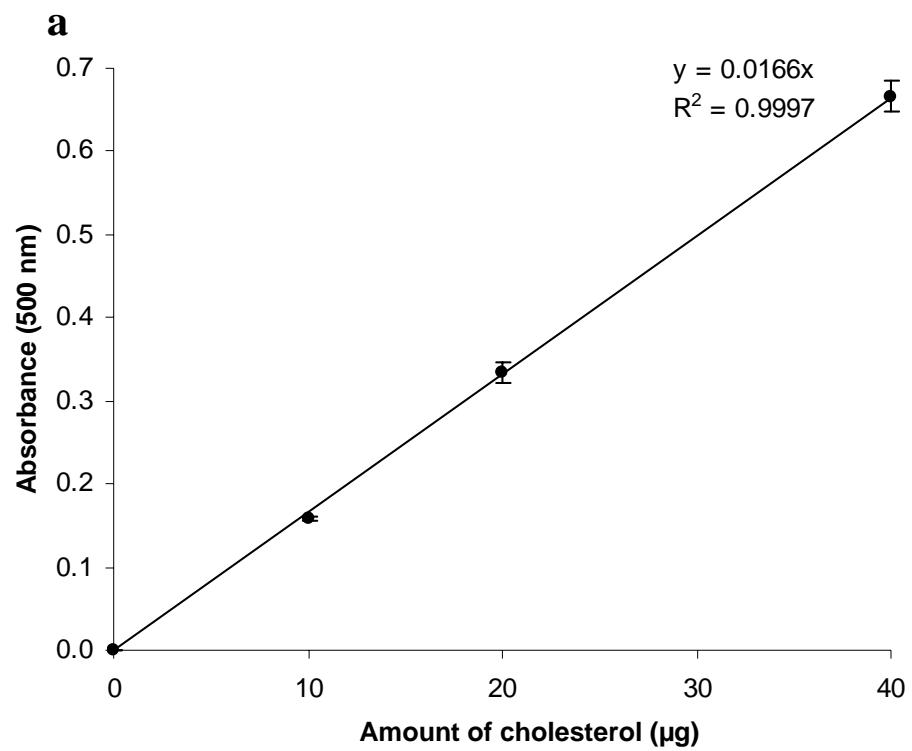
Fig. 4.1: Cholesterol calibration curves using Infinity® Cholesterol Reagent based on the methods for a) cuvette, and b) microtiter plate. Vertical bars denote SE (n=3).

hydrogen peroxide produced. Reactivation of the peroxidase through oxidation of an indicator molecule produced a chromogen which, when measured, facilitated an indirect estimation of total cholesterol. Infinity$^®$ Cholesterol Reagent was based on the formulation of Allan *et al.* (1974) (Figure 4.2). The reagent allowed the direct measurement of the amount of cholesterol reduced in cholesterol-reduction experiments without the hassle of extracting sterols from reaction mixtures.

Cholesterol esters $\xrightarrow{\text{CE}}$ Cholesterol $\xrightarrow{\text{CO}}$ H_2O_2 $\xrightarrow{\text{POD}}$ Quinoneimine Dye

    +   + O_2 +   + HBA +AAP   +

Fatty acids   4-cholesten-3-one H_2O
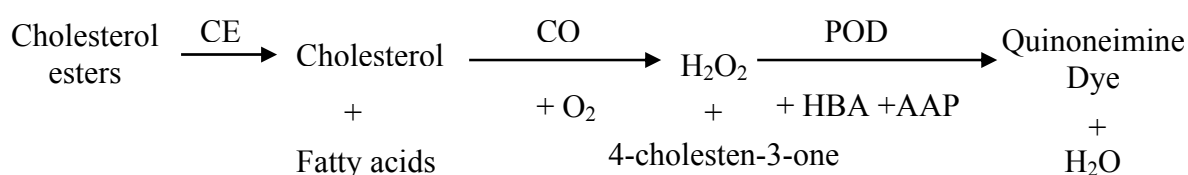
Fig 4.2: Reaction of Infinity$^®$ Cholesterol Reagent. CE= Cholesterol esterase; CO= Cholesterol oxidase; POD= peroxidase; HBA= hydroxybenzoic acid; AAP= 4-aminoantipyrine.

4.3.1.2 Analysis of cholesterol reduction using TLC

Cholesterol and coprostanol were resolved on TLC plate eluted with hexane: ethyl acetate (80:20, v/v) (Figure 4.3). The proposed intermediates for cholesterol-reduction pathway in *E. coprostanoligenes*, 5-cholesten-3-one, 4-cholesten-3-one and coprostanon-3-one (Ren *et al.*, 1996), were also resolved (Figure 4.3). Cholesterol and coprostanol of as low as 1 μg could be detected on TLC (Table 4.1). The sensitivity of cholesterol, coprostanol and the intermediates is tabulated in Table 4.1. 5-Cholesten-3-one, 4-cholesten-3-one and coprostan-3-one less than 100, 10 and 50 μg, respectively could not be detected.

Tan *et al.* (1970) reported that coprostanol was not well separated from cholesterol on TLC eluted with solvent systems such as chloroform: ether (9:1, v/v), chloroform: methanol (9:1, v/v), hexane: ethyl acetate (1:1, v/v) and benzene: acetone
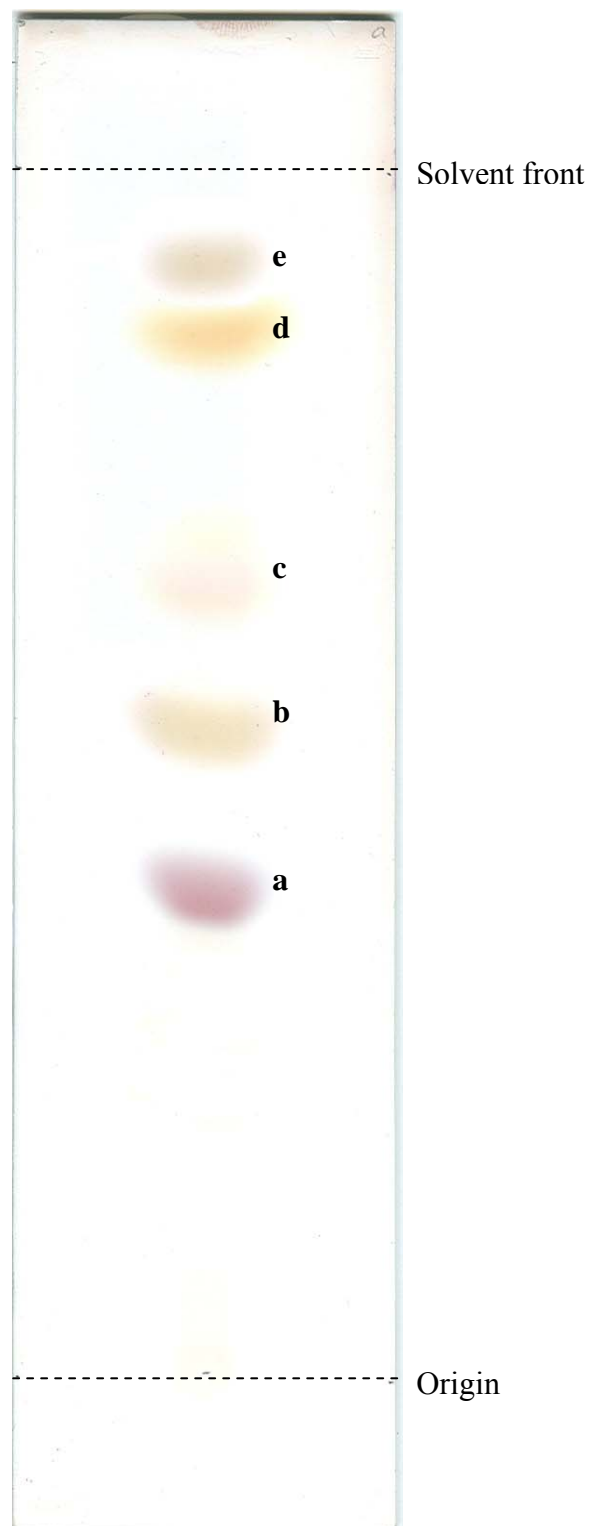
Fig 4.3: TLC of cholesterol, coprostanol, 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one eluted with hexane: ethyl acetate (80:20, v/v). a, b, c, d and e are spots of cholesterol, coprostanol, 4-cholesten-3-one, 5-cholesten-3-one and coprostan-3-one, respectively.

Table 4.1: Relative mobility and sensitivity of cholesterol, coprostanol, 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one eluted with hexane: ethyl acetate (80:20, v/v) on TLC.

Substance	Relative mobility, R_m	Sensitivity (μg)
Cholesterol	0.47	≥ 1
Coprostanol	0.59	≥ 1
4-Cholesten-3-one	0.68	≥ 10
5-Cholesten-3-one	0.84	≥ 100
Coprostanon-3-one	0.86	≥ 50

(4:1, v/v). On the other hand, Domnas *et al.* (1983) found that hexane/ ethyl acetate (9:1, v/v) was most effective in resolving cholesterol and coprostanol. A TLC plate, when pre-eluted with diethyl ether and using chloroform as developing solvent, was found to resolve cholesterol and coprostanol (Bethell *et al.*, 1994). In a study of cholesterol utilization by mycobacterium, cholesterol degradation could be clearly shown on TLC plates eluted with cyclohexane/chloroform (1:1, v/v) (Av-Gay and Sobouti, 2000). However, all the TLC methods discussed above had not studied the resolution of cholesterol and coprostanol together with the three proposed intermediates, which was already achieved in our method.

The TLC method could be utilized to study cholesterol reduction activity in *E. coprostanoligenes*. One drawback of qualitative TLC is that the absolute amount of substances on TLC plates cannot be determined as spots were difficult to quantify. In addition, the spots did not remain for a long time and would fade off after 1 day.

4.3.1.3 Analysis of cholesterol reduction using GC

Cholesterol, coprostanol and coprostan-3-one were resolved as single and sharp peaks on GC chromatogram (Figure 4.4). On the other hand, 5-cholesten-3-one and 4-cholesten-3-one appeared as a single peak. Attempts to resolve these two compounds by reducing the flow rate from 1.7 to 1 ml/ min and oven temperature from 230 to 200 °C were not successful. This might be attributed to highly similar structure between the two compounds, which differs only in the position of C=C double bond at C-5 and C-4, respectively. In the GC, coprostan-3-one was eluted first, followed by coprostanol, cholesterol and lastly the two cholesten-3-ones. The retention time and relative retention time (R_t) of these sterols are tabulated in Table 4.2. The calibration curves of cholesterol and coprostanol showed a linear pattern over
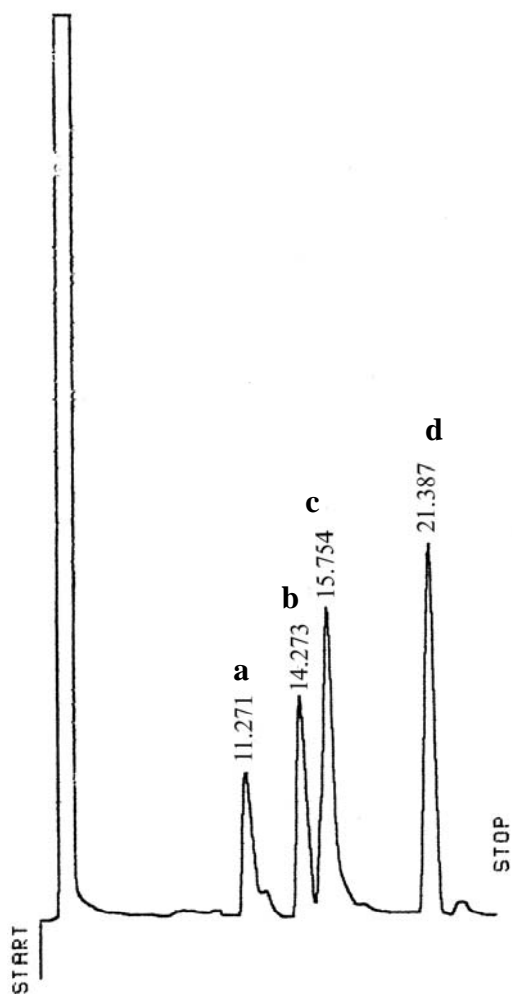
Fig 4.4: GC chromatogram showing peaks of sterol standards. Peaks a, b, c and d are coprostan-3-one, coprostanol, cholesterol and choleste-3-ones (4-cholesten-3-one and 5-cholesten-3-one), respectively. 4-Colesten-3-one and 5-cholesten-3-one were not resolved. 0.5 µl of sterol standards containing 1 mM of each sterol were injected to HP5890 Series II gas chromatography and resolved using HP-5 column. Injector/ detector temperature: 280 °C/ 300 °C; Oven temperature: isothermal 230 °C. Helium carrier gas: 1.7 ml/min.

Table 4.2: Relative retention times of cholesterol, coprostanol, 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one resolved with HP-5 capillary column in GC. Injector/ detector temperature: 280 °C/ 300 °C; Oven temperature: isothermal 230 °C. Helium carrier gas: 1.7 ml/min.

Substance	Retention Time (min)	Relative retention time, R_t
Coprostanon-3-one	11.27	0.72
Coprostanol	14.27	0.91
Cholesterol	15.75	1.00
4-Cholesten-3-one	21.39	1.36
5-Cholesten-3-one	21.39	1.36

these two concentration ranges: 0 to 2.5 nmol and 0 to 1 nmol, respectively (Figure 4.5). The method developed is sensitive as it can detect cholesterol and coprostanol of as low as 0.1 nmol.

Successful separation of cholesterol and coprostanol based on GC required a high degree of efficiency because the two sterols differ in their molecular structure merely by the presence of a double bond (Tan *et al.*, 1970). Cholesterol and coprostanol were only partially resolved in glass column packed with 3 % SE-30 on 100-140 mesh Gas Chrom P (Rosenfeld *et al.*, 1961) and 100-120 mesh Gas Chrom Q (Hassett and Lee, 1977). An almost complete resolution between cholesterol and coprostanol was achieved with the GC method developed by Tan *et al.* (1970) using combined OV^{TM}-1 and OF^{TM}-1 phases on a single column. GC equipped with glass column packed with 1.5% OV^{TM} 17 on Chromosorb$^®$ W 80/100 mesh was used in the study of microbial degradation of sterols (Marsheck *et al.*, 1972).

The GC method developed by Marriott *et al.* (1998) had greatly improved the separation and resolution of cholesterol, coprostanol and plant sterols. The protocol involved supercritical fluid extraction, derivatization and GC analysis using a BPX5 capillary column and electron capture detector. Complete separation was also achieved using HiCap CBP-1 capillary column (Yamaga *et al.*, 2002) with 19-hydroxycholesterol as the internal standard.

