THE SUBMERGED-CULTURE FERMENTATION OF *CORIOLUS VERSICOLOR* IN MILK PERMEATE BASED MEDIA AND THE CHARACTERISATION OF ITS BIOACTIVE POLYSACCHARIDES

JIAN CUI

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ABSTRACT

The protein-bound polysaccharides of *Coriolus versicolor* (CPS) are known to improve human immune functions against cancers and various infectious diseases. With the increasing concern for longer lifespan and better quality of life, CPS may find new application in the food industry as a novel nutriceutical. The commercial therapeutic CPS are the intracellular polysaccharides of *Coriolus versicolor* (IPS) produced either through the submerged-culture fermentation or from the extract of the fruit body of *Coriolus versicolor*. The objective of this study was to characterise the IPS and EPS (the extracellular polysaccharides) produced by *C. versicolor* through submerged-culture fermentation that was based on cost-effective milk permeate (MP) as a medium component. Ten *C. versicolor* species were screened and the strain Wr-74 was found to be the most comparable to the patented ATCC-20545 strain in terms of EPS/IPS production, morphology of the fruit bodies and weight-average molecular weight distributions of EPS/IPS. In addition, *in vitro* physiological activities of EPS, IPS and mushroom extracts on cytokine production were investigated using murine splenocytes.

The growth medium was optimised using MP as a base component, indicating the 50%MP-YEG medium was comparable to YEG medium in terms of EPS/IPS levels. When the lactose in MP was hydrolysed (HMP) by 0.04 % (v/v) Maxilact® lactase, a 40-60 % increase in EPS/IPS levels was observed in the 50%HMP-YEG medium compared to 50%MP-YEG medium. Approximately twice the amounts of EPS (3.2 mg/mL) and IPS (0.3 mg/mL) were obtained in the 100%HMP-YEG medium compared to the 50%HMP-YEG medium. Different nitrogen sources were screened and yeast extract supplied by DIFCO was found to be the most suitable for EPS/IPS production. Though glucose was the main carbon source consumed, excess amount of glucose would retard EPS/IPS production. The optimum carbon to nitrogen ratio (C/N) ratio was approximately 70.

Submerged-culture fermentation was conducted using a modified *impeller-assistant airlift fermenter*. This fermenter was equipped with an inner draught tube and helical impeller to produce an efficient circulation of broth and dissolved oxygen.

Wr-74 produced higher levels of EPS than ATCC-20545 in the fermenter. An approximately 74 % increase in EPS level was obtained in the 50%HMP-YEG medium compared to 50%MP-YEG medium. The EPS levels were close to those obtained in the shake flasks (~2.5 mg/mL). The effects of antifoaming agents and salt addition were also investigated in the fermenter. Agitation with the helical impeller appeared to improve the EPS production, but resulted in lower biomass levels.

Studies on the EPS/IPS compositions using HPLC indicated that the EPS from both strains only contained glucose. However, the IPS probably contained galactose, mannose or/and xylose in addition to glucose. Ratios of polysaccharide to protein were approximately 15:1 and 25:1 for EPS from ATCC-20545 and Wr-74, respectively. The EPS from both strains contained about 80 % of pure polysaccharides. Approximately 82 % of IPS and 32 % of EPS from both strains could be obtained in the water-soluble form. Results of the amino acid analysis showed that the IPS from both strains contained higher levels of amino acids (16 %, w/w) than the EPS (2 % to 4 %, w/w). The EPS and IPS from both strains had very similar molecular weight distributions. The weight-average molecular weights (Mw) of IPS from both strains were approximately ten times higher (10^5 Da) than EPS (10^4 Da) in the major elution range of 9.8 to 10.3 mL. The largest Mw fractions of EPS and IPS from both strains were about 10^6 Da in the elution range of 8.2 to 8.7 mL.

All samples of EPS, IPS and mushroom extracts stimulated cytokine production from murine splenocytes. Generally, lower polysaccharide levels (0.1 to 2.0 μg/mL) induced higher levels of cytokines. The mushroom extract from ATCC-20545 induced higher levels of IL-12 and IFN-γ than that from Wr-74. The IPS from the mycelia of Wr-74 induced higher IL-12 production, but lower levels of IFN-γ than that from ATCC-20545. The EPS from both strains were comparable in terms of IL-12 and IFN-γ production.

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CHAPTER I INTRODUCTION

The biopolymers produced by *Coriolus versicolor* are protein-bound polysaccharides (CPS), known to function as anticancer agents, immunopotentiators and biological response modifiers (Cho et al., 1988; Ueno et al., 1989; Sakagami et al., 1991; Wang et al., 1996; Yang et al., 1992a; Fisher and Yang, 2002). Belonging to the higher class of fungi-*Basidiomycetes*, *Coriolus versicolor* is a medicinal mushroom known for over a thousand years in the East. Its fruit bodies grow on tree trunks and stumps all year round in the temperate zones throughout the world. Of more than 200 medicinal mushrooms, the polysaccharides from *C. versicolor* are commercially the most established (Johl et al., 1996). In China and Japan, *C. versicolor* mushrooms were dried and the extracts were obtained either by decoction or by grinding the fruit-body into powder to be used as a herbal tea. During the 1970s and the 1980s, Japanese and Chinese scientists discovered the biological activities of *C. versicolor* extracts and began an extensive controlled clinical research on *C. versicolor* polysaccharides.

PSK (polysaccharides-Krestin) were the first commercial CPS preparation of *C. versicolor*, produced by Kureha Chemical Industries Co. Ltd in Japan through batch submerged-culture fermentation. Subsequently, another similar product called PSP was isolated by Chinese scientists. Both PSK and PSP were extracted from *C. versicolor* mycelia. PSK were produced from CM-101 and CM-103 (ATCC-20545, Hotta et al., 1981) and PSP was produced from Cov-1 strains of *C. versicolor* (Yang et al., 1992). All the strains used for the commercial production of CPS were patented.

Though the products have been marketed for years, their potential physiological activities and applications in the clinical area continued to interest scientists worldwide. In New Zealand, other medicinal mushroom polysaccharides are currently available on the market. However, they do not include *C. versicolor* polysaccharides. As a very promising health-care product, CPS would have broad applications in the food industry as a nutriceutical.

The objectives of the project are as follows:

- To select a local strain of *C. versicolor* that is comparable to the patented strain, ATCC-20545 through a screening process;
- To use milk permeate, a waste stream of the dairy industry, as a low cost base medium for CPS production;
- To optimize CPS production through the submerged-culture fermentation using a suitable bioreactor;
- To characterize the CPS isolated from the broth and biomass in terms of molecular weights and chemical compositions;
- To investigate the physiological activities of CPS based on cytokine production and the effect on cancer cell proliferation.

CHAPTER II LITERATURE REVIEW

2-1 Background of CPS development

Medicinal mushrooms have been commonly used as herbal medicine for prevention and treatment of various diseases for hundreds of years. *Coriolus versicolor* is one such medicinal mushroom. In Japan it is known as *Kawaratake* and in China it is called *Yun Zhi*. This mushroom has long been treasured in the East for its medicinal values. According to the Materia Medica of the Ming dynasty edition volume 28, "The black and green *Yun zhi* are beneficial to one's spirit and vital energy, and strengthen one's tendon and bone. If *Yun zhi* is taken for a long time, it will make one vigorous and live long."

History of polysaccharides isolated from fungi, as nonspecific immunostimulants, dates back to the 1960's when Japanese scientists began screen *Polyporaceae* and other species for their antitumor activities. Among the various extracts from the class *Basidomycetes*, the polysaccharides from *Coriolus versicolor* were found to be the most outstanding due to its high antitumor activity *in vivo* with minimal toxicity. The polysaccharides were stable during serial cultivation and processing (Fisher and Yang, 2002). It was then named as Polysaccharide Krestin (PSK) and produced through industrial submerged-culture fermentation by Kureha Chemical Industries Co Ltd in the 1970s. After extensive clinical trials, PSK was approved for use in Japan in 1977 by the government's Health and Welfare Ministry (equivalent to America FDA) as a chemoimmunotherapy agent in the treatment of cancer (Yang *et al.,* 1992). By 1985, it ranked nineteenth on the list of the world's most commercially successful drugs. Annual sales of PSK were worth US$ 255 million in 1985 and US$ 357 million in 1987 (Yang *et al.,* 1992). About 10-years later after PSK, another *C. versicolor* product known as polysaccharidopeptides (PSP) was developed in China in 1983. PSK, PSP and numerous other polysaccharide extracts of *C. versicolor* mushroom are currently sold in the form of capsules, ground biomass tablets, syrups, beverages and teas. These products are claimed to possess remarkable antitumor effects via immunomodulation. Over the last 2-3 decades, scientific and medical studies carried out in Japan, China, Korea and more recently in the US, have increasingly demonstrated the potent and unique health enhancing properties of the polysaccharides. In spite of the long history of the recognition for its efficacy, the application of CPS beyond the area has not been fully explored (Johl *et al.,* 1996).

2-2 Physiological activities of CPS

2-2-1 Efficacy of CPS on human immune systems

The human immune system consists of a highly complex constellation of immune cells and molecules, which circulate through the bloodstream and lymphatic system. Their functions are to patrol the body and destroy or inactivate any foreign invaders that may cause infections and abnormal cells that may cause cancer. The three kinds of immune cells, T and B-lymphocytes and macrophages, cooperate and interact in a highly complex way to produce an immune response. CPS with large molecular weights (10,000 to 100,000 Da) can enhance the immune function of a normal body by acting as an antigen to stimulate or activate these immune cells to produce molecules, such as antibodies, interlukin-2 (IL-2), IL-6, interferons (IFN) and immunoglobulin-G (gl.-G). Furthermore, CPS helps to counter immunosuppressive effects of chemotherapy, radiotherapy and blood transfusion; antagonize immunosuppression induced by tumors. Therefore, CPS has become recognized as an immuno-potentiator and biological response modifier that serves as an adjunct to conventional therapy (Fisher and Yang, 2002).

Fung and co-workers (1996) reported that PSK stimulates the synthesis of various cytokines including tumor necrosis factor-α (TNF-α) IL-1, IL-1R, IL-2, IL-4, IL-6, IFN- α and IFN-γ. The use of anti-cytokine antibodies did not abrogate PSK-induced enhancement of natural killer cell (NK cell) activity, which suggests that the enhanced tumoricidal cytotoxicity was caused by more than just manipulation of cytokine production, secretion and binding (Fisher and Yang, 2002). Both intracellular CPS (IPS) and extracellular CPS (EPS) induced production of human serum interferon (Chen *et al.,* 1986) and potentiate the immune system (Wang *et al.,* 1996). Compared with the intracellular polymers, the interferon inducing activity of the extracellular polymers appeared to be more potent (Chen *et al.,* 1986).