Each compound analyzed with GC could be quantified. This would be useful in studying the conversion of cholesterol into coprostanol as well as the kinetics of cholesterol reduction reaction.

a

$$y = 3.2428x$$
$$R^2 = 0.9997$$

Area under peak (counts $\times$ 10^7)

Cholesterol (nmol)

b

$$y = 2.4313x$$
$$R^2 = 0.9899$$

Area under peak (counts $\times$ 10^7)
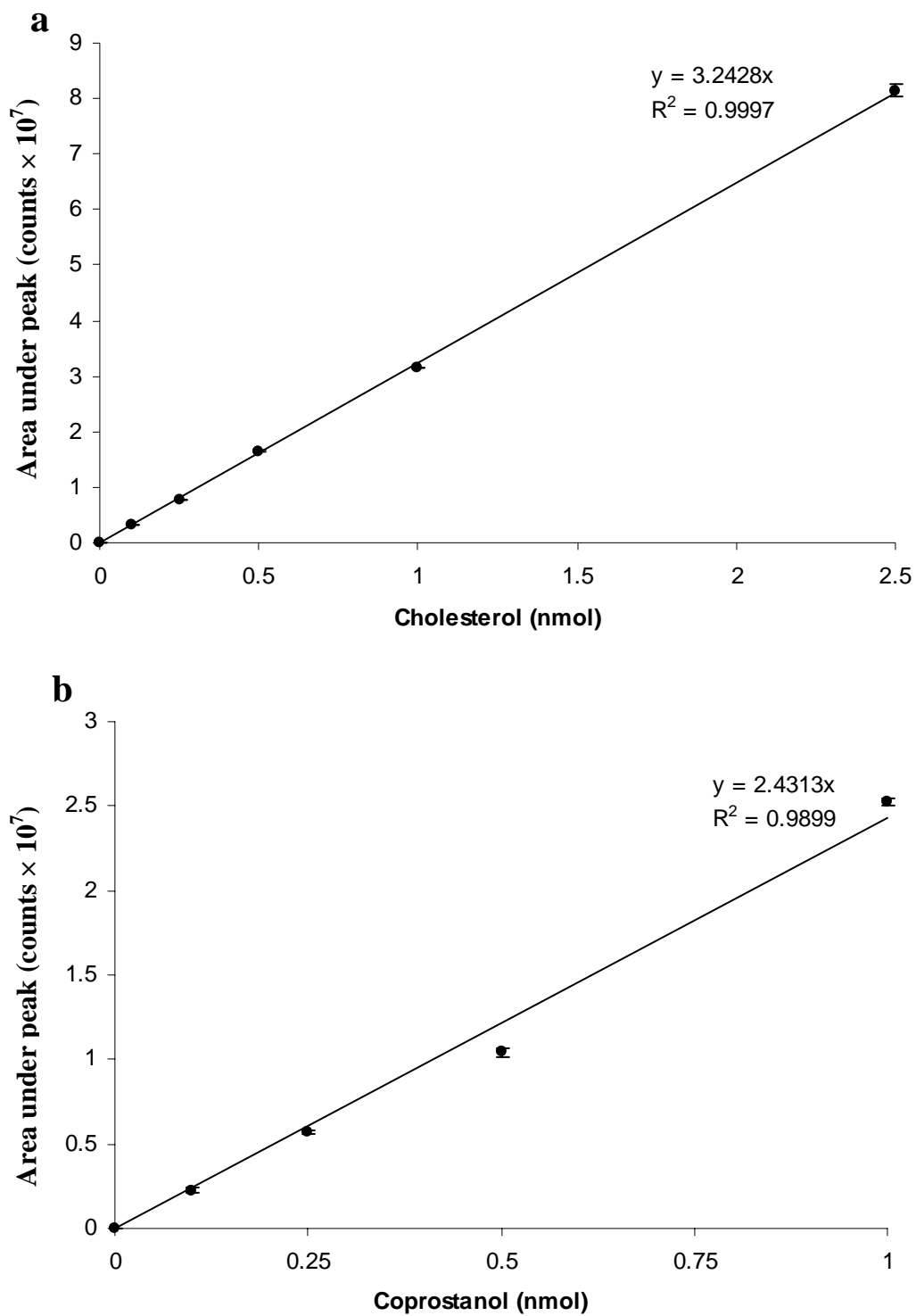
Coprostanol (nmol)

Fig. 4.5: GC calibration curves for a) cholesterol, and b) coprostanol. Vertical bars denote SE (n=3).

4.3.1.4 Summary of methods development

A summary of spectrophotometric and chromatographic methods for cholesterol-reduction study is shown in Table 4.3. Spectrophotometric and chromatographic approaches were investigated and compared for their suitability in cholesterol reduction study. Spectrophotometric method utilizing the Infinity® Cholesterol Reagent is useful because it is simple, direct and quantitative. However, the reagent cannot measure coprostanol, the end product of cholesterol reduction in *E. coprostanoligenes*. From the perspective of this project, it is seen as a disadvantage of using Infinity® Cholesterol Reagent.

TLC and GC are able to detect cholesterol, the proposed intermediates and coprostanol in a sample. TLC has better throughput because as many as 20 samples can be analyzed at any one time. In contrast, samples in GC have to be run consecutively, which greatly reduces its efficiency. In our study, the time taken for each sample in TLC was lesser compared to GC even though post-elution treatment was required for spots visualization. In TLC, each sample took approximately 6 min when considering 70 and 50 min for running and post-elution treatment, respectively. Analysis of one sample alone in GC required approximately 25 min.

Despite a lower efficiency and longer analysis time in GC, it represented a better choice in cholesterol reduction study because each substance in a sample could be quantified. With quantitative values, results obtained would be more accurate and reliable. Differences between treatments in an experiment could also be compared and reported.

Table 4.3: Summary of spectrophotometric and chromatographic methods for cholesterol-reduction study

| **Analysis** | Detectable substance | | | | | |
	Cholesterol	Coprostanol	4-Cholesten-3-one 5-Cholesten-3-one Coprostan-3-one	Quantitative	Sensitivity* (μg)	Analysis time (min/ sample)
Spectrophotometry^	Yes	No	No	Yes	1	5
Chromatography						
TLC	Yes	Yes	Yes	No	1	6~
GC	Yes	Yes	Yes	Yes	0.2	25

*Based on coprostanol

^Infinity® Cholesterol Reagent

~Based on a maximum of 20 samples in 120 min of running and post-elution treatment.

4.3.2 Cholesterol reduction activity of *E. coprostanoligenes*

E. coprostanoligenes was found to reduce cholesterol when cultured in BCM containing 1 mM cholesterol. Using GC, it was observed that cholesterol reduction was accompanied with coprostanol formation (Figure 4.6a). Approximately 65 % of the conversion took place during the first two days of culture. Cholesterol reduction continued from day-3 to day-5 but the amount of conversion was not significant, with another 3 % of cholesterol being reduced. Active cholesterol reduction reaction took place at the exponential growth phase (Figure 3.3). Our findings re-affirmed the cholesterol reduction ability of *E. coprostanoligenes* reported by Freier *et al*. (1994).

Conversion of cholesterol to coprostanol involves the reduction of the double bond at C-5 of the A ring of cholesterol and it is the most common reduction reaction that occurs with cholesterol (Hylemon and Harder, 1999). Anaerobic faecal bacteria from human intestine had been found to modify bile acids and steroids by deconjugation, dehydration, reduction and dehydroxylation (Holland *et al.*, 1987). Besides *E. coprostanoligenes*, a denitrifying bacterium strain 72Chol was found to be able to convert cholesterol completely under anaerobic condition to carbon dioxide (Hylemon and Harder, 1999).

Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic bacteria has been reviewed (Hylemon and Harder, 1999). Bile acids that were not reabsorbed through enterohepatic circulation were exposed to up to 400 different kinds of mostly obligate anaerobes in the colon. The predominant species are members of genera *Bacteroides*, *Fusobacterium*, *Eubacterium* and *Clostridium* which generated 15 to 20 different bile acid metabolites (Hylemon and Harder, 1999).

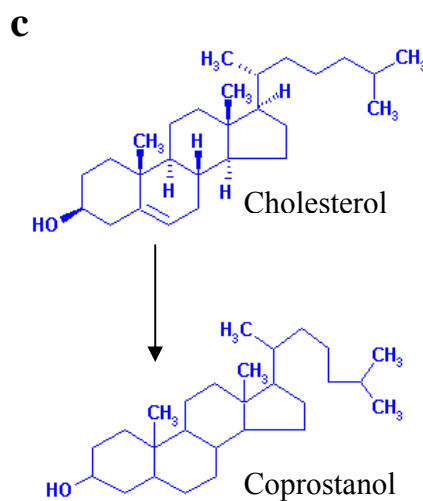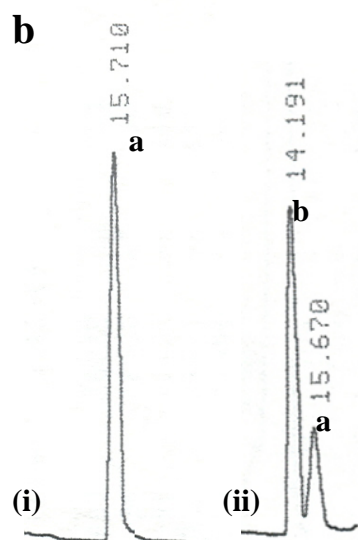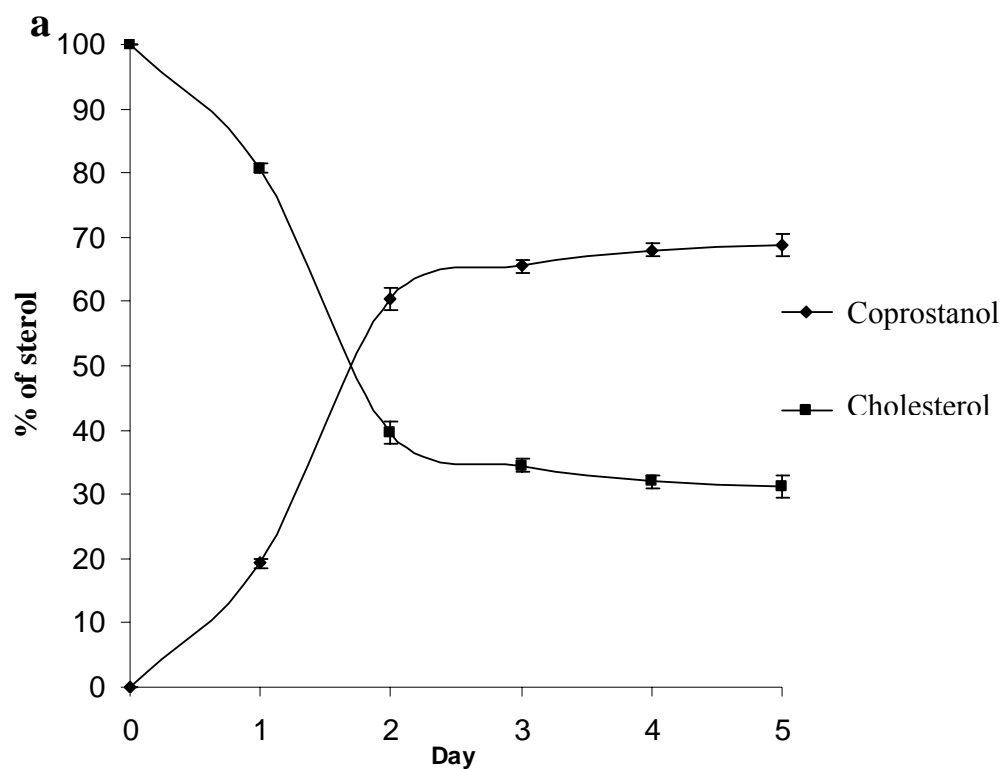**Fig.** 4.6: Cholesterol reduction activity of *E. coprostanoligenes* at 1 mM of cholesterol. a) Graph showing the conversion of cholesterol to coprostanol. Vertical bars denote SE (n=3). b) GC chromatogram showing the action of *E. coprostanoligenes* (i) before, and (ii) after the inoculation in BCM. Peaks a and b are cholesterol and coprostanol respectively. c) Molecular structures showing the reduction of cholesterol to coprostanol.

The conversion of cholesterol to coprostanol was thought to be carried out by cholesterol reductase (Dehal *et al.*, 1991), which is yet to be characterized. However, many other steroid transforming reactions and enzymes had been studied in anaerobic bacteria particularly in the genus *Eubacterium* (Feighner *et al.*, 1979; Glass and Burley, 1984; Winter *et al.*, 1984; Oda *et al.*, 2001). For example, 21-dehydroxylase, extracted from *Eubacterium lentum*, is known to catalyze the conversion of 11-deoxycorticosterone to progesterone (Feighner *et al.*, 1979). A 16- dehydroprogesterone reductase was assumed to be involved in the biotransformation of 16-dehydroprogesterone to isoprogesterone in intestinal *Eubacterium* sp. 144 (Glass and Burley, 1984). Another example is 7β-Hydroxysteroid dehydrogenase produced by *Eubacterium aerofaciens*, which was reported to reduce a double bond in methyl 7-ketolithocholate to methyl ursodeoxycholate (Oda *et al.*, 2001). All these examples may indicate the possible existence of a sterol reductase in *E. coprostanoligenes*.

4.3.3 Factors affecting cholesterol reduction activity

4.3.3.1 Effect of Lecithin

Cholesterol reduction activity, as indicated by coprostanol production, increased with increasing lecithin concentration. In the absence of lecithin, conversion of cholesterol to coprostanol was not detected (Figure 4.7). Maximum coprostanol production was achieved when lecithin was increased to 5 g/l, which was up to 43 % increase compared to lecithin at 1 g/l (Figure 4.7). When the lecithin concentration was doubled to 10 g/l, the media became more viscous, but no further increase in coprostanol production was observed.

Fig 4.7: Effect of lecithin on cholesterol reduction activity of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate relative activity with respect to that of 1 g/l (taken as 1). Different letters (above each bar chart) indicate significant different between treatments (one-way ANOVA Tukey's Test, 95 % confidence level).

As discussed in Chapter 3, optimal bacterial growth was actually achieved at a lecithin concentration of 1 g/l. Lecithin concentration above that resulted in a reduction in the number of viable cells (Figure 3.5). This thus gives rise to the speculation that the increased cholesterol reduction at lecithin above 1 g/l was the effect of increased lecithin. Freier *et al.* (1994) had suggested that increased lecithin could increase lecithin digestion by the bacteria which could in turn increase cholesterol reduction activity.

4.3.3.2 Effect of CaCl$_2$

Increasing CaCl$_2$ concentration caused an increase in cholesterol reduction with the optimum achieved at 1 g/l where 60 % of cholesterol was reduced. A further increased in CaCl$_2$ concentration to 5 g/l showed no significant increase in cholesterol reduction (Figure 4.8). Cholesterol reduction was severely affected at 10 g/l CaCl$_2$, with only 25 % of cholesterol being reduced. As discussed in Chapter 3, growth of *E. coprostanoligenes* was reduced at CaCl$_2$ above 2.5 g/l (Figure 3.6). However, this reduction in growth did not affect the cholesterol reduction at 5 g/l CaCl$_2$.