In vitro studies reveal that PSP acts selectively on HL-60 leukemic cells, arresting the cell in the G-phase of the cell cycle and inducing apoptosis (Hsieh *et al.,* 2002). However, lymphocytes are not affected by PSP (Hsieh *et al.,* 2002).

2-2-2 Application of CPS as an anticancer reagent

C. versicolor has been widely reported to inhibit the proliferation of various cancers by inducing production of superoxide dismutase, glutathione peroxidase and general immune enhancement (Cho *et al.,* 1988; Sakagami *et al.,* 1991; Yang *et al.,* 1992; Dong *et al.,* 1996; Mao *et al.,* 1996, 1998, Johl *et al.,* 1996). Oral administration of PSK or PSP has successfully controlled various carcinomas in experimental animals and humans (Ng, 1998). Yang and co-workers (1992) reported that PSP is active against Ehrlich ascites carcinoma, P388 leukemia, and sarcoma 180. Although CPS suppresses proliferation of some human cancer cell lines (Yang *et al.,* 1992; Ng, 1998), not all the cancer cells are affected by CPS (Wang *et al.,* 1996; Dong *et al.,* 1997).

Dong *et al.* (1996) observed that CPS dose-dependently inhibited the proliferation of a human hepatoma cell line (HEPG2). Samples with the concentrations of 20 to 2000 μg/ml were tested and the concentrations of less than 1000 μg/ml were found able to directly inhibit the proliferation of a human hepatoma cell line (HEPG2) without significant effects on the corresponding normal human fetal liver cell line (QZG). In nude mice, the progression of sarcoma 180 was measurably reduced by oral administration of CPS. Data obtained from *in vitro* and *in vivo* studies suggest that PSP can slow progression of murine H238 tumors (Mao *et al.,* 1996). PSP has been observed to enhance the transcription of tumor necrosis factor gene in mouse peritoneal macrophages, indicating an immuno-modulatory effect of PSP (Liu *et al.,* 1993).

Inhibition of DNA synthesis in MCF-7 (a human breast cancer cell line) by PSK appeared to be dose dependent. A PSK dose of 200 μg/mL caused a 50% inhibition of DNA synthesis *in vitro* (Aoyagi *et al.,* 1997). In the case of intra-peritoneal administration and oral administration, a dose of 10 mg/kg and 1000 mg/kg per administration was given to mice transplanted with Sarcoma-180 solid tumour cells once a day for 20 days, respectively. The tumors were enucleated on the 25th day after transplantation (Ueno *et al.,* 1989). In addition, CPS has also been reported to be effective in complementary treatment of gastric and other intestinal cancers (Nakazato *et al.,* 1994).

Concurrent use of CPS with monoclonal antibody IgG2a could enhance the *in vitro* antibody-independent macrophage-mediated cytotoxicity as well as the *in vivo* antitumor activity. This suggests that the combined use of a monoclonal antibody and CPS, with each having different modes of action, may be useful in the treatment of cancer (Kanoh *et al.,* 1994).

Therefore, CPS have long been used in the clinic for the cancer patients who received chemotherapy or radiation. Clinical research has constantly demonstrated the ability of CPS to double and even triple survival rates for these patients (JHS Natural Products, 2001). The anticancer activity of CPS are closely associated with its outstanding immuno-enhancing properties, in which the CPS-activated immune cells and cytokines inhibit the growth of various cancers and even kill the cancer cells (Chen *et al.,* 1986; Sakagami *et al.,* 1991; Yang *et al.,* 1992; Liu *et al.,* 1993; Wang *et al.,* 1996; Qian *et al.,* 1997; Ooi and Liu, 1999; Chu *et al.,* 2002).

2-2-3 Other physiological activities of CPS

In addition to the anticancer activity, reports have claimed that CPS can improve the appetite and liver function; calm the central nervous system and enhance pain threshold. In addition, CPS can remedy intestinal disorders and are beneficial in preventing opportunistic microbial infections that suppress the immune response (Yang *et al.,* 1998).

Furthermore, CPS may benefit general health by inducing enzymes that mop up free radicals and mitigate oxidative damage (Chu *et al.,* 2002). The enzyme superoxide dismutase (SOD) counters the tissue damaging effects of free radials. Intraperitioneal administration of CPS increased the SOD activity in lymphocytes and thymus of normal mice. Similar enhancements in SOD activity and suppression of tumors were observed in tumor-bearing mice administered with CPS (Wei *et al.,* 1996). Activity of SOD on cancer cell lines was enhanced by the presence of CPS *in vitro*, leading to the suppression of cancer cell growth (Kobayashi *et al.,* 1994a, b). Pang *et al.* (2000) reported that glutathione peroxidases play an important role in the defense against oxidative injury. Its production in mouse peritoneal macrophages can be induced by PSK. This effect was ascribed to transcriptional induction of expression of mRNA (Pang *et al.,* 2000). In another study, treatment of mouse peritoneal macrophages by CPS not only enhanced glutathione peroxidase activity of the cells, but also prevented inhibition of respiratory burst by tert-butylhydroxide (Jun *et al.,* 1993).

The *in vitro* anticancer activity of cisplatin in chemotherapy was augmented by the presence of CPS (Kobayashi *et al.,* 1994c). In the presence of CPS, the cytotoxicity of cisplatin toward healthy cells was reduced (Kobayashi *et al.,* 1994c).

Hepatic lesions and mortality induced by intravenous injection of influenza virus to mice were reduced by oral or peritoneal administration of intracellular CPS to the animals (Chen *et al.,* 1986).

The inhibitory effect of CPS on HIV-1 reverse transcriptase and α-glucosidase that is associated with viral glycosylation was enhanced after chemical modification with chlorosulfonic acid (Wang and Ng, 2001). CPS has also shown to inhibit cytopathic effects of the HIV that infects cells of the CD_4 positive human T-cell line by inhibiting the binding of HIV to the cells (Hirose *et al.,* 1987). The high water solubility, heat-stability and low cytotoxicity of CPS, make it a potential compound for further studies on its possible use as an anti-viral agent (Collins and Ng, 1997).

In the experiments with fruit flies (*Drosophila melanogaster*), intake of CPS increased the frequency of mating and the lifespan of flies (Li *et al.,* 1993). Compared to controls, administration of CPS enhanced recovery of mice following gamma-irradiation-induced spleen damage (Lin *et al.,* 1996). Analgesic (pain-releasing) activity of PSP has also been reported (Ng and Chan, 1997; Gong *et al.,* 1998).

2-2-4 Safety of CPS

The CPS was a so-called ‘green medicine’ (naturally derived medicine) in the clinical application. In animal studies, PSK is effective taken orally, intravenously or intraperitoneally (Yang *et al.,* 1992). In human therapy, CPS are generally administered orally. They can be used either alone or in admixture with other medicines, such as painkillers, tranquilizers, anti-tumor drugs, vitamins, antibiotics, digestives, anti-inflammatory drugs and hormones. For immune response and tumor treatment, the proposed dosage ranges from 0.5 to 30g per adult per day, preferably from 1 to 6 g per adult per day (Ueno *et al.,* 1989). Accumulating evidence suggests that the CPS are non-toxic even when administered several times above the therapeutically effective dosage and over extended periods. Extended use of PSP at 100-fold the normal clinical dosage has not induced acute and chronic toxicity in animals. The use of CPS appears to be safe during pregnancy with no adverse effects observed in the reproductive and embryonic development in mice (Ng and Chan, 1997). Therefore, PSP is non-teratogenic (*i.e.* without causing abnormalities in embryo or fetus). However, the use of CPS appears to be contraindicated when immune suppression is desired. CPS can reduce the potency of immunosuppressants such as cyclosporine. Administration of PSP partly restores immunosuppression induced by cyclophosphamide in rats (Qian *et al.,* 1997).

2-2-5 Possible anticancer pathways stimulated by CPS

Research on CPS elucidated many biochemical actions responsible for its anticancer and immunopotentiating properties. In addition to the action of simulating the cells to secrete cytokines, CPS are capable of increasing the activities of immune cells, such as T and B-lymphocytes and macrophages against cancer cells and various infectious diseases. CPS has also been found to have direct antineoplastic effects, including the ability to suppress the progression of cancer by inhibiting the process of metastasis (Kobayashi H *et al.,* 1995). The various immuno-modulating and anticancer properties of CPS are shown in Fig. 2.1 (Fisher and Yang, 2002).

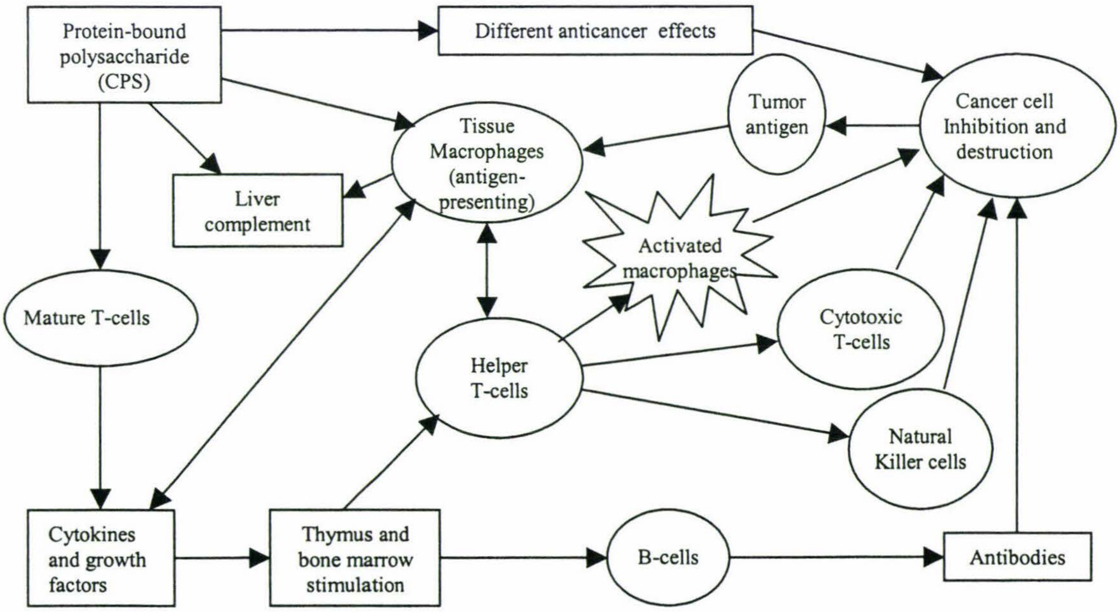


Fig. 2.1. Proposed immunomodulatory and anticancer pathways of CPS

2-3 *Coriolus versicolor* – the strain used for CPS production

The visible form of *C. versicolor* is a fan-shaped mushroom with wavy margin and colored concentric zones (see Fig. 2.2). *C. versicolor* is an obligate aerobe that is commonly found all year round on dead logs, stumps, tree trunks and branches. The fungus, which belongs to the family *Basidiomycotina* occurs throughout the wooded temperate zones of Asia, Europe and North America and may be the most common shelf fungus in the Northern Hemisphere.