Freier *et al.* (1994) suggested that calcium ions supplied a net positive charge to lecithin which functioned as activator of phospholipase for lecithin hydrolysis. The metabolites of lecithin hydrolysis in turn played a role in cholesterol reduction. In present study, CaCl$_2$ at 1 g/l could be just optimal to supply the net positive charge. CaCl$_2$ concentration above that would therefore not enhance cholesterol reduction.
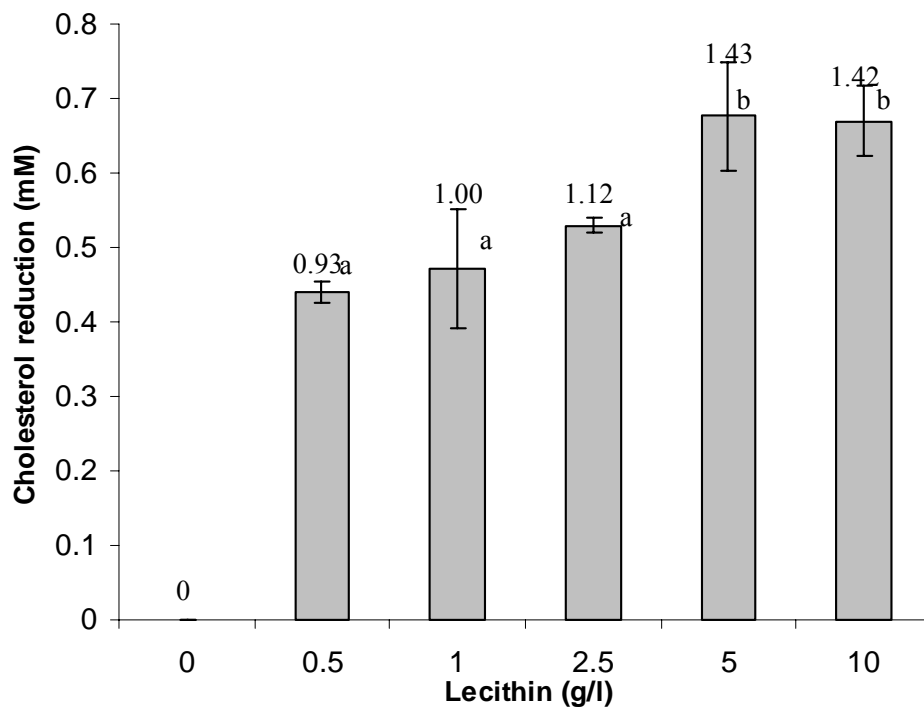
Fig 4.8: Effect of CaCl$_2$ on cholesterol reduction activity of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate relative activity with respect to that of 1 g/l (taken as 1). Different letters (above each bar chart) indicate significant different between treatments (one-way ANOVA Tukey's Test, 95 % confidence level).

4.3.3.3 Effect of pH

Cholesterol reduction activity was found to take place between pH 5 to 9. Optimal cholesterol reduction took place when BCM was adjusted to pH 7, with approximately 75 % of cholesterol undergoing reduction (Figure 4.9). This observation agreed with that reported by Freier *et al.* (1994). While the activity was reduced almost by half when pH was adjusted to 5 from 7, no significant difference in cholesterol reduction was found in BCM at pH 7, 7.5 and 8. In addition, no cholesterol reduction was detected at pH 4 and 10 (Figure 4.9), where bacterial growth was not evident (Figure 3.7). The discrepancy of the present findings with that of Freier *et al.* (1994) who found no cholesterol reduction taking place at pH 5.5 or 8 suggest that *E. coprostanoligenes* may be stable enough over a range of pH values to carry out cholesterol reduction. The stability of *E. coprostanoligenes* to carry out cholesterol reduction reaction over a wide range of pH is an advantage for its future application.

4.3.4 Cholesterol reduction activity of *E. coprostanoligenes* under aerobic condition

No significant difference was found in cholesterol reduction for *E. coprostanoligenes* cultured in BCM with or without sodium thioglycollate, either under aerobic or anaerobic conditions. However, bacterial culture in BCM without sodium thioglycollate tended to reduce approximately 10 % more cholesterol than those cultured in media containing sodium thioglycollate (Figure 4.10).
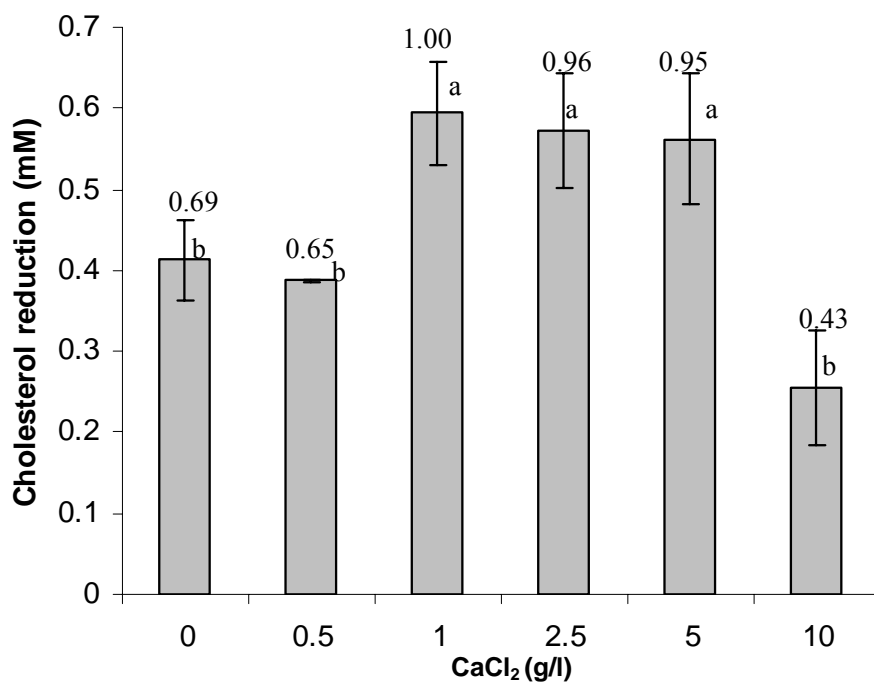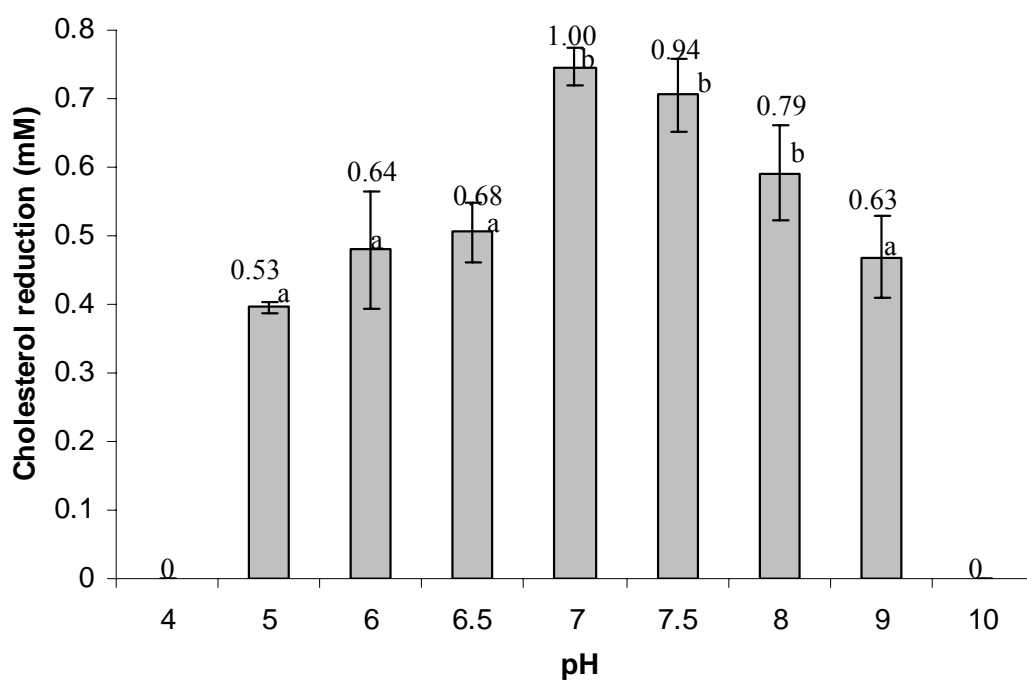
Fig 4.9: Effect of pH on cholesterol reduction activity of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate relative activity with respect to activity at pH 7 (taken as 1). Different letters (above each bar chart) indicate significant different between treatments (one-way ANOVA Tukey's Test, 95 % confidence level).
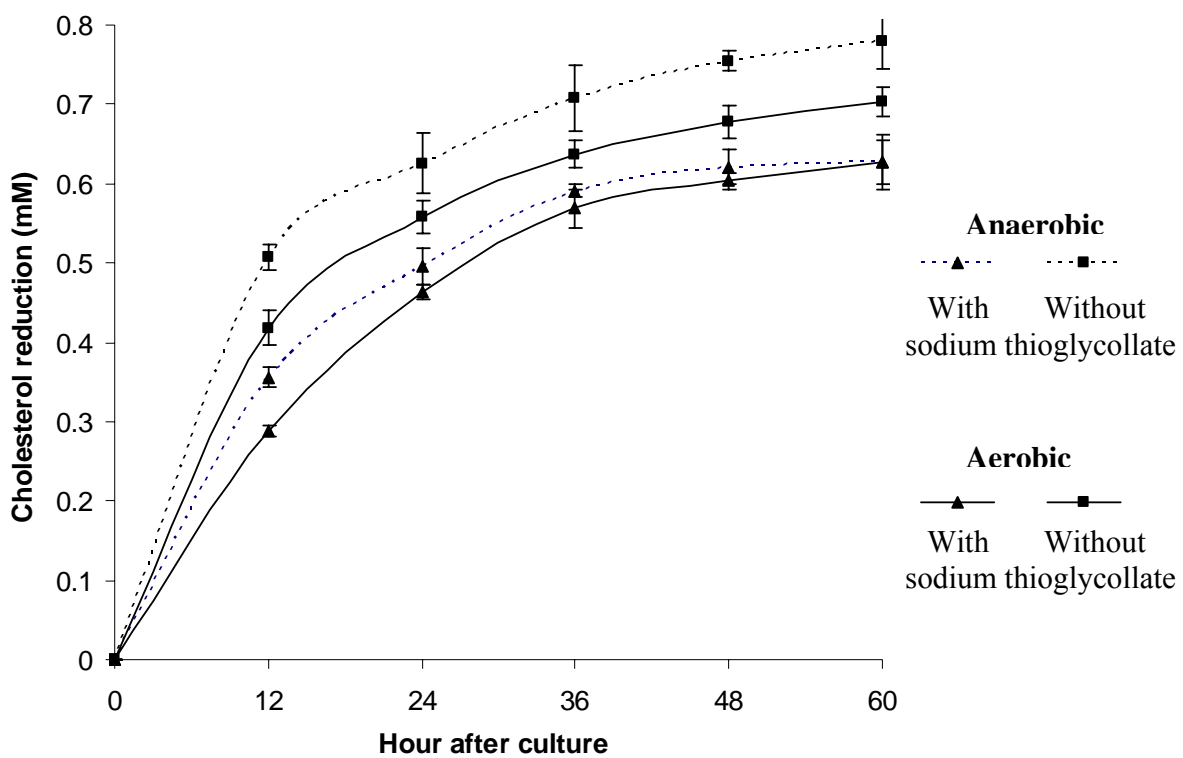
Fig 4.10: Cholesterol reduction activity in *E. coprostanoligenes* cultured in BCM with and without sodium thioglycollate, under aerobic and anaerobic conditions. Vertical bars denote SE (n=3).

4.4 Concluding Remarks

Spectrophotometric determination of cholesterol reduction activity using Infinity$^{®}$ Cholesterol Reagent and chromatographic (TLC and GC) approaches in detecting cholesterol reduction activity were developed and compared. Each method had its advantages and disadvantages. After considering for reliability and accuracy, GC was evaluated as the best method despite requiring a longer analysis time.

E. coprostanoligenes were found to convert 65 % of cholesterol to coprostanol in BCM containing 1 mM cholesterol. This re-affirmed the cholesterol reduction property of *E. coprostanoligenes* reported by Freier *et al.* (1994). Lecithin, $CaCl_2$ and pH of medium were found to affect cholesterol reduction activity. The activity increased with increasing lecithin concentration and maximum cholesterol reduction was achieved at 5 g/l of lecithin. $CaCl_2$ of 1 g/l was found to be optimum for cholesterol reduction activity. In addition, the reaction could occur over a wide range of pH from 5 to 9, as well as in aerobic condition.

The results obtained from this chapter have provided useful information on the cholesterol reduction properties of *E. coprostanoligenes* and formed a fundamental for its future application. Further studies, however, are essential in order to comprehend the cholesterol reduction mechanisms in relation to the overall metabolism of the bacteria.

5 PROPERTIES OF PUTATIVE CHOLESTEROL REDUCING ENZYME(S)

5.1 Introduction

Literature on cholesterol reduction reaction of *E. coprostanoligenes* has been scarce since its isolation and characterization (Freier *et al.*, 1994; Li *et al.*, 1995b; Ren *et al.*, 1996). An enzyme designated as cholesterol reductase was suggested to carry out the conversion of cholesterol to coprostanol in *E. coprostanoligenes* (Dehal *et al.*, 1991). To date, it has not been characterized. Although the mechanisms underlying bacterial cholesterol reduction have been studied (Schoenheimer *et al.*, 1935; Rosenfeld *et al.*, 1955; Björkhem and Gustafsson, 1971; Ren *et al.*, 1996), two pathways of cholesterol reduction, the direct and the indirect pathways were proposed to take place in *E. coprostanoligenes* (Ren *et al.*, 1996). In the direct pathway, cholesterol is converted directly to coprostanol. On the other hand, in the indirect pathway, it is transformed via 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one to coprostanol.

The objectives of this chapter are to study the properties of putative cholesterol reducing enzyme(s) as well as cholesterol reduction pathway in *E. coprostanoligenes*.

5.2 Materials and Methods

5.2.1 Kinetics of cholesterol reduction activity

BCM containing cholesterol of concentrations ranging from 0 to 2 mM were prepared (50 ml of each concentration) and autoclaved. The media were then reduced in an anaerobic chamber for 2 hours. The different media were dispensed into 10 ml per tube, respectively in triplicate, and inoculated with 1 ml of 24-hour-old culture

(containing approximately 10^6 cells) and incubated at 37 °C in anaerobic chamber. For each concentration, 1 ml of culture was withdrawn daily for sterol extraction and analysis using GC. Extraction and analysis were carried out for a period of 5 days.