Fig. 2.2 A photograph of fruit body of *Coriolus versicolor*

Many different names have been used in the literature for *C. versicolor*, including *Agaricus versicolor, Boletus versicolor, Polyporus versicolor, Polystictus versicolor, Poria versicolor, Trametes versicolor, Yun-Zhi* (Chinese) and *Kawaratake* (Japanese). In North America, *C. versicolor* is commonly known as "turkey tail" mushroom. The morphological characteristics of C. versicolor fruiting body have been described (Soothill and Fairhurst, 1977; Orson and Miller, 1970). The dimension across the brackets that is semicircular, flattened, thin and tough is typically 3-5 cm. Young brackets are flexible and usually occur in tiers, spread along branches. The upper surface is velvety and attractively marked with concentric zones of varying colors (brown, yellow, gray, greenish or black). The margin is usually wavy. Mature mushrooms are stalkless and overlapping, and are too leathery to be eaten. On the reverse side of the brackets, delicate polypores can be observed by using a hand lens. The mushroom has white spores that are oblong and cylindrical (4–6 × 2–2.5 μm) (Soothill and Fairhurst, 1977). In agitated submerged-culture, the fungus grows as dispersed or pelleted mycelium.

More than 120 strains of *C. versicolor* have been recorded in the *Compendium of Materia medica* in China thousands of years ago. Commercial PSK are obtained from the mycelia of the CM-101, CM-102 and CM-103 strains, and PSP from Cov-1. Currently, there are more 40 strains recorded by America Type Culture Collection (ATCC). The productivities of the CM-101, CM-103 and CM-103 strains are compared in Table 2.1 (Hotta *et al.,* 1981).

Table 2.1. The productivity of strains of CM-101, CM-102 and CM-103

Content	CM-101	CM-102	CM-103
Dry biomass content in the fermentation broth (mg/mL)	20.0-21.5	10.0-7.5	13.5-16.0
IPS yield on biomass ($Y_{p/x}$)	0.7-0.9	0.3	0.4

2-4 Production of *C. versicolor* Biomass

Commercially, CPS were extracted from mushrooms or mycelia cultivated on solid substrates (Yadav and Tripathy, 1991; Park *et al.,* 1994) and mycelial biomass produced in submerged-culture fermentations (Yoshikumi *et al.,* 1978b; Ueno *et al.,* 1980a, b; Chen *et al.,* 1981; Zhou *et al.,* 1994; Cheng *et al.,* 1998; Wang *et al.,* 1996). Owing to the advantages of higher mycelial production in a compact space, at a shorter time with lower rate of contamination, the submerged-culture fermentation has been widely employed in the industry.

2-4-1 Mushroom cultivation of *C. versicolor*

Historically, mushrooms were gathered from the wild for consumption and for medicinal use, mostly in the Far East. Over 200 species of mushroom have been recorded for such purposes. However, only about 20 mushroom species to date have been cultivated on an industrial scale for commercial purposes. The output yield of *C. versicolor* mushroom in the world has reached 130,500 metric tons and made up 2.1% of the total world mushroom production in 1997. By comparison with the output yield of 40,000 metric tons in 1986, there has been a three-fold increase in mushroom production worldwide (Chang, 1988). The *C. versicolor* mushroom is a wood utilizer and can be easily grown by adopting the methods used for growing Shiitake mushrooms (*Lentinus edodes*). Cultivation of mushrooms is carried out in three steps: preparation of growing medium, development of spawn / inoculum and crop management for mushroom production. Failure of any phase will result in a decreased yield or total loss. An overview of the various techniques for growing mushrooms is shown in Fig. 2.3.

- Preparation of growth medium

Growth medium may be regarded as the heart of the process and therefore the most important. The main components in the medium usually consist of sawdust, cottonseeds, wheat straw, horse manure, poultry manure, and fertilizers. Most medicinally important mushroom species can grow as saprophytes on dead wood – primary decomposers. In an innovative approach, various hardwood sawdust or wood chips supplemented with nitrogen-rich additives such as rice bran (though other cereal brans work adequately) are mixed together and then compacted into autoclavable polypropylene bags of various dimensions (Stamets, 1993; Yamanake, 1997). The bags are then autoclaved to ensure complete internal sterility, allow cool to 20 °C and then aseptically inoculated with the desired amount of spawn.

- Development of spawn/inoculums

Such cultures have originally been derived from single or multi-spore cultures or by tissue culture from a mushroom of a high yielding and vigorous strain. The purpose of the grain spawn is to boost the mycelium to a state of vigour so that it will rapidly colonize the substrate. The grain is an important nutrient source as well as a vehicle for even distribution in the substrate. All operations from pure culture isolation through spawn preparation must be conducted under sterile conditions and performed as rapidly as possible to lessen the possibility of contamination.

- Crop management for mushroom production

In this stage, the inoculated bags, sometimes known as space bags or artificial logs, can then be moved to growing rooms with controlled humidity (>80%) and

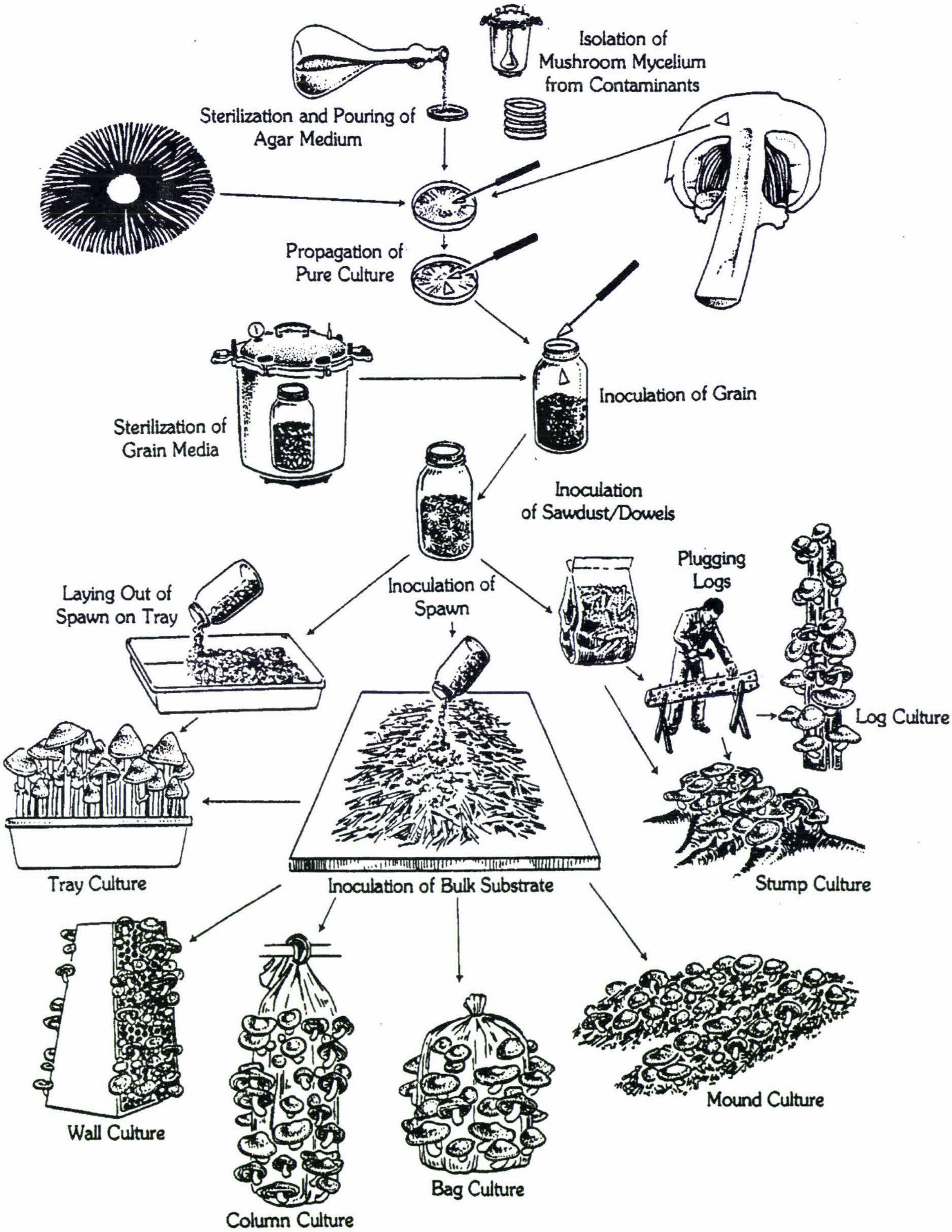


Fig 2.3. Diagram illustrating overview of general techniques for the cultivation of mushrooms (Stamets and Chilton, 1983).

temperature (15-20 ˚C). Correct control of potential microbiological contamination especially from other fungi is also highly critical for mushroom production. Though pesticides, especially fungicides are employed in many areas, the high premium paid for organic mushrooms necessitates good management practices to avoid the need for pesticides.

2-4-2 Submerged-culture fermentation of *C. versicolor*

Since *C. versicolor* is an aerobic fungus, the submerged-culture fermentation involves aerating the liquid medium. Therefore, *C. versicolor* is usually cultured in shake flasks held on a rotary shaker (150 rpm) to ensure sufficient dissolved oxygen is maintained. The culture needs a well-controlled temperature of around 27 °C for 7-days. The pH is usually not controlled and during fermentation pH typically declines from about 6.5 to 2.5. However, when pH is maintained between 4 to 5, both EPS and biomass increase, but IPS decreases (You *et al.,* 2001).

Yoshikumi *et al.* (1978a) used a 50 L stirred fermenter for the submerged-culture fermentation of *C. versicolor* under 25 °C. The medium consisted of glucose and yeast extract. Additional medium was fed stepwise during the 7-day culture. The final concentration of the biomass was ~23 $kg{\cdot}m^{-3}$.

Larger scale submerged-culture fermentation was carried out by Sugiura *et al.* (1980) in a 250 L stirred fermenter. The seed culture was prepared in a shake flask at 30 ˚C for 7 days and then transferred to a 15 L jar fermenter. After culturing at an agitation rate of 200 rpm and aeration of 1:1 v/v/m for two days, the broth of the jar fermenter was transferred as inoculum into 250 L fermenter. The fermentation process was controlled at an agitation rate of 200 rpm and aeration of 1:0.5 v/v/m, 30 ˚C for only 48 hours. During the fermentation, the pH of broth gradually decreased from 5.5 to ~ 3.5. Fermentation was ceased when the culture reached a specific viscosity of 3.0, pH 4.0 and mycelial mass 10 mg/mL. The medium used is described in section 2-5-1.

Hotta *et al.* (1981) cultured the mycelium on the surface of a static nutrient broth using strain CM-103. The 30 mL broth in a 200 mL Erlenmeyer flask was left in the static state for 10 days at 25–27 °C. The mycelial mat that formed on the surface of the broth was collected as seeds and homogenized with saline solution. The seeds were then inoculated a Erlenmeyer flask containing 200 mL medium. After 25-days of surface growth, only 3.2 g of biomass was obtained per flask. This clearly showed that surface culture on a static liquid medium is not a satisfactory method for commercial production of CPS.

In submerged-culture fermenter, *C. versicolor* can be grown as mycelial pellets or predominantly dispersed mycelium. Conditions favoring one morphology over the other are unclear, but hydrodynamic shear forces appear to play a role (Chisti, 1999). Zhou *et al.* (1994) observed that the broths posses a yield stress and discussed the factors that influence broth rheology.

2-4-3 Solid-substrate fermentation of *C. versicolor*

In nature, *C. versicolor* grows on solid substrates and not in liquid media. Solid-substrate fermentation of *C. versicolor* is used mainly in bioremediation applications. However, only a few have reported using this method of cultivation for producing CPS. Yadav and Tripathy (1991) used wheat bran and wheat straw to culture the fungus. The solid substrates were supplemented with water, 1.0% (w/v) superphosphate, and 1.5% (w/v) urea. The process was based on shallow trays where moist air was blown through the substrate bed. The optimal fermentation conditions were 55% moisture, pH 5.5 and 30 °C over 21 days of culture.

Inert solids (zeolite, orchid soil) moistened with solutions of carbon (glucose, sucrose, starch) and nitrogen sources have been used to grow *C. versicolor* (Park *et al.,* 1994). Although the large-scale production of mushrooms using solid substrate is well established (Flegg *et al.,* 1985), this method provides limited control and is labor intensive and prone to contamination. However, solid-substrate fermentation techniques have the advantage of low capital cost, low energy expenditure, low wastewater output and absence of foaming problems in liquid-fermentation.

2-5 Nutrition requirements of *C. versicolor*

2-5-1 Basic nutrition sources

Generally, glucose and sucrose are the preferred carbon sources in submerged-culture fermentation. Other carbon sources, such as starch and dextrin can also be taken up by *C. versicolor*. They are metabolized and converted to exopolysaccharides with high efficiency. In such case, transport mechanisms must be present in the cell membrane to permit substrate uptake (Berkeley *et al.,* 1979). The concentration of the carbon source also plays an important role in promoting growth and polysaccharide production.

For the nitrogen sources, *C. versicolor* is able to utilize simple inorganic and organic nitrogen sources, such as ammonium and amino acids. However, the utilization of ammonia by most fungi is accompanied by a drop in pH of the culture medium. Amino acids usually serve as the nitrogen donors for synthesizing more complex molecules. Thus, the particular nitrogen source in the culture medium is usually converted intracellularly to an amino acid before it is actually utilized by the fungi (Garraway *et al.,* 1984). *C. versicolor* can utilize a wide range of nitrogen sources for growth, which include peptone, yeast extract, yeast powder, peanut flour, soybean flour, and soy sauce (*shoyu*).

Inorganic salts are critically important for the growth and production of most fungi though they are often added in small quantities of the culture medium. Phosphorus is an integral component of cell macromolecules and phosphates are involved with the storage and transfer of energy. Magnesium has a wide variety of regulatory roles, such as stabilizing enzymes. The level of its retention or uptake by the cells is closely associated with the effect of phosphorus. Potassium, by shunting back and forth across the cell membrane, maintains the electrical and osmotic balances of the cell. Sulphur is an important component for synthesis of certain amino acids, vitamins, and metabolites (Garraway *et al.,* 1984). Other micronutrients may also be required by *C. versicolor* but in quantities too small to detect.

C. versicolor is usually grown in defined media or complex media in the laboratory. Wang *et al.,* (1996) used a medium of pH 6 for growing *C. versicolor,* which consists of 24 g potato dextrose broth, 5 g peptone, 0.46 g KH_2PO_4, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$ and 20 mg vitamin B1 in one liter of distilled water. Yoshikumi, *et al.,* (1978a) conducted a series of medium optimization experiments on glucose and yeast extract and found the combination of 12.5 % (w/v) glucose and 1.5 % (w/v) of yeast extract could achieve the best yield of mycelia. The growth medium reported by Hotta, *et al.* (1981) contained 5% (w/v) glucose, 0.2% (w/v) peptone, 0.3% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ (w/v). Sugiura *et al.* (1980) achieved a mycelial yield of 10 mg/mL using a medium consisting of 5 % (w/v) sucrose, 5 % (w/v) soy sauce, 0.67 % (w/v) onion extract, 0.05 % (w/v) $MgSO_4$, 0.1 % (w/v) KH_2PO_4 and 0.1 % (w/v) silicone.

The complex media derived from the waste products of some bioprocesses (e.g. brewing and sugar refining) are good growth substrates for many fungi. Experiments utilizing different ratios of molasses to brewery cake in the growth media suggested that the carbon to nitrogen ratio could influence both the biomass and CPS production (Archibald *et al.,* 1990).

2-5-2 Milk Permeate as a base medium

Milk permeate is a by-product of dairy industry and presents a serious disposal problems due to its unacceptably high Biological Oxygen Demand (BOD), ranging from 29,000 –72,000 g/m^3 (Clarke 1999). This could lead to the contamination of ground water.

The composition of a typical batch of milk permeate is listed in Table 2.2 (Clarke 1999). Milk permeate contains 5% (w/v) lactose, traces of proteins and many other micronutrients. Conversion of the lactose into a sugar syrup by the enzymatic hydrolysis of lactose with the galactosidase, Maxilact® is one way of utilizing the permeate stream. The sugar syrups have been used to sweeten foods, retard crystallization, alter viscosity and stability, contribute to nutrition and enhance flavors.

Table 2.2. Chemical Composition of Milk Permeate

Components		Contents
Water	% (w/w)	94.6
Fat	% (w/w)	<0.010
Protein	% (w/w)	0.220
Ash	% (w/w)	0.486
Energy	KJ / 1000 g	83
Carbohydrate	g / 100 g	4.7
Vitamin C	mg / 100 g	0.09
Sodium	mg /100 g	41
Potassium	mg / 100 g	150
Calcium	mg / 100 g	29
Total solids	% (w/w)	5.37
Magnesium	mg / 100 g	6.5
Phosphorus	mg / 100 g	39
Lactose monohydrate	% (w/w)	4.95

To date, however, no information is available with respect to CPS production using milk permeate as a base medium though it could be a potential carbon source for the CPS biosynthesis.

2-6 Fermenter used for fungi culture

Most submerged-culture fermentations of *C. versicolor* used the stirred bioreactors. Although some impeller designs such as the Rushton turbine impeller (a disc turbine with six flat blades) have effectively improved the rate of oxygen transfer necessary for high cell density fermentations (Wainwright, 1992), they imparted high shear stress to the culture medium (Lawford and Rousseau, 1991). The high shear caused by high-speed agitation was found to have a negative impact on the CPS production and growth of *C. versicolor* (Cui, 2002). Lawford and Rousseau (1991) modified a conventional stirred fermenter for the fermentation of *Alcaligenes faecalis* and *Agrobacterium radiobacter* to produce curdlan, a β, 1,3-glucan exopolysaccharide. They found that low shear mixing achieved through the replacement of the radial-flow flat-blade impellers by low shear (high-pumping) axial-flow helical impellers led to an increase in the quality of the exopolymer recovered during the stationary-phase of batch fermentation. The high volumetric oxygen transfer can be improved by the use of sparging devices of microporous materials (sintering glass sparger).

Airlift bioreactors are also considered to provide conditions of low shear (Christi, 1989) and there is evidence to suggest that some fungi produce a higher level of exopolysaccharides under these conditions (Gibbs *et al.,* 1992). The looped airlift bioreactor contains either an internal or external draught tube that is often baffled, resulting in increased mixing by forcing a directional flow of the bulk medium. The driving force for circulation is created by the difference in density (which results from the amount of dispersed air bubbles) between the riser and downflow section. Therefore, as a low-shear mixing fermenter, an airlift bioreactor is also advantageous in terms of lower maintenance and operating cost. However, the lower biomass tolerance of airlift fermenter as compared to the stirred tank limits its industrial application.

2-7 CPS recovery

EPS is directly isolated from the broth by means of ethanol precipitation and purification to remove the impurities, such as inorganic salts and low molecular weight species such as sugars and peptides. Recovery of IPS consists of two steps, extraction of IPS from biomass and isolation of IPS from the extract. The extract are concentrated by evaporation under vacuum or ultrafiltration and then precipitated by ammonium sulfate or alcohol. Precipitates of both EPS and IPS are re-dissolved, dialyzed, and may be further purified by chromatographic methods. Solution of the purified product is concentrated and spray-dried. Removal of low molecular weight contaminants appears to be important because they do not contribute to physiological activity and may cause bitter taste and disagreeable odor in the final product (Hotta *et al.,* 1981).

2-7-1 Extract of IPS by hot water

A multi-step hot water extraction of *C. versicolor* biomass appears to be necessary to recover the IPS in sufficient amounts. Ueno *et al.* (1980a) extracted IPS from 200 g biomass (dry weight) in 3-liter hot water (90±2 °C) for 2–3 hours. Two subsequent extractions of the filtered biomass residue were carried out using less water (e.g. 2 liter per step). Extraction rate of IPS may be increased by disrupting the mycelial biomass in a bead mill (Chisti and Moo-Young, 1986) prior to or during extraction with hot water. The crude product extracted from the milled biomass is likely to contain many intercellular low molecular weight contaminants but these are readily removed by the purification methods such as ultra-filtration or dialysis.

Extraction of polysaccharides under acidic conditions is obviously to be avoided if possible so that inadvertent cleavage of glycosidic bonds does not occur. Dilute alkali has been used extensively for polysaccharide extraction, but there is now a more general awareness of the possible structural modifications or base-catalyzed degradations that may occur (Gerald, 1982). Ueno *et al.* (1980a, b) used sequential extraction of the mycelial biomass with aqueous alkaline solutions (0.1–1.0 M sodium hydroxide) at 90–95 °C. Extraction periods were different for different concentrations of alkali. Treatment with >2 M alkali was not recommended as it could degrade the IPS. The extracts were neutralized with mineral acid, pooled, and concentrated by ultrafiltration or reverse osmosis to remove the low molecular weight contaminants.

The concentrated solution of CPS was spray-dried or freeze-dried to yield a brown powder. The process patents (Ueno *et al.,* 1980 a, b) specify extraction temperature and times of 80–90 °C and 20–600 min, respectively. Similar recovery methods have been used by others (Hotta *et al.,* 1977; Wada *et al.,* 1977; Yoshikumi *et al.,* 1978b; Sugiura *et al.,* 1974, 1980; Wang *et al.,* 1996).