5.2.2 Induction of putative cholesterol reducing enzyme(s)

E. coprostanoligenes was sub-cultured ten times in BCM without cholesterol. At every sub-culture, 200 ml fresh BCM were inoculated with 20 ml of 2-day-old culture (containing approximately 10^7 cells/ml). For the eleventh sub-culture, bacterial culture was incubated at 37 °C anaerobically for 24 hours followed by the addition of cholesterol suspension (193 mg of cholesterol and 97 mg of lecithin were boiled and mixed in 100 ml milli-Q water as stock cholesterol suspension of 5 mM) at a final concentration of 1 mM. The bacterial culture (1 ml) was then withdrawn at 0, 0.5, 1 and 2 hours of incubation for sterol extraction and analysis using GC. The test was carried out in triplicate.

5.2.3 Secretion of putative cholesterol reducing enzyme(s)

BCM (50 ml) with (1 mM) and without cholesterol were prepared and autoclaved. The media were then reduced in an anaerobic chamber for 2 hours. The different media were dispensed into 10 ml per tube in triplicate and inoculated with 1 ml of 24-hour-old culture (containing approximately 10^6 cells) and incubated at 37 °C in anaerobic chamber. After 24 hours of incubation, tubes of culture were centrifuged at 14,000 g for 20 min. Supernatants harvested from culture with and without cholesterol were labeled as "Supernatant W" and "Supernatant W/O", respectively. Cholesterol suspension and NADH (10 mg of NADH was dissolved in 2.56 ml of milli-Q water to form 5 mM

NADH solution) were then added to both types of supernatants at a final concentration of 1 mM of cholesterol and NADH. The mixtures were incubated at 37 °C under anaerobic condition. After 24 hours of incubation, 1 ml of mixture was withdrawn for sterol extraction and analysis using GC. Bacterial culture (1 ml) from BCM containing 1 mM of cholesterol was used as control.

5.2.4 Elucidation of cholesterol reduction pathway

Sterol media (50 ml) containing 1 mM 4-cholesten-3-one, 5-cholesten-3-one or coprostan-3-one were prepared according to the procedure outlined in Section 3.2.1 by replacing cholesterol with the respective sterols. The prepared media were dispensed into 10 ml per tube in triplicate and inoculated with 1 ml of 24-hour-old culture (containing approximately 10^6 cells) and incubated at 37 °C in an anaerobic chamber. From each type of sterol medium, 1 ml of culture was withdrawn daily for a period of 4 days for sterol extraction and analysis using GC.

5.2.5 Inhibition of putative cholesterol oxidase activity

Cholesterol oxidase inhibitors (tridemorph, fenpropidin and fenpropimorph) were purchased from Sigma-Aldrich®. BCM (150 ml) containing 1 mM cholesterol was prepared and autoclaved. The media were then dispensed into 3 bottles of 50 ml/bottle and reduced in an anaerobic chamber for 2 hours. Tridemorph was then added to the media in the three separate bottles at three different concentrations of 50 mg/L, 100 mg/L and 200 mg/L, respectively. Medium in each bottle was then dispensed into 10 ml per tube in triplicate and inoculated with 1 ml of 24-hour-old culture (containing

approximately 10^6 cells) and incubated at 37 °C in an anaerobic chamber. After 24 hours of incubation, 1 ml of culture was withdrawn from each tube for sterol extraction and analysis using GC. Plate counting for viable cells was done at the same time in triplicate for each concentration. The same procedures were repeated for fenpropidin or fenpropimorph. Bacterial culture without inhibitor was used as control.

5.3   Results and Discussion

5.3.1   Kinetics of cholesterol reduction activity

The kinetics of cholesterol reduction activity in *E. coprostanoligenes* was investigated in the present study. Cholesterol reduction activity was found to increase with increasing cholesterol concentration (Figure 5.1). Active cholesterol reduction took place during the first two days of incubation corresponding to the exponential growth phase of the bacteria after which the reduction activity tapered off. In our study, 73.6 % and 42.5 % of cholesterol were reduced in BCM containing 0.25 and 2 mM cholesterol, respectively. Freier *et al.* (1994) had reported the conversion of up to 90 % of cholesterol to coprostanol in BCM containing 5.2 mM cholesterol.

Cholesterol was not completely reduced even when incubation was extended to 5 days most likely because the bacteria have entered death phase after 4 days of culture and the number of cells would have declined sharply. As the growth of *E. coprostanoligenes* was not affected by cholesterol (Figure 3.3), the difference in the rate of cholesterol reduction is likely to be a direct effect of different in cholesterol concentrations.

The Lineweaver-Burk plot was constructed based on the cholesterol reduction activity at day-1 (Figure 5.2). V_{max} was calculated to be approximately 14 µM cholesterol
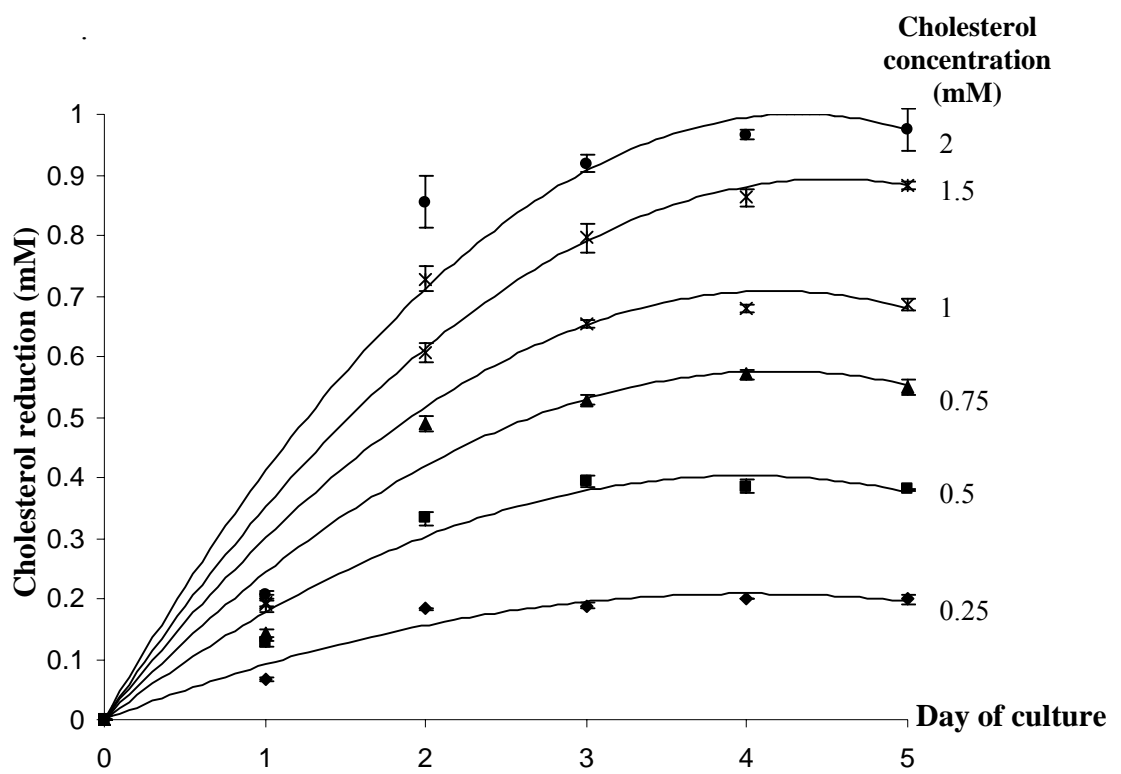
Fig. 5.1**:** Kinetics of cholesterol reduction of *E. coprostanoligenes* at different cholesterol concentrations. Vertical bars denote SE (n=3).
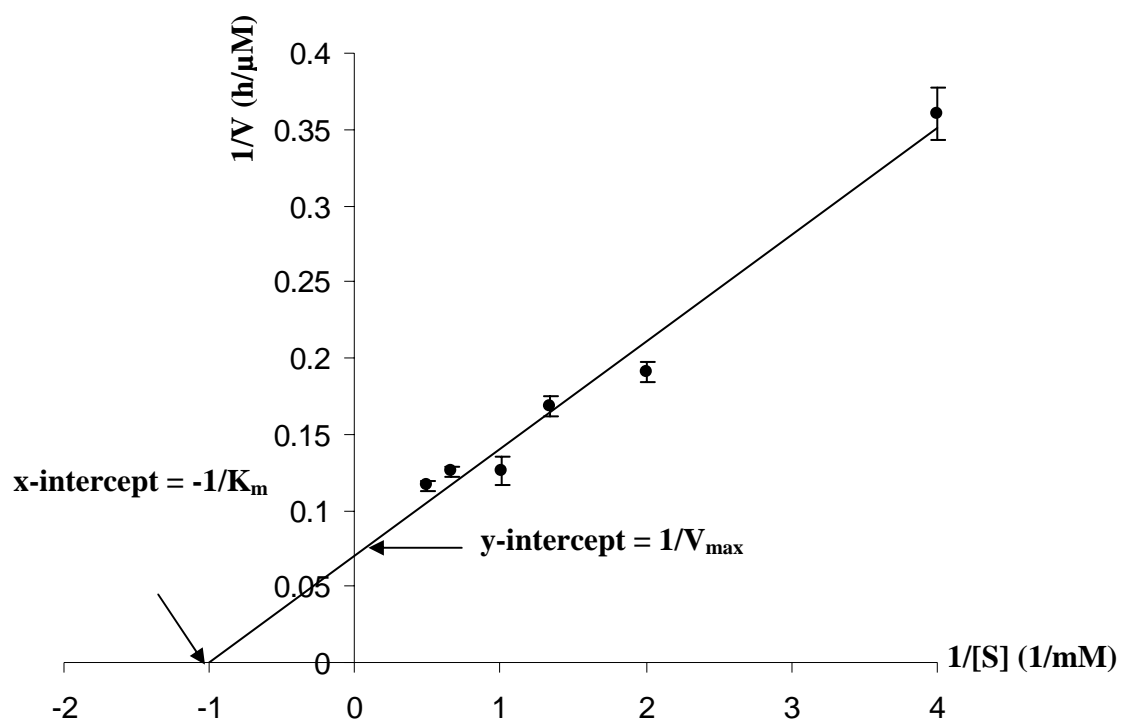
Fig. 5.2: Lineweaver-Burk plot for cholesterol reduction in *E. coprostanoligenes*. V and [S] denote initial velocity of cholesterol reduction and concentration of cholesterol, respectively. Vertical bars denote SE (n=3).

reduced/h. It was found that any further increase in cholesterol concentration did not increase the rate of cholesterol reduction. K_m for cholesterol reduction in *E. coprostanoligenes* was calculated to be 1 mM cholesterol.

5.3.2 Induction of putative cholesterol reduction enzyme(s)

The enzyme(s) responsible for cholesterol reduction appeared to be constitutively produced. This is postulated based on the fact that cholesterol reduction activity has already taken place as early as 30 min of incubation (Figure 5.3). The activity of inducible enzyme would only be detected 3 to 6 hours after the addition of substrate (Glass and Burley, 1984). The reduction reaction appeared to proceed at a constant rate with 50 μM of cholesterol being reduced at 30 min and making up to 170 μM of cholesterol reduced in 2 hours. The present study showed that *E. coprostanoligenes* had not lost its capability for cholesterol reduction after being sub-cultured 10 times in cholesterol-free media.

5.3.3 Secretion of putative cholesterol reduction enzyme(s)

Cholesterol reducing enzyme(s) appeared to be produced and retained intracellularly. Only 8 to 9 % of cholesterol was reduced in supernatants W and W/O compared to almost 60 % reduction in bacterial culture (Figure 5.4). Plate counting with the supernatants after 24-hour of incubation revealed the presence of *E. coprostanoligenes* at a magnitude of hundreds to a thousand cells. The insignificant cholesterol reduction in the supernatants could be attributed to the residual cells retained. Cholesterol did not induce extracellular secretion of enzyme(s) because no
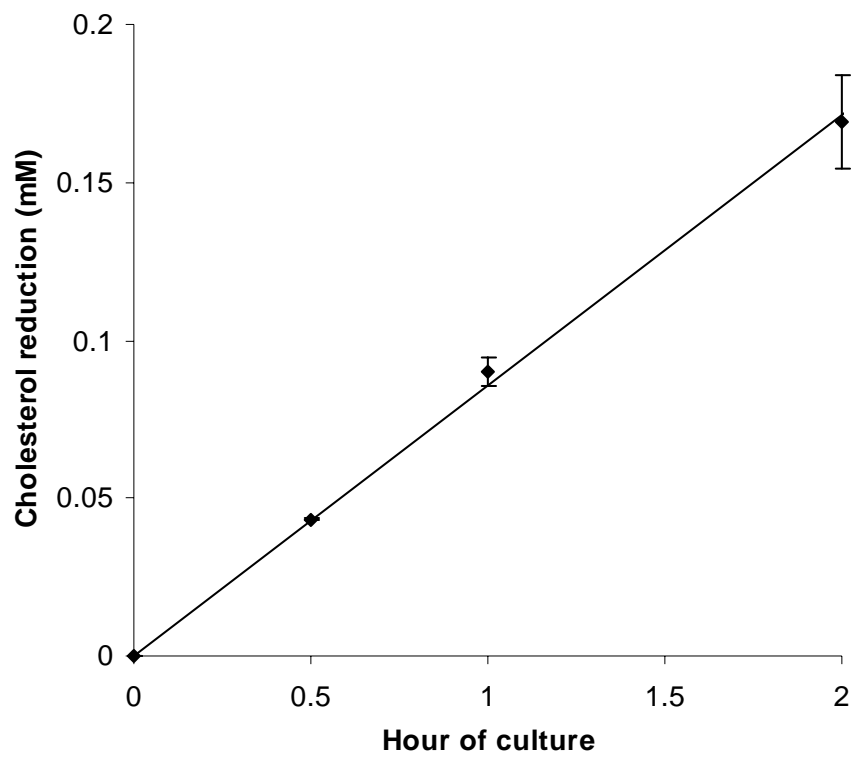
Fig. 5.3: Constitutive secretion of cholesterol reducing enzyme(s) by *E. coprostanoligenes*. Vertical bars denote SE (n=3).
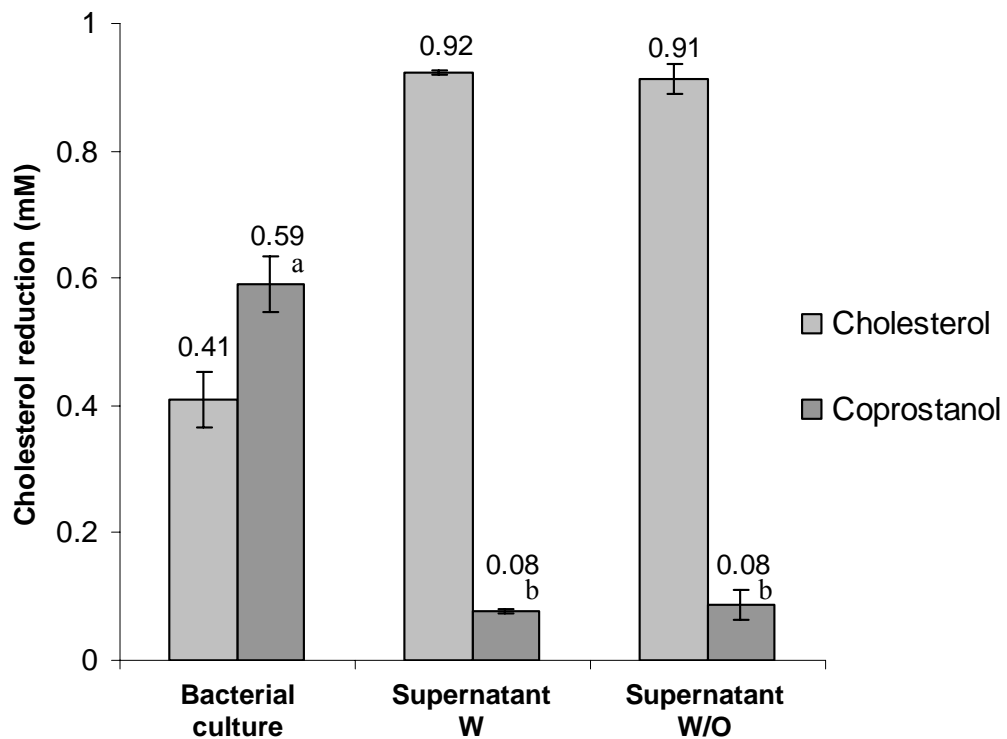
Fig. 5.4: Intracellular secretion of cholesterol reducing enzyme(s) by *E. coprostanoligenes*. Supernatants W and W/O were harvested from bacterial culture with and without cholesterol, respectively. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate concentrations. Different letters (above each bar chart) indicate significant difference between treatments (one-way ANOVA. Tukey's Test, 95 % confidence level).

significant difference in cholesterol reduction was observed in supernatants obtained from media with (Supernatant W) and without (Supernatant W/O) cholesterol (Figure 5.4).