Extraction of IPS from the biomass was successful using hot aqueous solutions containing a surfactant (2% Triton X-100) (Park *et al.,* 1992). Atomachi (1988) treated the mycelial extract with aqueous lead acetate. The precipitate produced was dissolved in water and lead was removed by precipitating with sulfuric acid or hydrogen sulfide. The filtrate was treated with water-miscible organic solvents to recover the IPS.

2-7-2 Isolation and purification of IPS and EPS

Isolation of IPS and EPS is commonly achieved by precipitation from the concentrated extract and biomass-free broth, respectively. It is generally suitable for the separation of rather large quantities of polysaccharides. Ethanol precipitation (Sugiura *et al.,* 1980; Chen *et al.,* 1981; Kim *et al.,* 2001) and ammonium sulfate fractionation (Hotta *et al.,* 1981) are used frequently.

In a typical processing scheme, Hotta *et al.* (1981) recovered the IPS by agitating the mycelia with distilled water at 98 ± 2 °C for 3 hours. The biomass slurry was cooled and filtered. The solid residue was further extracted as above. The extracts were combined and concentrated by evaporation under vacuum. The concentrated solution was saturated with ammonium sulphate to precipitate the IPS. The precipitate was dissolved in water and desalted by dialysis using a cellulose acetate membrane. The IPS solution thus obtained was concentrated to 5% (v/v) of its original volume. Further ammonium sulfate precipitation steps followed. The final precipitate was desalted by dialysis, and purified by DEAE-cellulose chromatography. One further ammonium sulphate fractionation step was conducted and the desalted precipitate solution was concentrated and spray-dried.

The removal of low molecular weight impurities is readily performed by dialysis, ion exchange chromatography or gel filtration. The last two techniques are also used extensively for the separation of the desired polysaccharides from contaminant macromolecules. High performance liquid chromatography (HPLC) has also been used in the final recovery stages to purify PSP through C-18 semi-preparative reversed-phase column (Yang *et al.,* 1998), but this is generally impractical in large-scale processing. Conventional ion exchange chromatography on DEAE-Sephadex and DEAE-cellulose columns has been also used effectively (Hotta *et al.,* 1981; Park *et al.,* 1992).

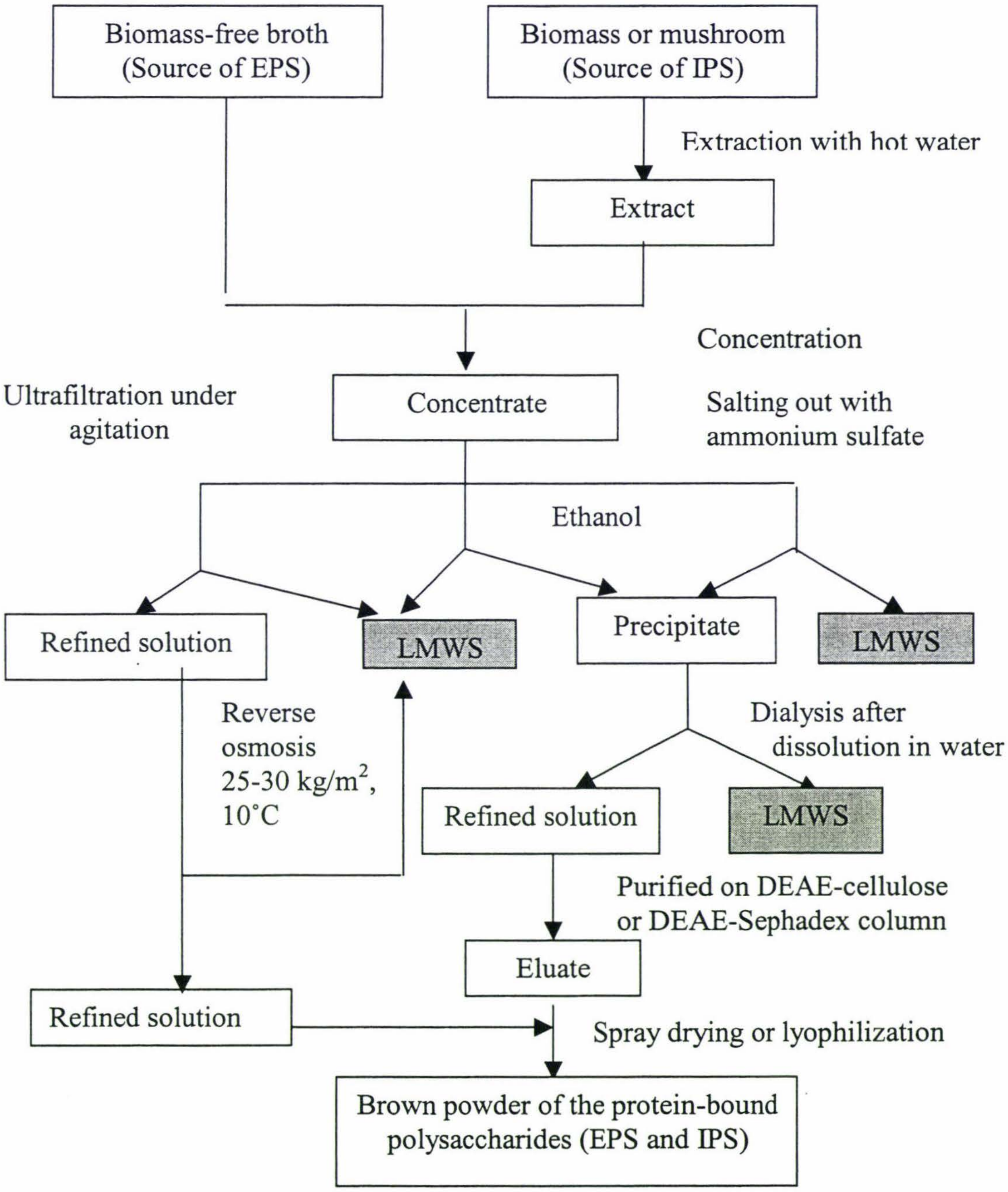


Fig 2.4 Different pathways for CPS isolation and purification. LMWS means low molecular weight substances

Different pathways for isolating and purifying CPS are summarized in Fig. 2.4. In commercial CPS production, the number of steps should be kept to a minimum wherever possible. Precipitation and dialysis steps should be repeated a minimum number of times and no more than one chromatography step is recommended for the product intended for oral consumption. Spray drying of the final solution is preferred to freeze-drying to reduce cost.

2-8 Characteristics of CPS

2-8-1 The physical properties of CPS

Brown powders of PSP and PSK are soluble and stable in hot water. They are odorless and tasteless (Ueno *et al.,* 1980a, b; Hotta *et al.,* 1981). The compounds do not have a definite melting point. The polysaccharides can withstand steam-sterilization at 120 °C for 30 minutes without showing any decrease in the antitumor activity. No hydrolysis of the polysaccharides occurs in 0.5 N sulphuric acid at room temperature with stirring for 24 hours (Sugiura *et al,* 1980). However, heating to more than about 120 °C caused browning of CPS. The PSP/PSK polymers are insoluble in methanol, pyridine, chloroform, benzene and hexane (Hotta *et al.,* 1981). Aqueous solutions of PSP (1 g per 100 mL water) have a pH value of between 6.6 and 7.2. The $[\alpha]_D^{25}$ value of the PSP solution is in the range of 0 to 30°.

2-8-2 The structure and composition of CPS

All polysaccharides contain repetitive features, often a regular periodic repeating unit. Notably, for some microbial polysaccharides whose biosynthesis involves polymerization of pre-assembled oligosaccharide units, the regularity of structural features is less apparent. While still subject to dispute, IPS structures mostly appear to comprise α-1,4 and β-1,3 linked glucosides in their polysaccharide fractions with branches at the 3- and 6- positions (Fisher and Yang, 2002). However, the EPS contain only β-1, 3 linked glucosides with β-1,3 and β-1,6 branches (You *et al.,* 2001).

Glucans with high molecular weight and water solubility seem to have the greatest antitumor activity, with some exceptions. Also a triple-helical structure has been identified as being important for antitumor activity (Fisher and Yang, 2002). The chemical modification of various polysaccharides has demonstrated that various biological activities may be enhanced or attenuated and might be an effective approach to improve the biological activity of these diverse polysaccharides (Fisher and Yang, 2002).

As for the composition of IPS, the water extract of *C. versicolor* mycelia contains many kinds of complex glycoproteins and no single antitumor-active substance could be identified (Sugiura et al, 1980). The EPS from mycelia -free broth contains only glucan (You *et al.,* 2001). Elemental analysis of IPS reveals the following composition: oxygen 47.5 %, carbon 40.5 %, hydrogen 6.2 %, and nitrogen

Table2.3 Comparison of mycelium extract and mushroom extract of *C. versicolor*

	MYCELIUM EXTRACT	MUSHROOM EXTRACT
Description	Fine brown powder	Fine brown powder
Storage	5-10°C, sealed	5-10°C, sealed
Technology	Submerged fermentation	Mushroom culture
Final processing	Spray-dried	Spray-dried
Total polysaccharides (%)	30.2	58.8
Moisture (%)	< 7.0	< 7.0
Protein (%)	12.1	15.1
Lipids (%)	0.14	1.3
Carbohydrates (%)	64.6	68.7
Ash (%)	16.5	7.9
Phosphorus (μg/g)	19800	6730
Potassium (μg/g)	29400	14400
Magnesium (μg/g)	10620	1850
Lead (μg/g)	<1.0	
Cadmium (μg/g)	0.16	0.4
Arsenic (μg/g)	<2.0	
Mercury (μg/g)	Negative	Negative

5.2 % (Ueno *et al.,* 1980a). The powdered extract typically contains 34–35 % (w/w) soluble carbohydrate (91–93 %, w/w, β-glucan), 28–35 % (w/w) protein, ~7 % (w/w) moisture, 6–7 % (w/w) ash, and remainder as free sugars and amino acids (Ueno *et al.,* 1980a). The mycelia-derived IPS and the mushroom-derived IPS have almost the same physiological activities. Their compositions have been compared by Garuda International, Inc., USA (1998), as shown in Table 2.3.

PSP and PSK are chemically similar and posses similar physiological activity profiles. Each is a mixture of polysaccharides that are covalently linked to a number of proteins (Yang *et al.,* 1992). D-glucose is the major monosaccharide present, which accounts for 99% of the total saccharides (Hotta *et al.,* 1981). Arabinose and rhamnose are the other principal monosaccharides in PSP, while PSK contains mannose and xylose, galactose and fructose (Wang *et al.,* 1996; Cheng *et al.,* 1998). At present, polysaccharides which contain more than six different types of monosaccharide units have not been found (Kennedy and White, 1983).