Enzyme that converts cholesterol to coprostanol has not been characterized. Since the cholesterol reducing enzyme(s) of *E. coprostanoligenes* was observed to be not secreted extracellularly, we postulated that the cholesterol reduction reaction took place either on the bacterial membrane (by membrane-bound enzyme) or within the bacterial cell (by cytoplasmic enzyme). Bacterial cells must be broken in order to isolate the enzyme(s). However, we lost the cholesterol reduction activity in our attempts to isolate the enzyme(s) using sonication, passage through a French pressure cell or enzymatic digestion of the bacterial membrane. The loss of activity could be due to disrupted membrane integrity.

5.3.4 Cholesterol reduction pathway of *E. coprostanoligenes*

The cholesterol reduction reaction with the intermediates of 5-cholesten-3-one, 4-cholesten-3-one and coprostan-3-one as intermediates was verified in *E. coprostanoligenes*. Each of these intermediates was converted to coprostanol when incorporated in BCM in place of cholesterol (Figure 5.5). The reaction profile appeared to follow such a sequence: cholesterol → 5-cholesten-3-one → 4-cholesten-3-one → coprostan-3-one → coprostanol. This profile agreed with the indirect pathway of cholesterol reduction proposed by Ren *et al.* (1996). On the other hand, direct conversion of cholesterol to coprostanol could not be excluded in view of the fact that the intermediates were not detected in our experiments when cholesterol was reduced.
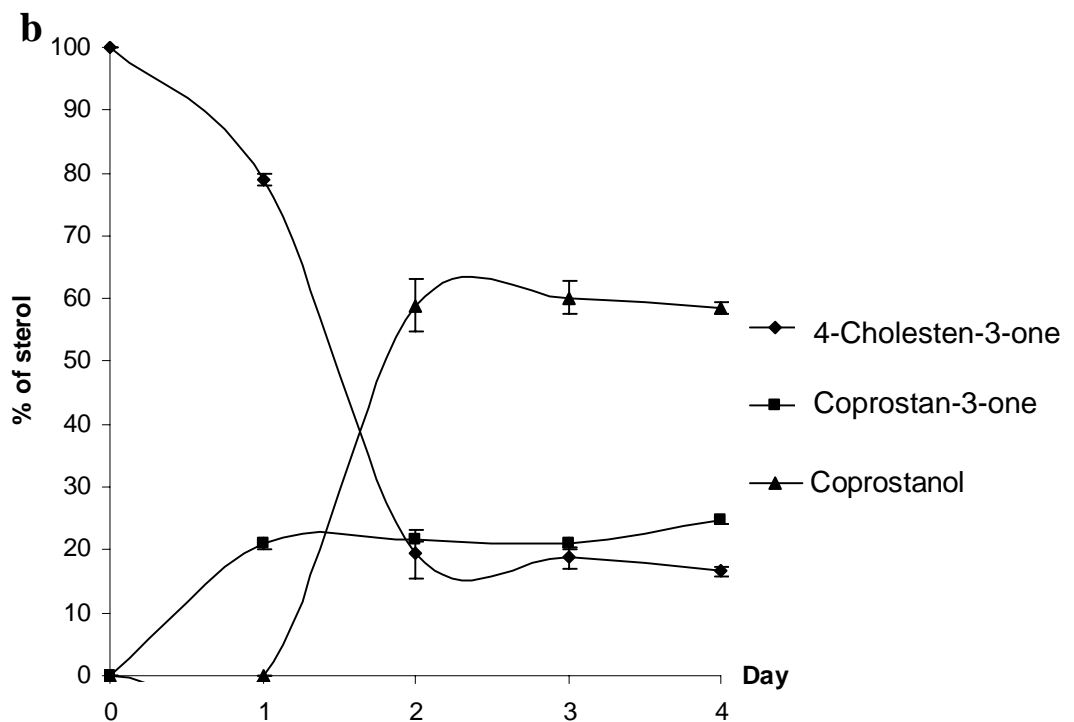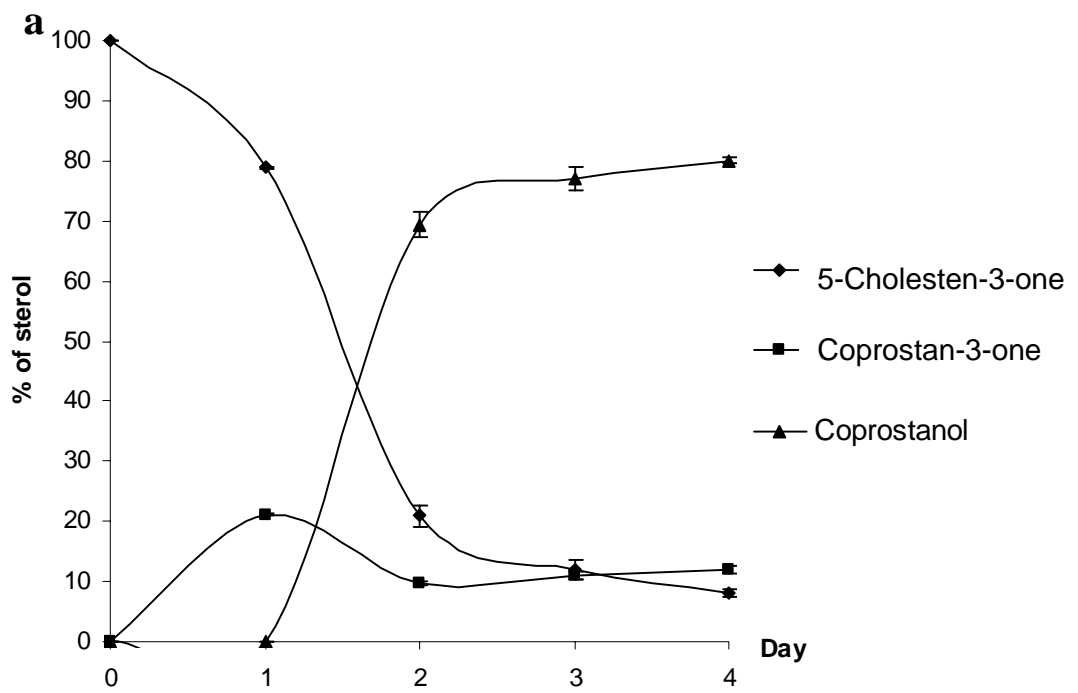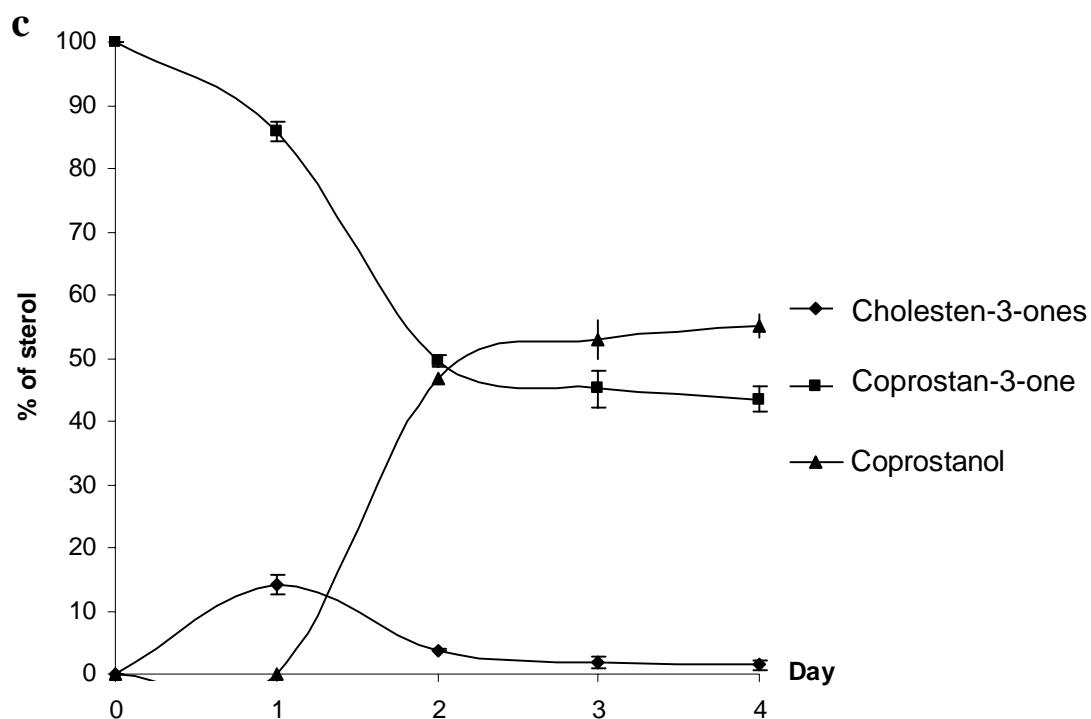
Fig. 5.5: Reduction of a) 5-cholesten-3-one; b) 4-cholesten-3-one; and c) coprostan-3-one to coprostanol by *E. coprostanoligenes*. The indirect pathway of cholesterol reduction was verified. Verticals bars denote SE (n=3).

In the reduction of 5-cholesten-3-one and 4-cholesten-3-one, coprostan-3-one was first detected followed by coprostanol. The reaction reached steady state after 2 days of incubation with a constant pool of coprostan-3-one. In the case of reduction of coprostan-3-one, there was a transient increase of cholesten-3-ones before it was converted to coprostanol.

The results suggested the possible roles of enzymes that catalyzed the conversion of each intermediate to the subsequent one until coprostanol was formed. In the conversion of plant sterol to stanol, various enzymes have been reported for the conversion of the intermediates (Li *et al.*, 1996b; Klahre *et al.*, 1998; Noguchi *et al.*, 1999; Venkatramesh *et al.*, 2003). A scheme for the conversion of plant sterol to stanol involving 3 different enzymes was proposed (Figure 5.6) (Venkatramesh *et al.*, 2003). It is likely that these enzymes are also present in *E. coprostanoligenes*.

Cholesterol oxidase from *Brevibacterium sp.* was found to have cholesterol reduction potential and could reduce up to 85.6 % of cholesterol in egg yolk (Lv *et al.*, 2002). This enzyme is well characterized to catalyze the conversion of cholesterol to 4-cholesten-3-one via 5-cholesten-3-one (MacLachlan *et al.*, 2000). It is likely to exist in *E. coprostanoligenes* catalyzing the conversion of cholesterol to 4-cholesten-3-one in the indirect pathway of cholesterol reduction. However, cholesterol oxidase does not convert cholesterol to coprostanol. Other enzyme(s) may catalyze the conversion of 4-cholesten-3-one leading to the formation of coprostanol after the initial action of cholesterol oxidase.
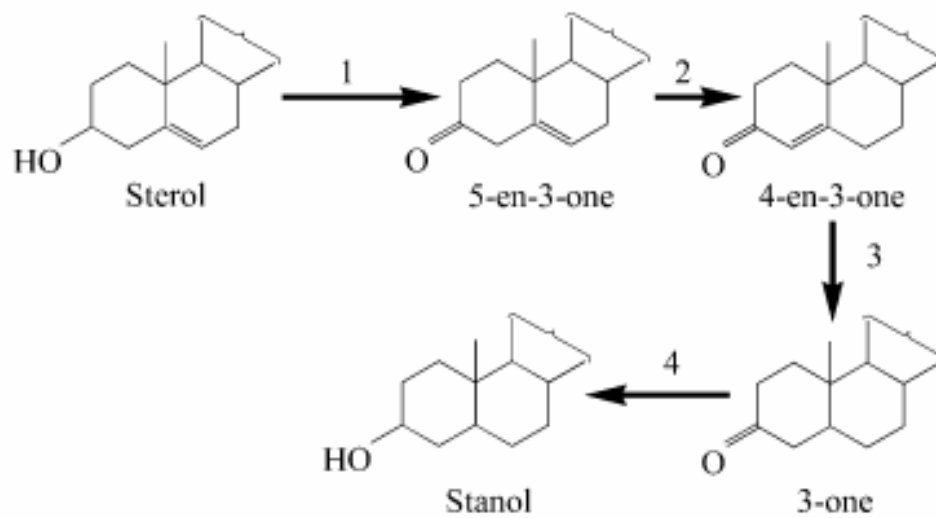
Fig 5.6: Proposed scheme for conversion of sterol to stanol in plants. Reactions 1 and 2 are catalyzed by 3-hydroxysteroid oxidase, whereas reactions 3 and 4 are catalyzed by steroid 5α-reductase and 3-keto reductase, respectively (Venkatramesh *et al.*, 2003).

5.3.4 Inhibition of putative cholesterol oxidase activity

Tridemorph, fenpropidin and fenpropimorph were reported to inhibit the conversion of cholesterol to 4-cholesten-3-one (Hesselink *et al.*, 1990; MacLachlan *et al.*, 2000). In our experiment, Tridemorph at a concentration of up to 200 mg/l did not affect the activity significantly. Fenpropidin and fenpropimorph at 200 mg/l and 100 mg/l, respectively were found to reduce cholesterol reduction activity by 28 % (Figure 5.7).

Plate counting, however, showed that bacterial growth was also inhibited by these inhibitors (Figure 5.8). Therefore, we could not confirm whether the depression of cholesterol reduction activity was due to the inhibition of cholesterol oxidase activity. Further increased of fenpropimorph to 2 g/l did not abolish cholesterol reduction activity (27 % remained). Cholesterol in this case might have been converted to coprostanol via the direct pathway of cholesterol reduction.

5.4 Concluding Remarks

Cholesterol reduction activity in *E. coprostanoligenes* was found to increase with increasing cholesterol concentration. V_{max} and K_m of cholesterol reduction activity in these bacteria were calculated to be 14 μM cholesterol reduced/h and 1 mM cholesterol, respectively. Cholesterol reducing enzyme(s) was shown to be secreted constitutively and intracellularly. Hence, the reaction site for cholesterol reduction was deduced to take place either in cytoplasm or bacterial membrane. However, attempts to isolate the enzyme(s) by disrupting *E. coprostanoligenes* cells were not successful.

The indirect pathway of cholesterol reduction was verified in *E. coprostanoligenes*. Based on this pathway, cholesterol oxidase is likely to exist in these
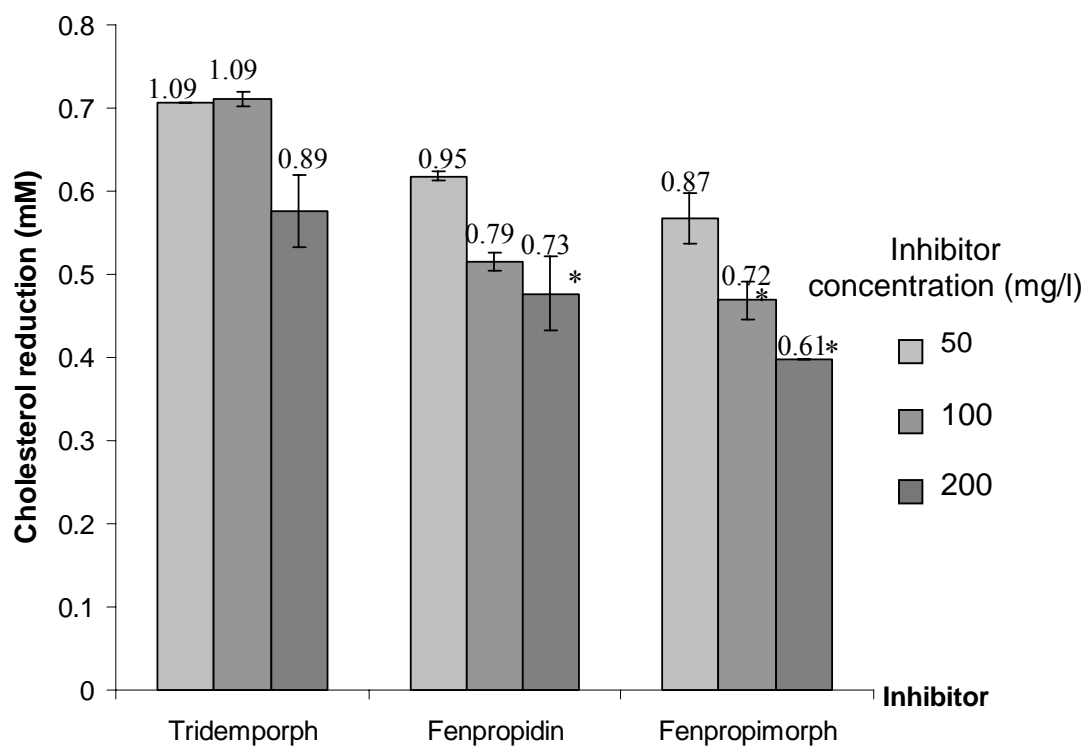
Fig. 5.7: Inhibition of putative cholesterol oxidase activity in *E. coprostanoligenes*. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate cholesterol reduction activity relative to that of control (taken as 1). Asterisk (above each bar chart) indicates significant different between treatment and control (one-way ANOVA. Tukey's Test, 95 % confidence level).
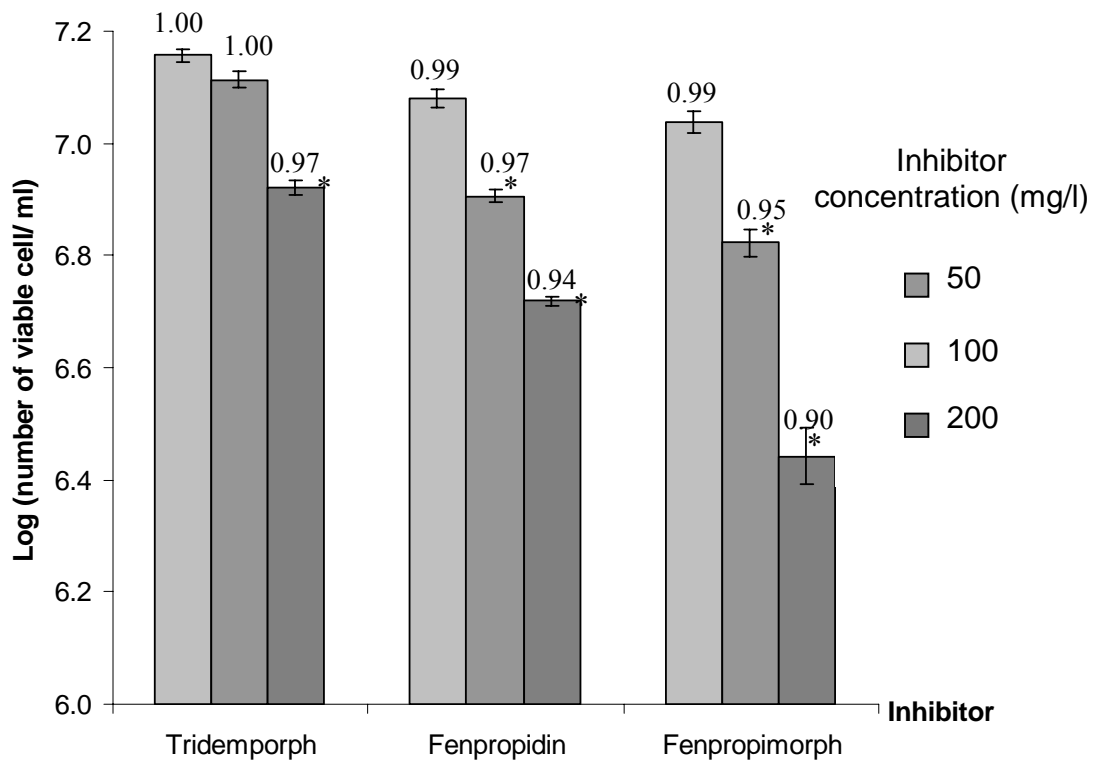
Fig. 5.8: Effect of tridemorph, fenpropidin and fenpropimorph on growth of *E. coprostanoligenes*. Vertical bars denote SE (n=3). Numbers (above each bar chart) indicate number of viable cell/ml relative to that of control (taken as 1). Asterisk (above each bar chart) indicates significant different between treatment and control (one-way ANOVA. Tukey's Test, 95 % confidence level).

bacteria. However, this postulation could not be confirmed by inhibitor study. Further studies, however, are necessary in order to characterize the putative enzyme(s). Molecular cloning of cholesterol oxidase gene in *E. coprostanoligenes* could be a possible approach.

6 CONCLUSION

This study is the first detailed investigation on the growth and cholesterol reduction activity of *E. coprostanoligenes*. The investigation began with the development of solid plate counting method to monitor growth of the bacteria, together with GC method to study its cholesterol reduction activity. Based on these methods, it was found that lecithin, $CaCl_2$ and pH of culture medium affected growth and cholesterol reduction activity of *E. coprostanoligenes* differently. The bacteria showed optimal growth at 1 g/l of lecithin, 0.5 g/l of $CaCl_2$ and at a wide pH range of 6 to 9. Maximum cholesterol reduction was found to take place at 5 g/l of lecithin, 1 g/l of $CaCl_2$ and at pH 7. Besides, growth of these cholesterol-reducing bacteria was not induced by cholesterol, thereby ruling out the role of cholesterol as an energy source. *E. coprostanoligenes* was also found to survive exposed to ambient air for at least 60 hours retaining its cholesterol-reducing ability at the same time.

The morphology of *E. coprostanoligenes* was re-affirmed with the aid of confocal and transmission electron microscopy. These bacteria were coccobacilloid cells of 0.5 to 0.7 μm in diameter and 1 to 1.2 μm in length.

The cholesterol reduction activity in *E. coprostanoligenes* was further explored so as to obtain more knowledge for its future application. A kinetics study of cholesterol reduction activity in these bacteria showed a V_{max} of 14 μM cholesterol reduced/h and K_m of 1 mM cholesterol. Secretion of the putative cholesterol reducing enzyme(s) appeared to be constitutive and intracellular. Attempts made to isolate these enzyme(s) by lysing the bacterial cells were not successful. On the other hand, cholesterol reduction pathway

in *E. coprostanoligenes* was elucidated in the sequence of cholesterol → 5-cholestern-3-one → 4-cholesten-3-one → coprostan-3-one → coprostanol. Postulation of cholesterol oxidase in the bacteria has yet to be confirmed.

Further investigations could be carried out to confirm the existence of cholesterol oxidase in *E. coprostanoligenes*. Molecular cloning of cholesterol oxidase gene could be a possible approach. It would also be useful to isolate and characterize the enzyme(s) catalyzing the conversion of 4-cholesten-3-one to coprostanol. Encapsulation of these enzymes for hypercholesterolemia treatment could then be made possible if the enzyme(s) could be successfully isolated.

REFERENCES

Allain, C.C., Poon, L.S., Chan, C.S., Richmond, W., Fu, P.C. 1974. Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, 20: 470-475.

Armstrong, M.J., Carey, M.C. 1982. The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities. *Journal of Lipid Research*, 23: 70-80.

Artaud-Wild, S.M., Conner, S.L., Sexton, G., Conner, W.E. 1993. Differences in coronary mortality can be explained by differences in cholesterol and saturated fat intakes in 49 countries but not in France and Finland. A paradox. *Circulation*, 88: 2771-2779.

Ascherio, A., Rimm, E.B., Giovannucci, E.L., Spiegelman, D., Stampfer, M., Willet, W.C. 1996. Dietary fat and risk of coronary heart disease in men: cohort follow up study in the United States. *British Medical Journal*, 313: 84-90.

Ashes, J.R., Gulati, S.K., Cook, L.J., Mills, S.C., Scott, T.W. 1978. Ruminal hydrogenation of cholesterol. *Journal of Lipid Research*, 19: 244-249.

Ast, M., Frishman, W.H. 1990. Bile acid sequestrants. *Journal of Clinical Pharmacology*, 30: 99-106.

Av-Gay, Y., Sobouti, R. 2000. Cholesterol is accumulated by mycobacteria but its degradation is limited to non-pathogenic fast-growing mycobacteria. *Canadian Journal of Microbiology*, 46: 826-831.

Bae, S.H., Lee, J.N., Fitzky, B.U., Seong, J., Paik, Y.K. 1999. Cholesterol biosynthesis from lanosterol. Molecular cloning, tissue distribution, expression, chromosomal localization, and regulation of rat 7-dehydrocholesterol reductase, a Smith-Lemli-Opitz syndrome-related protein. *Journal of Biological Chemistry*, 274: 14624-14631.

Beerens, H., Gavini, F., Neut, C. 2000. Effect of exposure to air on 84 strains of bifidobacteria. *Anaerobe*, 6: 65-67.

Benitez, M., Romero, C., Mas, R., Fernandez, L., Fernandez, J.C. 1997. A comparative study of policosanol versus pravastatin in patients with type II hypercholesterolemia. *Current Therapeutic Research*, 58: 859-867.

Bethell, P.H., Goad, L.J., Evershed, R.P. 1994. The study of molecular markers of human activities: the use of coprostanol in the soil as an indicator of human faecal material. *Journal of Archaeological Science*, 21: 619-632.

Bhattacharyya, A.K. 1986. Differences in uptake and esterification of saturated analogues of cholesterol by rat small intestine. *American Journal of Physiology*, 251: G495.

Björkhem, I., Gustafsson, J.A. 1971. Mechanism of microbial transformation of cholesterol into coprostanol. *European Journal of Biochemistry*, 21: 428-432.

Bosner, S.M., Lange, L.G., Stenson, W.F., Ostlund, R.E. 1999. Percent cholesterol absorption in normal women and men quantified with dual stable isotopic tracers and negative ion mass spectroscopy. *Journal of Lipid Research*, 40: 302-308.

Boststo, E., Ghiselli, G.C., Gallikienl, M., Galli, G., Sirtori, C.R. 1981. Effects of dietary soy protein on liver catabolism and plasma transport of cholesterol in hypercholesterolemic rats. *Journal of Steroid Biochemistry*, 14: 1201-1207.

Bottema, C.K., Parks, L.W. 1978. Delta14-sterol reductase in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*, 531: 301-307.

Brinkley, A.W., Gottesman, A.R., Mott, G.E. 1980. Growth of Cholesterol-reducing *Eubacterium* on cholesterol-brain agar. *Applied Environmental Microbiology*, 40: 1130-1132.

Brinkley, A.W., Gottesman, A.R., Mott, G.E. 1982. Isolation and characterization of new strains of cholesterol-reducing bacteria from baboons. *Applied Environmental Microbiology*, 43: 86-89.

Canetti, M.M., Moreira, M., Mas, R., Illnait, J., Fernandez, L., Fernandez, J.C. 1997. Effects of policosanol on primary hypercholesterolemia: a 3-year open-extension follow-up. *Current Therapeutic Research*, 58: 868-875.

Chan, K., Lam, M.H.W., Poon, K., Yeung, H., Chiu, T.K.T. 1998. Application of fecal stanols and sterols in tracing sewage pollution in coastal waters. *Water Research*, 32: 225-235.

Christian, U., Jacobsen, W., Floren, L.C. 1998. Metabolism and drug interaction of 3-Hydroxy-3-Methylglutaryl coenzyme A reductase inhibitors in transplant patients: are the statins mechanistically similar? *Pharmacology and Therapeutics*, 80: 1-34.

Clarke, R., Frost, C., Collins, R., Appleby, P., Peto, R. 1997. Dietary lipids and blood cholesterol: quantitative and meta-analysis of metabolic ward studies. *British Medical Journal*, 314: 112-117.

Connor, W.E., Lin, D.S. 1981. Absorption and transport of shellfish sterols in human subjects. *Gastroenterology*, 81: 276-284.

Crouse, J.R., Grundy, S.M. 1979. Effects of sucrose polyester on cholesterol metabolism in man. *Metabolism*, 28: 994-1000.