The polypeptide fractions of IPS were closely bounded to the polysaccharide and not separated by native PAGE and chromatographic methods. They are small molecular proteins (polypeptide). According to Yang (1997), only those fungal polysaccharides that bound with proteins produce anti-tumor effect after oral administration to patients. They contain large amounts of aspartic acid and glutamic acid (Ng, 1998). Acidic and neutral amino acids such as aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine account for 70 % of all the 18 amino acids present (Hotta *et al.,* 1981). The IPS resists enzymatic proteolysis (Hotta *et al.,* 1981). Therefore, these polysaccharides are not affected by the digestive process (JHS Natural Products, 2001).

2-8-3 Molecular weight of CPS

Since polysaccharides are polydisperse, only an average molecular weight can be estimated (Gerald 1982). Both PSK and PSP have an average molar mass of approximately 100 kDa (Yang *et al.,* 1992; Ng, 1998). A wide range between 10,000 and 100,000 Da has been reported by Hotta *et al.*(1981) and Yoshikumi *et al,* (1986). Fractionation of PSK by successive filtration has revealed at least 4 sub-fractions, with the highest molecular weight fraction showing the greatest biological activity (Sakagami *et al.,* 1991).

The low molecular weight fractions (<5,000 Da) discharged from refining process did not demonstrate inhibitory effect against Sarcoma-180 solid tumours in mice upon intraperiotoneal administration. Furthermore, they contributed to bitterness and disagreeable odor and induced side effects (Ueno *et al.,* 1980a). Therefore, specific biological activities of CPS are somehow related to the molecular weights of the polymer.

2-8-4 Color reaction tests on IPS

Hotta *et al.* (1981) and Ueno *et al.* (1980b) conducted a series of color reaction tests on the aqueous solution of ISP to indicate the existence of saccharides and proteins in the substances and to confirm that the substances were nitrogen-containing polysaccharides. The results of these tests are summarized in Table 2.4

Table 2.4 Color reaction tests on IPS

COLOR REACTION	COLOR	RESULTS
α- naphthol-sulfuric acid reaction (Molish reaction)	Purple	Saccharides
Indole-sulfuric acid reaction (Dische reaction)	Brown	Saccharides
Anthrone-sulfuric acid reaction	Greenish blue	Saccharides
Phenol-sulfuric acid reaction	Brown	Saccharides
Tryptophane-sulfuric acid reaction	Purplish brown	Saccharides
Lowry-Folin assay	Blue	Peptide bonds, tyrosine, tryptophane, cysteine
Ninhydrin reaction after hydrochloric acid hydrolysis (6N HCl, 110 °C, 20 hrs)	Purplish blue	α-amino acids

2-9 Concluding remarks

For years, CPS have been used as immunopotentiators in therapeutics and are regarded as a useful adjunct to conventional therapy of cancers and other diseases. Their clinical bioactivities are primarily dependent on the polysaccharide structures, fermentation conditions, culture media and isolation processes. Within the host immune system, CPS potentiate the antitumor activity of natural killer cells, T cells, lymphokine-activated killer cells and macrophages under normal conditions. This action results in an increased activity of these immune cells that are vitally important for the maintenance of homeostasis. Nowadays, CPS are used clinically in East Asia for the cancer patients who undergo supplement surgery, chemotherapy and radiotherapy. Because of their immunopotential role, CPS are also sold commercially as a health supplement (immunoceuticals) and have been clamed to be effective against infectious diseases such as heptatitis and pneumonia that are opportunistic infections, acquired when the immune system is weakened. CPS are nontoxic over prolonged use and could benefit general well-being. Further work is also necessary to establish the mechanisms of CPS against tumor cells.

CPS can be obtained from the mushrooms of *C. versicolor* harvested in the wild or cultivated commercially. Alternatively, mycelia growth of *C. versicolor* in submerged-culture fermentation provides a viable method for commercial production of CPS. Batch culture of *C. versicolor* requires a well-balanced medium, sufficient aeration and an initial pH of 5.5±0.5. The optimal culture temperature is 25 to 27 °C. The fermentation lasts 5 to 7 days. An airlift bioreactor can be used to carry out the fermentation and applies less shear on mycelia. However, the fermenter design needs to be improved to overcome its limitation with viscous ferment. The polymers produced in submerged culture can be extracted from the mycelial biomass (IPS) and the biomass-free culture broth (EPS). The CPS isolated from different sources (mushroom, mycelium, biomass-free broth) differ somewhat in structure, composition and physiological activity according to various reports.

C. versicolor can utilize a wide range of nutrient sources. Glucose is a preferred carbon source for most CPS productions. Nitrogen source and proper carbon/nitrogen ratio in the culture medium are considered as crucial factors for the optimal growth of the fungi. Milk permeate from waste stream of biological processing contains mainly lactose, proteins and other micronutrients and may potentially be used as a base medium for CPS production.

IPS is heat stable and is easily recovered by extraction of the biomass (mushrooms or mycelia) with hot water or dilute alkaline solutions. EPS can simply be recovered form the biomass-free broth by precipitation method. Both IPS and EPS are concentrated and the polymers are precipitated by salting out using ammonium sulphate or precipiting by organic solvents. Further purification is achieved by dialysis, ultrafiltration and chromatographic methods. The purified CPS are made into a powder by lyophilization or spray drying.

The structure of IPS has generally been considered as a triple-helical structure with α-1,4 and β-1,3 glucoside and branches at 3- and 6- positions along the 1-4 glucan backbones. The proportion of side chains is one per several backbone glucose units. Glucan makes up the backbone of the polysaccharide moieties with small amount of other monosaccahrides, including arabinose, rhamnose, galactose, mannose and xylose. Their polypeptides are covalently bounded to the polysaccharide backbones. Acidic and neutral amino acids account for 70% of all the 18 kinds of amino acids present. On the other hand, the EPS was reported to contain only glucan and β-1, 3 linked glucosides with β-1,3 and β-1,6 branches. The reported weight average molecular weights of IPS ranged from 10,000 to 100,000 Da.

CHAPTER III MATERIALS AND METHODS

3-1 Materials

3-1-1 Media

Potato Dextrose Agar (PDA) and malt extract were obtained from MERCK KgaA, Darmstadt, Germany; yeast extract, Bacto^{TM} casitone peptone, Bushnell-HAAS Broth (Table 3.1) and YM broth (Table 3.2) were obtained from DIFCO Laboratory, Detroit, Michigan, U.S.A.; Milk permeate (MP) and Ypp3 (yeast extract) were obtained from Fonterra Co-operative Group Ltd, New Zealand;

Table 3.1 Formula of Bushnell-HAAS broth (BH broth)

Components	Concentration (gL^{-1})
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.02
KH_2PO_4	1.0
$(NH_4)_2HPO_4$	1.0
KNO_3	1.0
$FeCl_3$	0.0 5

Table 3.2 Formula of YM broth

Components	Concentration (gL^{-1})
Bacto-Yeast Extract	3
Malt Extract-Difco	3
Bacto-Peptone	5
Bacto-Dextrose	10

Fungi salt solution (Table 3.3) and Fungi Growth Trace Element solution (Table 3.4) were used as basic mineral sources in the media. Main growth media used for culturing *Coriolus versicolor* in the various experiments are shown in Table 3.5. All media and solutions were made to volume with distilled water.

Table 3.3 Fungi Salt Solution (FS solution)

Components	Concentration (gL^{-1})
$(NH_4)_2SO_4$	3.0
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.5
KCl	0.5

Table 3.4 Fungi Growth Trace Element solution (FGTE solution)
(Mandel' medium, Hendy, 1981)

Components	Concentration (gL^{-1})
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.3
$CaCl_2$	0.4
$FeSO_4.7H_2O$	0.005
$MnSO_4.H_2O$	0.016
$(CH_3.COO)_2Zn$	0.014
$CoCl_2$	0.02

Table 3.5 Main growth media of *Coriolus versicolor* used in the project

Components	MEG	YEG	50% MP-MEG	50% MP-YEG	50% HMP-YEG	100% HMP-YEG
Glucose (gL^{-1})	40	40	20	20	20-25	15-20
Yeast extract (gL^{-1})	2	7	2	6	6	6
Malt extract (gL^{-1})	12.5	_	6			
MP/HMP * (mLL^{-1})			500	500	500	1000

All media in Table 3.5 contain FS solution (Table 3.3)
*MP means milk permeate and HMP means hydrolysed milk permeate

Table 3.6 Tissue Culture Medium (TCM)

RPMI 1640 base medium (Sigma)	
Foetal bovine serum (Gibco)	10 %
L-glutamine	100 mM
2-mercaptoethanol	2 µm
Penicillin	100 U/mL G
Streptomycin sulfate	100 µg/mL

3-1-2 Sugars

Sugars used in the experiments were all of analytical grade. Arabinose and rhamnose were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Glucose, fructose, xylose, galactose, mannose and lactose were all obtained from BDH laboratory supplies, Poole, England.

3-1-3 Chemicals

Chemicals used in fermentation and analysis were all of analytical grade. Their sources and chemicals were:

- MERCK KgaA, Darmstadt, Germany

  calcium chloride.
- May&Baker Ltd. Dagenham, England

  ferous sulfate.
- Sigma Chemical Co., St Louis, Missouri, U.S.A.

  3.5-dinitrosalicylic acid
- AJAX Chemicals, Auburn, N.S.W. Australia

  sodium sulfate and ammonia sulfate.
- BDH Laboratory Supplies, Poole, England

  potassium dihydrogen orthophosphate, magnesium sulphate 7-hydrate, potassium chloride, urea, manganese sulphate 1-hydrate, zinc acetate, potassium sodium tartrate, cobaltous chloride, phenol, sulphuric acid, chlorhydric acid and sodium hydroxide pellets.

3-1-4 Enzyme

The enzyme used for hydrolysing the lactose in the milk permeate was Maxilact® L2000 lactoase, activity > 2000 NLU (Neutral Lactase Units) /g. The International Enzyme Number is EC 3.2.1.23. It is a purified lactase preparation provided by DSM Food Specialties, the Netherlands, isolated from a special strain of the dairy yeast *Saccharomyces (Kluyveromyces) marxianus var. lactis*. Its main application areas include milk hydrolysis; whey hydrolysis and animal feed applications. It hydrolyses the milk sugar, lactose, into the two monosaccharides, glucose and galactose.

3-2 Organisms

Stock cultures of *Coriolus versicolor* used in this work were obtained from the following sources:

- American Type Culture Collection, Rockville, Maryland, U.S.A.

  Coriolus versicolor ATCC 20545, or CM-103 (named by depositor, Kureha Chemical Industry Co., Ltd.)

- Forest Research Institute of New Zealand

  C. versicolor 15D

  C. versicolor 75

  C. versicolor 75 A

  C. versicolor 75 B

  C. versicolor 75 C

  C. versicolor 75 D

  C. versicolor 75 E

  White rod 87

  White rod 74

  White rod 61

  White rod 45

  White rod 35

All cultures were maintained by subculturing once every 3 months on Potato Dextrose Agar (PDA) slants, which were incubated at 27°C for 5 days and then stored at 4 °C.

3-3 Sterilization

3-3-1 General Sterilization

All the media, chemicals, miscellaneous glassware used for culturing the fungi were sterilized in an autoclave at 121 °C for 15 min. All media for inoculum preparation and batch flask fermentations were autoclaved immediately prior to use. Aluminum foil covers on top of the containers were used as a moisture barrier.

In some cases, certain solutions (*i.e.* enzyme preparation) were sterile-filtered through a single-use 0.2-µm-membrane filter prior to their aseptic addition to cultures.

3-3-2 Fermenter sterilization

An empty 7-litre fermenter was autoclaved at first at 121 °C for 15 minutes with all open-ended tubes attached to the vessel covered with aluminum foil. 5-lter freshly prepared growth medium (Table 3.5) was then transferred into the vessel and autoclaved once more at 121 °C for 15 minutes. Immediately upon cooling to below approximately 30 °C, the fermenter was inoculated and attached to all the accessories and the air pump. A Mettler Toledo pH electrode that was separately sterilized (module: InPro 3030/325; Mettler Toledo International Inc., Columbus, USA) was installed into the fermenter before all the operations.

3-4 Cleaning of glassware

All glassware was washed in hot Pyroneg® solution (Diversey Wallace Company, Auckland, New Zealand), rinsed in tap water and hot-air dried. The glassware used in the analysis of sugars or polysaccharides were soaked in 5% (v/v) dilute nitric acid after the detergent wash and tap water rinse. They were then rinsed twenty times with distilled water and hot air dried.

Fermenter and all tubings were cleaned at the end of each run of fermentation by soaking in hot Pyroneg ® solution followed by hot water rinse. The single-use air filter was discarded after each run of fermentation.

3-5 Culture methods

3-5-1 Inoculum preparations

About 0.8 cm^2 of mycelium mat from a PDA mycelium slant was aseptically transferred into baffled 500 mL Erlenmeyer flasks containing 100 mL of YEG medium. The flask culture was agitated at 180 rpm on Environ-Shaker (model 3597, Lab-line Instruments, Inc., Melrose Park, Illinois, U.S.A.) at 27 ˚C for 4-5 days. When abundant mycelium pellets had formed, the inocula were ready for seeding the fermentation flasks or the fermenter. A 10 % (v/v) inoculum was used in both the shake flask and the fermenter.

3-5-2 Shake flask culture

Seed culture (10 mL) was inoculated to 100 mL of required medium in a 500 mL Erlenmeyer flask. Each flask was plugged with two layers of cotton cloth as a stopper that allowed airflow during the growth. The inoculated flasks were then incubated at 27 °C on the Environ-Shaker and agitated at 180 rpm for 5-7 days until levels of CPS yield, biomass and pH reach to the criterion required.

3-5-3 Batch fermenter culture

A modified 7-litre *impeller-assistant airlift bioreactor* was used for the batch fermentations (Fig. 6.1). After being autoclaved, the fermenter containing 5 L required medium (*i.e.* MP/HMP-based YEG medium) was aseptically inoculated with 500 mL of inoculum from the shake flasks. Culture temperature was maintained at 27°C by means of a heating element (a water-bath). Agitation was set up at 0 or 150 rpm according to the experiment requirements. Sterile air was sparged at a rate of 5 to 7.5 L/min) through a fine porous stainless steel sparger from the vessel base. pH was recorded base on the readings from the Mettler Toledo pH electrode that was calibrated using pH 4.0 and 7.0 buffer solution prior to each batch of fermentation. Occasionally the electrode required soaking in 0.1 M hydrochloric acid for 2 hours, after a wash in detergent solution, to remove contaminating proteins.

Samples were withdrawn from the fermenter every day during the fermentation for further analysis. Prior to sampling, the sample system was flushed with approximately 30 mL of the culture to remove any resident “dead” volume. The flush volume and sample volume were recorded to enable volumetric analysis of fermentation components. Duration of the fermentation was between 5-7 days, depending on the biomass and CPS yield achieved.

3-5-4 Data analysis

Batch culture data were analysed using the following conventions:

- EPS/IPS yield on biomass ($Y_{p/x}$): The value is calculated as EPS/IPS level (mg) divided by biomass dry weight (mg) produced in 1 mL of broth;
- EPS/IPS yield on substrate ($Y_{p/s}$): The value is calculated as EPS/IPS level (mg) produced divided by the amount of sugar utilized (mg) in 1 mL of broth;

- The amount of sugar utilized is calculated by total amount of sugar in the medium before inoculation minus the residual amount of sugar in the broth during fermentation. The values can be obtained in the units of mg/mL or percentage through Phenol/Suphuric acid Assay or HPLC.

- Maximum Specific growth rate (μ_{max}): It is the value of a plot of ln (X/X_0) versus culture t. X and X_0 are the biomass dry weights during the exponential growth period from time t to t_0;

- Calculation of the carbon/nitrogen (C/N) ratio was based on total amount of glucose and galactose (%, w/v) determined by HPLC divided by total amount of nitrogen component (yeast extract %, w/v × 10.9 %, w/w, OXOID manual, 1995) in the medium.

3-5-5 Mushroom cultivation

Strains adopted in the experiment were ATCC-25454 and White rot-74 (Wr-74). The seeds for mushroom cultivation came from the broth inoculums described in section of 3-5-1.

The compost for mushroom growth was made of 3 kg *Photinea* wood chips. Other components included 300 g maize starch and 600 g corn meal. About 4 kg water was needed for wetting the compost.

The well-made compost was pressed into the autoclavable plastic bags or 1,000 mL wide-mouth bottles prior to autoclaving. The bags or bottles need to be autoclaved twice at 120 °C for total of 30 minutes. After cooling, the inocula were aseptically added into these bags or bottles under sterile conditions.

The inoculated compost bags or bottles were then incubated at about 25 °C for 10-15 days to allow the mycelia to grow. After white mycelia covered the entire solid substrate, the bags/bottles were transferred to a moist area and opened for aeration. The ambient temperature was approximately 15-20 °C. The fruit bodies of *C. versicolor* usually appeared 30 days later, the period depending on the species and environmental conditions. The appearance (size and surface colour) of the ATCC and Wr-74 fruit bodies were compared.

3-6 Separation method

3-6-1 Extraction of IPS from biomass

Biomass was collected by filtering a known volume of the broth form fermenters or flasks through No.541 hardened ashless filter paper (Whatman International Ltd, Maidstone, England) on a Buchner funnel. The biomass was thoroughly washed with ten-times its volume of distilled water and then re-suspended in the distilled water or 0.2-0.6 M aqueous sodium hydroxide solution, and heated in a boiling water bath for 3 hours. After cooling, the volume of whole slurry was recorded and then the slurry was subjected to suction filtration using a Buchner funnel to separate the biomass and extract. For the aqueous alkaline extract, neutralisation to pH 5-7 with hydrochloric acid was necessary prior to filtration. The residual biomass was washed with a small amount of water and the wash water was added to the extract.

3-6-2 Isolation of IPS from the extract

Four volumes of 95 % (v/v) ethanol were added with agitation into the extract solution that was then left at 4 °C overnight. The precipitate was then collected by centrifugation at 4000 rpm for 20 minutes (the centrifuge, BHG HERMLE Z320 was made in Germany and supplied by John Morris Scientific PTY, LTD, New Zealand) and subjected to freeze-drying on a Virtis model 10-020 bench-top freeze-drier (Virtis Company Inc. Gardiner, N.Y. U.S.A.). The light brown freeze-dried powder (IPS) was collected and the weight was recorded.

3-6-3 Isolation of EPS from biomass-free broth

Biomass-free broth was obtained by removing the biomass by filtration as described in section 3-6-1. Four volumes of 95 % ethanol were added to the broth which was then stored at 4 °C overnight. The resulting precipitate was collected by centrifugation at 4000 rpm for 20 minutes and subjected to freeze-drying. The light brown freeze-dried powder (EPS) was collected and the weight was recorded.

3-6-4 Dialysis

Dialysis tubing (molecular weight cut off of 6-8 kDa) needed a pre-treatment prior to use. The tubing was soaked in distilled water and boiled for 20 minutes. One end was closed with an overhand knot. The bag was then filled with distilled water to check the leakage. After the crude EPS or IPS solution was filled in the dialysis tubing, the open end of the bag was fastened with a clip. The dialysis against distilled water was carried out with agitation at 4 °C for 48 hours with water changed twice a day.

After dialysis, the dialysed solution was centrifuged (4000 rpm for 20 minutes) to separate the water-soluble part (supernatant) and the water-insoluble part (sediment) of EPS or IPS. The sediment was resuspended in water and centrifuged until no soluble polysaccharide remained in the supernatant (checked with phenol/sulphuric acid reagent). All the supernatant were collected and subjected to precipitation by four volume of 95 % (v/v) ethanol at 4 °C overnight. The light brown colour powder of water-soluble EPS or IPS was obtained by lyophilization of the precipitate pellets as described above. The water-insoluble EPS or IPS was simply obtained by lyophilization of the washed sediment.

3-7 Physiological tests

3-7-1 Effects of EPS/IPS on proliferation of cancer cells

Two cancer cell lines were used in this test. One was YAC-1, a mouse lymphoma cell line and the other was K562, a human myelogenous leukemic cell line. Six samples including EPS and IPS were isolated from the fermentation broth of ATCC-25454 and Wr-74. IPS was extracted from mycelia by boiling in the water, precipitating with ethanol, dialysing and freeze-drying. EPS was separated by the similar procedures as IPS, but without the hot water extraction step. All samples were sterilised by passing through 0.02-μm sterile filters and diluted with water to required concentrations.

YAC-1 and K562 cancer cell lines were incubated in the TCM medium (Table 3.6) at approximately 38 °C for two days to achieve required cell count (5.0E+05 cells/mL). The cells were counted by a flow cytometer (Model: FACS Calibur, produced by Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Each sample (100 μl) was added to 900 μl of cell solution in a 24 well plate. For the control sample, 100-μl water was added. All samples were prepared in triplicate and incubated at 38 °C for 18 hours. The culture in each well was transferred to a 96-well plate with each sample in triplicate. Cells were then sucked onto a 96-well printed filtermat by a cell harvester (cell harvester 95®, produced by

Tomtec Inc. USA). Finally, the cell numbers on the filtermat were counted by a Liquid scintillation & luminescence counter (Walac, USA).