Crowther, J.S., Drasar, B.S., Goddard, P., Hill, M.J., Johnson, K. 1973. The effect of a chemically defined diet on the faecal floral and faecal steroid concentration. *Gut*, 14: 790-793.

Danijela, R.M., Vanja, R., Vesna, T., Marija, R., Gordana, R., Snežana, V., Ilona, E. 2003. Effect of soybean Leci-Vita product on serum lipids and fatty acid composition in patients with elevated serum cholesterol and triglyceride levels. *Nutrition Research*, 23: 465–477.

Dawber, T.R., Nickerson, R.J., Brand, F.N., Pool, J. 1982. Eggs, serum cholesterol, and coronary heart disease. *American Journal of Clinical Nutrition*, 36: 617-625.

de Leon, P.M., Iori, R., Barbolini, G., Pompei, G., Zaniol, P., Carulli, N. 1982. Influence of small-bowel transit time on dietary cholesterol absorption in human beings. *New England Journal of Medicine*, 307: 102-103.

de Macêdo Farias, L., de Carvalho, M.A.R., Houw, H., de Oliveira, A.A.P., Rodrigues, P.H., de Farias, F.F., Nicoli, J.R., da Costa, J.E. 1999. Atmospheric oxygen sensitivity of *Fusobacterium* strains. *Anaerobe*, 5: 157-159.

Dehal, S.S., Beitz, D.C., Young, J.W. 1988. Discovery of cholesterol reductase activity in plant leaves. *The Federation of American Societies for Experimental Biology Journal*, 2: A582.

Dehal, S.S., Freier, T.A., Young, J.W., Hartman, P.A., Beitz, D.C. 1991. A novel method to decrease the cholesterol content of foods. In: Haberstroh, C., Morris, C. (Editors), *Fat and cholesterol-reduced foods: technologies and strategies*, Portfolio Publishing Company, The Woodlands, Texas. pp: 202-220.

Dehal, S.S., Osslund, B.A., Young, J.W., Beitz, D.C. 1990a. Partial purification and characterization of cholesterol reductase from alfalfa leaves (*Medicago sativa*). *The Federation of American Societies for Experimental Biology Journal,* 4: A423.

Dehal, S.S., Young, J.W., Beitz, D.C. 1990b. Demonstration of cholesterol reductase in pea (*Pisum sativum*). *The Federation of American Societies for Experimental Biology Journal*, 4: A543.

Dietschy, J.M., Turley, S.D., Spady, D.K. 1993. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including human. *Journal Lipid Research*, 34: 1637-1659.

Domnas, A.J., Warner, S.A., Johnson, S.L. 1983. Reversed-phase thin layer chromatography of some sterols. *Lipids*, 18: 87-89.

Elkihel, L., Soustre, I., Karst, F., Letourneux, Y. 1994. Amino- and aminomethylcholesterol derivatives with fungicidal activity. *FEMS Microbiology Letters*, 120: 163-168.

Esrey, K.L., Joseph, L., Grover, S.A. 1996. Relationship between dietary intake and coronary heart disease mortality: lipid research clinics prevalence follow-up study. *Journal of Clinical Epidemiology*, 49: 211-216.

Eyssen, H.J., Parmentier, G.G., Compernolle, F.C. 1973. Biohydrogenation of sterols by *Eubacterium* ATCC21408-*Nova* Species. *European Journal of Biochemistry*, 36: 411-421.

Farias, F.F., Lima, F.L., Carvalho, M.A.R., Nicoli, J.R., Farias, L.M. 2001. Influence of isolation site, laboratory handling and growth stage on oxygen tolerance of *Fusobacterium* strains. *Anaerobe*, 7: 271-276.

Farnier, M., Salko, T., Isaacsohn, J.L., Troendle, A.J., Dejager, S., Gonasun, L. 2003. Effects of Baseline Level of Triglycerides on Changes in Lipid Levels from Combined Fluvastatin+ Fibrate (Bezafibrate, Fenofibrate, or Gemfibrozil). *American Journal of Cardiology*, 92: 794–797.

Feighner, S.D., Bokkenheuser, V.D., Winter, J., Hylemon, P.B. 1979. Characterization of a C_{21} neutral steroid hormone transforming enzyme, 21-dehydroxylase, in crude cell extracts of *Eubacterium lentum*. *Biochimica et Biophysica Acta*, 574: 154-163.

Flores-Díaz, M., Thelestam, M., Clark, G.C., Titball, R.W., Alape-Girón, A. 2004. effects of *Clostridium perfringens* phospholipase C in mammalian cells. *Anaerobe*, 10: 115-123.

Freier, T.A., Beitz, D.C., Li, L., Hartman, P.A. 1994. Characterization of *Eubacterium coprostanoligenes sp*. Nov., a cholesterol-reducing anaerobe. *International Journal of Systemic Bacteriology*, 44: 137-142.

Fromm, H. 1984. Gallstone dissolution and the cholesterol--bile acid--lipoprotein axis. Propitious effects of ursodeoxycholic acid. *Gastroenterology*, 87: 229-233.

Fukushima, R.S., Weimer, P.J., Kunz, D.A. 2002. Photocatalytic interaction of resazurin *N*-oxide with cysteine optimizes preparation of anaerobic culture media. *Anaerobe*, 8: 29-34.

Gaskell, S.J., Eglinton, G. 1975. Rapid hydrogenation of sterols in a contemporary lacustrine sediment. *Nature*, 254: 209-211.

Ginsberg, H.N., Karmally, W., Siddiqui, M., Holleran, S., Tall, A.R., Blaner, W.S., Ramakrishnan, R. 1995. Increases in dietary cholesterol are associated with modest increases in both LDL and HDL cholesterol in healthy young women. *Arteriosclerosis and Thrombosis*, 15: 169-178.

Ginsberg, H.N., Karmally, W., Siddiqui, M., Holleran, S., Tall, A.R., Rumsey, S.C., Deckelbaum, R.J., Blaner, W.S., Ramakrishnan, R. 1994. A dose-response study of the effects of dietary cholesterol on fasting and postprandial lipid an lipoprotein metabolism in healthy young men. *Arteriosclerosis and Thrombosis*, 14: 576-586.

Glass, T.L., Burley, C.Z. 1984. Biotransformation of 16-dehydroprogesterone by the intestinal anaerobic bacterium, *Eubacterium* sp. 144. *Journal of Steroid Biochemistry*, 21: 65-72.

Glatz, J.F., Turner, P.R., Katan, M.B., Stalenhoef, A.F., Lewis, B. 1993. Hypo- and hyperresponse of serum cholesterol level and low density lipoprotein production and degradation to dietary cholesterol in man. *Annals of New York Academy of Sciences*, 676: 163-179.

Goddard, P., Hill, M.J. 1974. The in vivo metabolism of cholesterol by gut bacteria in the rat and guinea-pig. *Journal of Steroid Biochemistry*, 5: 569-572.

Grunwald, C. 1975. Plant sterols. *Annual Review of Plant Physiology*, 26: 209-236.

Hassett, J.P., Lee, G.F. 1977. Sterols in natural water and sediment. *Water Research*, 11: 983-989.

Hefmann, E. 1984. Metabolism of cholesterol in plants. In: Nes, W.D., Fuller, G., Tsai, L.S. (Editors), *Isopentenoids in plants, biochemistry and function*, Mercel Dekker, New York. pp: 487-518.

Heftmann, E., Lieber, E.R., Bennett, R.D. 1967. Biosynthesis of tomatidine from cholesterol in *Lycopersicon pimpinellifolium*. *Phytochemistry*, 6: 225-229.

Hegsted, D.M., Ausman, L.M. 1988. Diet, alcohol and coronary heart disease in men. *Journal of Nutrition*, 118: 1184-1189.

Hesselink, P.G.M., Kerkenaar, A., Witholt, B. 1990. Inhibition of microbial cholesterol oxidases by dimethylmorpholines. *Journal of Steroid Biochemistry*, 35 (1): 107-113.

Holland, K.T., Knapp, J.S., Shoesmith, J.G. 1987. Types of anaerobic and microaerophilic bacteria. In: Holland, K.T. (Editor), *Anaerobic bacteria*, Chapman and Hall, New York. pp: 70-104.

Holmer, L., Pezhman, A., Worman, H.J. 1998. The human lamin B receptor/sterol reductase multigene family. *Genomics*: 54: 469-476.

Howard, B.V., Kritchevsky, D. 1997. Phytochemicals and cardiovascular disease. *Circulation*, 95: 2591.

Hu, F.B., Stampfer, M.J., Manson, J.E., Rimm, E., Coldiz, G.A., Rosner, B.A., Hennekens, C.H., Willett, W.C. 1997. Dietary fat intake and the risk of coronary heart disease in women. *New England Journal of Medicine*, 337: 1491-1499.

Hu, F.B., Stampfer, M.J., Rimm, E.B., Manson, J.E., Ascherio, A., Colditz, G.A., Rosner, B.A., Spiegelman, D., Speizer, F.E., Sacks, F.M., Hennekens, C.H., Willett, W.C. 1999. A prospective study of egg consumption and risk of cardiovascular disease in men and women. *Journal of American Medical Association*, 281: 1387-1394.

Hunninghake, D.B. 1990. Bile acid sequestrant therapy. *Journal of Drug Development,* 3: 205.

Hylemon P.B. and Harder J. 1999. Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems. *FEMS Microbiology Review*, 22: 475-488.

Ingraham, J.L. and Ingraham, C.A. 1995. The growth of microorganisms. In: Prentiss, H. (Editors), *Introduction to microbiology*, Wadsworth Publishing Company. pp: 191-213.

Istvan, E. 2003. Statin inhibition of HMG-CoA reductase: a 3-dimentional view. *Atherosclerosis Supplements*, 4: 3-8.

Jimenez, M.A., Scarino, M.L., Vignolini, F., Mengheri, E. 1990. Evidence that polyunsaturated lecithin induces a reduction in plasma cholesterol level and favorable changes in lipoprotein composition in hypercholesterolemic rats. *Journal of Nutrition*, 120: 659–667.

Kim, C.K., Jeon, K.I., Lim, D.M., Johng, T.N., Trzaskos, J.M., Gaylor, J.L., Paik, Y.K. 1995. Cholesterol biosynthesis from lanosterol: regulation and purification of rat hepatic sterol 14-reductase. *Biochimica et Biophysica Acta*, 1259: 39-48.

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S., Chua, N. 1998. The Arabidopsis *DIMINUTO/DWARF1* Gene Encodes a Protein Involved in Steroid Synthesis. *The Plant Cell*, 10: 1677-1690.

Knopp, R.H., Retzlaff. B.M., Walden, C.E., Dowdy, A.A., Tsunehara, C.H., Austin, M.A., Nguyen, T. 1997. A double-blind, randomized, controlled trial of the effects of two eggs per day in moderately hypercholesterolemic and combined hyperlipidemic subjects taught the NCEP step I diet. *Journal of American Collaborative Nutrition*, 16: 551-561.

Kromhout, D., Menotti, A., Bloembery, B., Aravanis, C., Blackburn, H., Buzina, R., Dontas, A.S., Fidanza, F., Giampaoli, S., Jansen, A. 1995. Dietary saturated and trans fatty acids and cholesterol and 25-year mortality from coronary heart disease: the Seven Countries Study. *Preventive Medicine*, 24: 308-315.

Kushi, L.H., Lew, R.A., Stare, F.J., Ellison, C.R., el Lozy, M., Bourke, G., Daly, L., Graham, I., Hickey, N., Mulcahy, R., Kevaney, J. 1985. Diet and 20-year mortality from coronary heart disease. *New England Journal of Medicine*, 312: 811-818.

Law, M.R., Wald, M.J, Thompson, S.G. 1994. By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischemic heart disease? *British Medical Journal*, 308: 367-372.

Li, L., Batt, S.M., Wannemuehler, M., Dispirito, A., Beitz, D.C. 1998. Effect of feeding of a cholesterol-reducing bacterium, of *Eubacterium coprostanoligenes*, to germ-free mice. *Laboratory Animal Science*, 48: 253-255.

Li, L., Baumann, C.A., Meling, D.D., Sell, J.L., Beitz, D.C. 1996a. Effect of orally administered of *Eubacterium coprostanoligenes* ATC 51222 on plasma cholesterol concentration in laying hens. *Poultry Science*, 75: 743-745.

Li, L., Buhman, K.K., Hartman, P.A., Beitz, D.C. 1995a. Hypocholesterolemic effect of *Eubacterium coprostanoligenes* ATC 51222 in rabbits. *Letters in Applied Microbiology*, 20: 137-140.

Li, L., Freier, T.A., Hartman, P.A. 1995b. A resting-cell assay for cholesterol-reductase activity in *Eubacterium coprostanoligenes* ATCC 51222. *Applied Microbiology and Biotechnology*, 43: 887-892.

Li, J., Nagpal, P., Vitart, V., McMorris, T.C., Chory, J. 1996b. A Role for Brassinosteroids in light-dependent development of *Arabidopsis*. *Science*, 272: 398-401.

Lin, D.S., Cornor, W.E. 1980. The long term effects of dietary cholesterol upon the plasma lipids, lipoproteins and cholesterol absorption, and the sterol balance in man: the demonstration of feedback inhibition of cholesterol biosynthesis and increased bile acid excretion. *Journal of Lipid Research*, 21: 1042-1052.

Lin, J.T., Palevitch, D., Heftmann, E. 1983. Reduction of androst-4-en-3,17-dione by growing cucumber plants. *Phytochemistry*, 22: 1149-1154.

Ling, W.H., Jones, P.J. 1995. Enhanced efficacy of sitostanol-containing versus sitostanol-free phytosterol mixtures in altering lipoprotein cholesterol levels and synthesis in rats. *Atherosclerosis*, 118: 319-331.

Low, M. 2000. Plant sterol and stanol margarines and health. *British Medical Journal*, 320: 861–864.

Lv, C., Tang, Y., Wang, L., Ji, W., Chen, Y., Yang, S., Wang, W. 2002. Bioconversion of yolk cholesterol by extracellular cholesterol oxidase from *Brevibacterium sp.*. *Food Chemistry*, 77: 457-463.

Macdonald, I.A., Bokkenheuser, V.D., Winter, J., Maclernon, A.M., Mosbac, E.H. 1983. Degradation of steroids in the human gut. *Journal of Lipid Research*, 24: 675-700.

MacLachlan, J., Wotherspoon, A.T.L., Ansell, R.O., Brooks, C.J.W. 2000. Cholesterol oxidase: sources, physical properties and analytical applications. *Journal of Steroid Biochemistry and Molecular Biology*, 72: 169-195.

Mahley, R.W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, 240: 622-630.

Manchenko, G.P. 1994. General principles of enzyme detection on electrophoretic gels. In: Manchenko, G.P. (Editor), *Handbook of detection of enzymes on electrophoretic gels*, CRC Press, Florida. pp: 3-13.

Mann, J.I., Appleby, P.N., Key, T.J., Thoorogood, M. 1997. Dietary determinants of ischaemic heart disease in health conscious individuals. *Heart*, 8: 450-455.

Maranhao, R.C., Quintao, E.C.R. 1983. Long term steroid metabolism balance studies in subjects on cholesterol-free and cholesterol-rich diets: comparison between normal and hypercholesterolemic individuals. *Journal of Lipid Research*, 24: 167-173.

Marriott, P.J., Jayasinghe, L.Y., Carpenter, P.D., Nichols, P.D., 1998. application of pentafluorophenyldimethylsilyl derivatization for gas chromatography- electron-capture detection of supercritically extracted sterols. *Journal of Chromatography A*, 809: 109-120.

Marsheck, W.J., Kraychy, S., Muir, R.D. 1972. Microbial degradation of sterols. *Applied Microbiology*, 23: 72-77.

März, W., Scharnagl, H., Winkler, K., Gierens, H., Nauck, M., Hoffmann, M., Klima, B., Schliack, M., Löser, R. Lang, G., Groβ, W., Wieland H. 1997. Lifibrol (K12.148): First member of a new class of hypolipidemic drugs? *Atherosclerosis,* 130 (Supp): S6.

McMurry, M.P., Cornor, W.E., Lin, D.S., Cerqueira, M.T., Cornor, S.L. 1985. The absorption of cholesterol and the sterol balance in the Tarahumara Indians of Mexico fed cholesterol-free and high cholesterol diet. *American Journal of Clinical Nutrition*, 41: 1289-1298.

McNamara, D.J. 2000. Dietary cholesterol and atherosclerosis, *Biochimica et Biophysica Acta*, 1529: 310-320.

McNamara, D.J., Davidson, N.O., Samuel, P., Ahrens, E.H. 1980. Cholesterol absorption in man: effect of administration of clofibrate and/or cholestyramine. *Journal of Lipid Research*, 21: 1058-1064.

Mesa, A.R., Mas, R., Nao, M., Hernandez, C., Rodeiro, I., Game, R., Garcia, M., Capote, A., Aleman, C.L. 1994. Toxicity of policosanol in beagle dogs: one-year study. *Toxicology Letters*, 73: 81-90.

Miller, K.W., Wood, F.E., Stuart, S.B., Alden, C.L. 1991. 20-Month olestra feeding study in dogs. *Food and Chemical Toxicology*, 29: 427-435.

Moebius, F.F., Fitzky, B.U., Lee, J.N., Paik, Y.K., Glossmann, H. 1998. Molecular cloning and expression of the human delta7-sterol reductase. *Proceedings of the National Academy of Sciences of the United States of America,* 95: 1899-1902.

Mott, G.E., Brinkley, A.W. 1979. Plasmenylethanolamine: growth factor for cholesterol-reducing Eubacterium. *Journal of Bacteriology*, 139: 755-760.

Najib, J. 2002. Fenofibrate in the treatment of dyslipidemia: A review of the data as they relate to the new suprabioavailable tablet formulation. *Clinical Therapeutic*, 24: 2022-2050.

Neil, H.A.W. and Huxley, R.R. 2002. Efficacy and therapeutic potential of plant sterols. *Atherosclerosis Supplements*, 3: 11-15.

Nestel, P.J., Schreibman, P.H., Ahrens, E.H. Jr. 1973. Cholesterol metabolism in human obesity. *Journal of Clinical Investigation*, 52: 2389-97.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, A., Yoshida, S., Li, J., Chory, J. 1999. Arabidopsis det2 is defective in the conversion of (24 R)-24-methylcholest-4-en-3-one to (24 R)-24-methyl-5a-cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiology*, 120: 833-839.

Oda, S., Sugai, T., Ohta, H. 2001. Synthesis of methyl ursodeoxycholate via microbial reduction of methyl 7-ketolithocholate with *Eubacterium aerofaciens* JCM 7790 grown on two kinds of carbon and hydride sources, glucose and mannitol. *Journal of Bioscience and Bioengineering*, 91: 178-183.

Packard, C.J. and Shepherd, J. 1982. The hepatobiliary axis and lipoprotein metabolism: effects of bile acid sequestrants and ileal bypass surgery. *Journal of Lipid Research*, 23: 1081-1098.

Pedersen, T.R., 1994. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*, 334: 1383-1389.

Pierce, H.D. Jr., Pierce, A.M., Srinivasan, R., Unrau, A.M., Oehlschlager, A.C. 1978. Azasterol inhibitors in yeast. Inhibition of the 24-methylene sterol delta24(28)-reductase and delta24-sterol methyltransferase of Saccharomyces cerevisiae by 23-azacholesterol. *Biochimica et Biophysica Acta*, 529: 429-437.

Price, N.C., Stevens, L. 1989. An introduction to enzyme kinetics. In: Price, N.C., Stevens, L. (Editors), *Fundamentals of Enzymology*. Oxford University Press, New York. pp: 136-180.

Prince, D.M., Welschenbach, M.A. 1998. Olestra: a new food addictive. *Journal of the American Dietetic Association*, 98: 565-69.

Quintao, E., Grundy, S.M., Ahrens, E.H. Jr. 1971. Effects of dietary cholesterol on the regulation of total body cholesterol in man. *Journal of Lipid Research*, 12: 233-247.

Ratledge, C. 1994. Biodegradation of oils, fats and fatty acids. In: Ratledge, C. (Editor), *Biochemistry of microbial degradation*, Kluwer Academic Publishers, Boston. pp: 89-128.

Ren, D., Li, L., Schwabacher, A.W., Young, J.W., Beitz, D.C. 1996. Mechanism of cholesterol reduction to coprostanol by *Eubacterium coprostanoligenes* ATCC 51222. *Steroids*, 61: 33-40.

Richmond, W. 1973. Preparation and properties of a cholesterol oxidase from *Nocardia sp.* and its application to the enzymatic assay of total cholesterol in serum. *Clinical Chemistry*, 19: 1350-1356.

Rocha, E.R., Herren, C.D., Smalley, D.J., Smith, C.J. 2003. The complex oxidative stress response of *Bacteroides fragilis*: the role of OxyR in control of gene expression. *Anaerobe*, 9: 165-173.

Ros, E. 2000. Intestinal absorption of triglyceride and cholesterol. Dietary and pharmacological inhibition to reduce cardiovascular risk. *Atherosclerosis*, 151: 357-379.

Rosenfeld, R.S., Hellman, L., Gallagher, T.F. 1956. The transformation of cholesterol-3*d* to coprostanol-*d*. Location of deuterium in coprostanol. *Journal of Biological Chemistry*, 222: 321-323.

Rosenfeld, R.S., Lebeau, M.C., Shulman, S., Seltzer, J. 1961. Analysis of fecal sterols by gas chromatography. *Journal of Chromatography*, 7: 293-296.

Sacks, F.M., Pfefer, M.A., Moye, L.A., Rouleau, J.L., Rutherford, J.D., Cole, T.G., Brown, L., Warnica, J.W., Arnold, J.M.O., Wun, C.C., Davis, B.R., Braunwald, E. 1996. The effect of pravastatin on coronary events after myocardial infraction in patients with average cholesterol levels. *New England Journal of Medicine*, 335: 1001-1009.

Sadzikowski, M.R., Sperry, J.F., Wilkins, T.D. 1977. Cholesterol-reducing bacterium from human feces. *Applied Environmental Microbiology*, 34: 355-362.

Salen, G., Ahrens, E.H. Jr., Grundy, S.M. 1970. Metabolism of β-sitosterol in man. *Journal of Clinical Investigation*, 49: 952-967.

Samuel, P., McNamara, D.J., Ahren, E.H. Jr., Crouse, J.R., Parker, T. 1982. Further validation of the plasma isotope ratio method for the measurement of cholesterol absorption in man. *Journal of Lipid Research*, 23: 480-9.

Schoenheimer, R., Rittenberg, D., Graff, M. 1935. Deuterium as an indicator in the study of intermediary metabolism. IV The mechanism of coprosterol formation. *Journal of Biological Chemistry*, 111: 183-192.

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J., Jurgens, G. 2000. *Genes and Development*: 14: 1471-1484.

Sedaghat, A., Samuel, P., Crouse, J.R., Ahrens, E.H. 1975. Effects of neomycin on absorption, synthesis and/or flux of cholesterol in man. *Journal of Clinical Investigation*, 55: 12-21.

Sewell, P.A., Clarke, B. 1991. Qualitative and quantitative analysis by chromatography. In: *Chromatographic Separations*. In: Kealey, D. (Editor), John Wiley & Sons, Singapore. pp: 147-191.

Shepherd, J., Cobbe, S.M., Ford, I., Isles, C.G., Lorimer, A.R., Macfarlane, P.W., McKillop, J.H., Packard, C.J. 1995. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *New England Journal of Medicine,* 333: 1301-1307.

Silve, S., Dupuy, P.H., Ferrara P., Loison, G. 1998. Human lamin B receptor exhibits sterol C14-reductase activity in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*, 1392: 233-244.

Smith, S. 1995. Cloning and sequence analysis of an ERG24 homolog from Schizosaccharomyces pombe. *Gene*: 155: 139-140.

Snog-kjaer, A., Prange, I., Dam, H. 1956. Conversion of cholesterol into coprostanol by bacteria. *Journal of General Microbiology*, 14: 256-260.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J.C. 1998. Mechanism of Action of Fibrates on Lipid and Lipoprotein Metabolism. *Circulation,* 98: 2088-2093.

Stanier, R.Y., Ingraham, J.L., Wheelis, M.L., Painter P.R. 1986. Microbial Growth. In: Stanier, R.Y., Ingraham, J.L., Wheelis, M.L., Painter P.R. (Editors), *The Microbial World*, Prentice-Hall, New Jersey. pp: 183-195

Stanton, T.B., Sellwood, R. 1999. Cloning and characterization of a gene encoding NADH oxidase, a major mechanism for oxygen metabolism by the anaerobic spirochete, *Brachyspira* (*Serpulina*) *hyodysenteriae*. *Anaerobe*, 5: 539-546.

Sundin, K.A., Leeming, R.L., Stenström, T.A.B. 1999. Degradation of faecal sterols in urine for assessment of faecal cross-contamination in source-separated human urine and urine storage tank sediment. *Water Research*, 33: 1975-1980.

Tan, B.Y. 2000. Highlights of the 1998 National Health Survey. *Statistics Singapore Newsletter*. 3-8.

Tan, L., Clemence, M., Gass, J. 1970. Gas chromatography analysis of fecal pollution sterols on a single combined packed column. *Journal of Chromatography*, 53: 209-215.

Taton, M., Rahier, A. 1991. Identification of delta 5,7-sterol-delta 7-reductase in higher plant microsomes. *Biochemical and Biophysical Research Communications*, 181: 465-473.

Tell, G.S., Evans, G.W., Folsom, A.R., Shimakawa, T., Carpenter, M.A., Heiss, G. 1994. Dietary fat intake and carotid artery wall thickness: the Atherosclerosis Risk in Communities (ARIC) Study. *American Journal of Epidemiology*, 139: 979-989.

Thurnhofer, H., Schnabel, J., Betz, M., Lipka, G., Pidgeon, C., Hauser, H. 1991. Cholesterol transfer protein located in the intestinal brush border membrane. Partial purification and characterization. *Biochimica et Biophysica Acta*, 1064: 275-286.

Trinh, S., Briolat, V., Reysset, G. 2000. Growth response of *clostridium perfringens* to oxidative stress. *Anaerobe*, 6: 233-240.

Vahouny, G.V., Tombes, R., Cassidy, M.M., Kritchevsky, D., Gallo, L.L. 1980. Dietary fibers. V. Binding of the bile salts, phospholipids and cholesterol from mixed micelles by bile acid sequestrants and dietary fibers. *Lipids*, 15: 1012-1018.

Venkatramesh, M., Karunanandaa, B., Sun, B., Gunter, C.A., Boddupalli, S., Kishore, G.M. 2003. Expression of a *Streptomyces* 3-hydrosteroid oxidase gene in oilseed for converting phytosterols to phytostanols. *Phytochemistry*, 62: 39-46.

Wassif, C.A., Maslen, C., Kachilele-Linjewile, S., Lin, D., Linck, L.M., Connor, W.E., Steiner, R.D., Porter, F.D. 1998. Mutations in the human sterol delta7-reductase gene at 11q12-13 cause Smith-Lemli-Opitz syndrome. *American Journal of Human Genetics*, 63: 55-62.

Waterham, H.R., Koster, J., Romeijn, G.J., Hennekam, R.C., Vreken, P., Andersson, H.C., FitzPatrick, D.R., Kelley, R.I., Wanders, R.J. 2001. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *American Journal of Human Genetics*, 69: 685-694.

Waterham, H.R., Wijburg, F.A., Hennekam, R.C., Vreken, P., Poll-The, B.T., Dorland, L., Duran, M., Jira, PE., Smeitink, J.A., Wevers, R.A., Wanders, R.J. 1998. Smith-Lemli-Opitz syndrome is caused by mutations in the 7-dehydrocholesterol reductase gene. *American Journal of Human Genetics*, 63: 329-338.

Watt, S.M. and Simmonds, W.J. 1984. Effect of taurin-conjugated bile acids on mucosal uptake and lymphatic absorption of cholesterol in rat. *Journal of Lipid Research*, 25: 448-455.

Wiggers, K.D., Jacobson, N.L., Getty, R., Richard, M. 1973. Mode of cholesterol ingestion and atherosclerosis in the young bovine. *Atherosclerosis*, 17:283-288.

Wiłkomirski, B., Goad, L.J. 1983. The conversion of (24S)-24-ethylcholesta-5,22,25-triene-3β-ol into poriferasterol, both *in vivo* and with a cell-free homogenate of the alga *Trebouxia* sp.. *Phytochemistry*, 22: 929-932.

Wilson, M.D., Rudel, L.L. 1994. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *Journal of Lipid Research*, 35: 943-955.

Wilson, T.A., Meservey, C.M., Nicolosi, R.J. 1998. Soy lecithin reduces plasma lipoprotein cholesterol and early atherogenesis in hypercholesterolemic monkeys and hamsters: beyond linoleate. *Atherosclerosis*, 140: 147–153.

Winter, J., O'Rourke-Locascio, S., Bokkenheuser, V.D., Mosbach, E.H., Cohen, B.I. 1984. Reduction of 17-keto steroids by anaerobic microorganisms isolated from human fecal floral. *Biochimica et Biophysica Acta*, 795: 208-211.

Yamaga, N., Ogura, Y., Islam, M.A., Matsuyama, H., Yamawaki, M., Kai, M., Yamada, K. 2002. Utility of 19-hydroxycholesterol as an internal standard compound for the quantitative determination of sterols using capillary gas chromatograph. *Yonago Acta medica*. 45: 27-33.

Yang, J.B., Beitz, D.C. 1992. A fluorometric assay for cholesterol reductase activity. *Analytical Biochemistry*, 206: 246-250.