3-7-2 Experiment of CPS activities on the production of cytokines

3-7-2-1 Preparation of murine splenocyte suspension

Four spleens obtained from mice were the source of immune cells used to investigate cytokine production. The mice were killed by inhalation of isoflurane (USP) and the removed spleens were washed three times, each with 2 mL TCM medium. The spleens were cut into small pieces using tweezers and a pipette to achieve a suspension of cells. The suspension was centrifuged (20 min, 1200 rpm) and the supernatant was discarded. The cell pellets were re-suspended in 5 mL ACK lysis buffer (150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA) and left for approximately 5 min to burst the red blood cells present. This was followed by centrifugation of the suspension (10 min, 1200 rpm), with the pellets containing mainly white blood cells. The cells were re-suspended in 5 mL TCM medium and centrifuged. The cells were then re-suspended in 3 mL TCM medium and the number of cells was counted using the flow cytometer (Model: FACSC, produced by Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Final cell concentration was adjusted to 2×10^6/mL in a total volume of 10 mL.

3-7-2-2 Sample preparations

Five EPS/IPS samples were isolated from the fermentation broth of ATCC-20545 and Wr-74. The freeze-dried IPS and EPS were obtained according to the procedures described in sections 3-6-1 and 3-6-3. Mushroom extracts of Wr-74 and ATCC-20545 were prepared by boiling the mushrooms in water for 3 hours. The extracts were precipitated with ethanol and freeze-dried. All samples were dissolved in distilled water, and then sterilized through cobolt-60 γ-radiation except for EPS from Wr-74 that was filter-sterilized using a 0.02-μm sterile filter.

3-7-2-3 Treatment of murine splenocytes with EPS, IPS and Mushroom extracts

The murine splenocytes harvested were treated with seven samples (Fig. 3.1). Each sample was prepared in four different concentrations, with each concentration prepared in triplicate. The samples were laid out in two 96-well plates as shown in

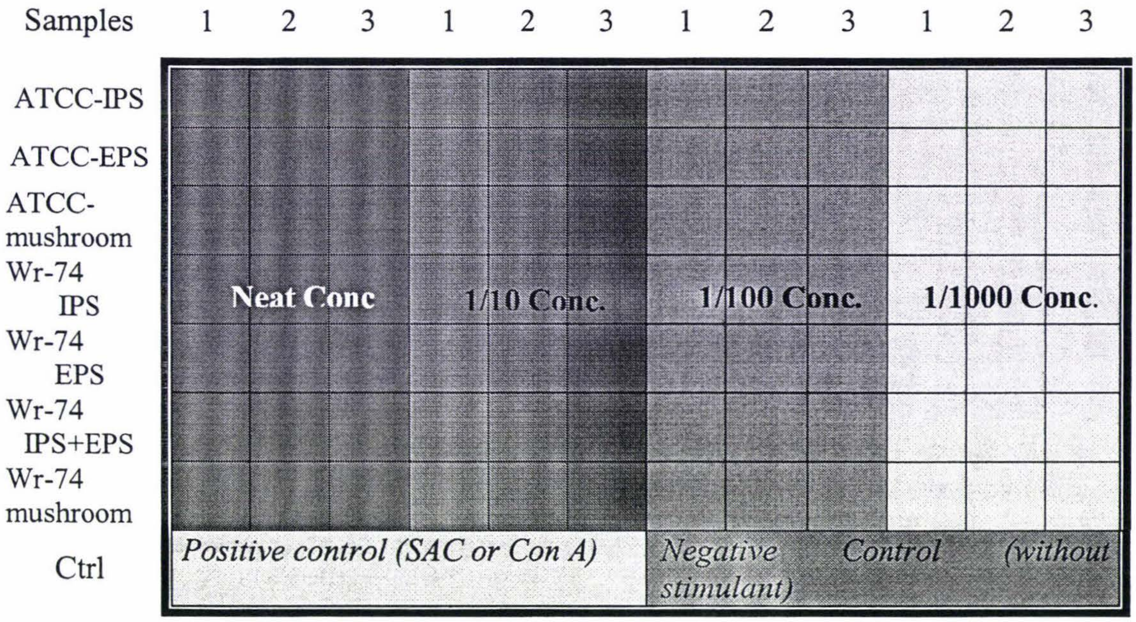


Fig. 3.1 Assay layout of seven samples with four dilutions plus positive and negative controls in a 96 well plate

Figure 3.1. Each well contained 50μL cells, 50μL sample solution and 50μL stimulant solution.

One 96-well plate included *Sarchromyces cerevisiae* (SAC) as a stimulant in all samples with a positive control sample (TCM medium used instead of sample), for the induction of interleukin-12 (IL-12). This plate was incubated for 20 hours at 37°C. Another 96-well plate had Concanavaline A (CON A) as stimulant in all samples with positive control (TCM medium was used instead of sample), for the induction of gamma-interferons (IFN-γ). This plate was incubated for 48 hours at 37°C. The negative controls in both plates contained only cells (50μL) in TCM medium (100μL). The supernatant from each well was collected and the level of IL-12 or IFN-γ was determined by ELISA.

3-7-2-4 Determination of cytokine production by ELISA

Enzyme-linked-immunosorbant assay (ELISA) is the most widely used type of Enzyme Immunoassays, which is based on an antigen-antibody interaction. The assay utilizes antibodies that are covalently linked to an enzyme. The enzyme causes a colour change when substrate is added. The detailed ELISA procedure used for detecting IFN-γ samples is described in the following.

A 96-well plate was coated with IFN-γ antibody (capture antibodies) specific to IFN-γ. The wells were sealed with block buffer and the supernatant (containing IFN-γ) to be tested was exposed to IFN-γ antibodies. The IFN-γ antibodies that combined with the antigens (IFN-γ) were detected by treating the test system with a conjugate, the IFN-γ antibody linked to HRPO enzyme (Horseradish peroxidase). This antibody enzyme complex served as a marker and reacted with the chromogenic substrate. When a substrate for the enzyme was added to the assay, a reaction between the substrate and the conjugate was indicated by a color change. If IFN-γ antibodies were present to bind with the antigen (IFN-γ), conjugate interaction would take place, and a blue color change would occur. The coloration was measured by a spectrophotometer. The levels of cytokine produced by the murine splenocytes were based on the absorbance, comparing it against the standard curves of IFN-γ.

Quantitative determination of IL 12 was conducted using the same procedure as that in the IFN-γ determination.

3-8 Analysis of broth samples

3-8-1 pH

The pH of the broth samples from shake flasks was measured using Mettle Toledo MP 220 pH metre (Mettler Toledo International Inc., Greifensee) that was calibrated daily with pH 4 and 7 buffer solutions prior to use.

3-8-2 Dry weight of biomass

The biomass was collected by filtration of a known volume (50-100 mL) of the broth sample through a Buchner funnel, on a No.541 hardened ashless filter paper. The biomass was washed thoroughly with distilled water and dried at 105 °C for 3-5 hours to a constant weight. The dry weight was expressed as mg/mL broth.

3-8-3 Relative viscosity

Relative viscosity of broth was determined by Fenske Routine Capillary Viscometer, module 100-821k, produced by Cannon Instrument Company, U.S.A. The measurements were carried out at 20 °C with water as the solvent. The relative viscosity was determined according to the equation:

$$RV=\frac{t_{sol}\cdot p_{sol}}{t_{solvent}\cdot p_{solvent}}$$

t_{sol} – the time for sample solution to flow from the position A to B of the viscometer; $t_{solvent}$ – the time for solution to flow from the position A to B of the viscometer; P_{sol} - solution density; $P_{solvent}$ – solvent density.

3-8-4 Monosaccharide determination

3-8-4-1 Phenol/Sulphuric acid method

Mono-, oligo- and poly-saccharides react with phenol and concentrated sulphuric acid at elevated temperatures resulting in the formation of colored substances with absorption maxima at 480-490 nm. The reaction mechanism reveals that, in the case of oligo- and polysaccharides, the external ether bridges are split. Parallel dehydration reactions take place yielding furfural derivatives, which condense with phenol to form triarylmethane dyes (Fig. 3.4). Pentoses, methylpentoses and hexuronic acids yield orange colored compounds with absorption maxima (λ_{max}) at 485 nm. (Scherz and Bonn, 1998).

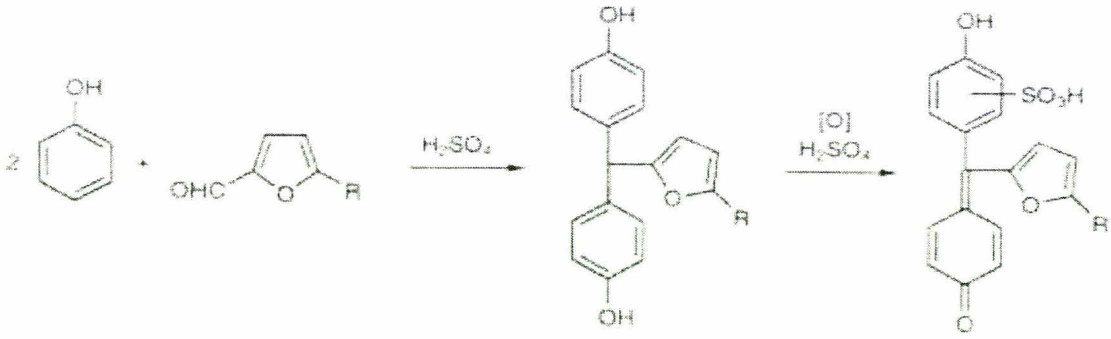


Fig. 3.2. Reaction between Phenol and Carbohydrates in the presence of concentrated sulphuric acid

- **Preparation of Phenol/Sulphuric acid reagent**

25 g phenol was dissolved and made to the volume with distilled water in 500 mL volumetric flask. The solution was kept from light and used within 3 months. Concentrated Sulphuric acid was used without any dilution. Careful handling of the chemical solutions is important during the operation because of their corrosive and toxic properties.

- **Standard curve**

The standard curve was based on glucose solutions (10 to 100 μg/mL). 1 mL of each standard solution was mixed with 1 mL of 5 % (w/v) phenol solution and then 5 mL of the concentrated sulphuric acid. The mixture was homogenized on a type 37600-vortex mixer (Barnstead Thermolyne Ltd, Dubuque, Iowa, USA) and left for at least 30 minutes prior to the determination. The absorbance of standard solutions was read against the blank at λ_{max} 485 nm from the UV/VIS spectrophotometer (model Ultrospec 2000, Cambridge, England). Plot of the glucose concentration versus absorbance was constructed in the linear range between 10 and 100 μg/mL of glucose.

- **Determination of total carbohydrates**

Samples were diluted with distilled water to concentrations ranging from approximately 10 and 100 μg/mL of glucose. The procedure for determining the total carbohydrate concentrations was similar to that for standard solution. The absorbance obtained was read against the standard curve and the dilution factors were taken into account. All determinations were performed in duplicate.

3-8-4-2 DNS method

Quantitative determination of glucose levels in broth was carried out using dinitrosalicylic acid (DNS) method. This colorimetric method involves the reaction of reducing sugar in an alkaline medium with dinitrosalicylic acid to give a brown-colored complex that absorbs strongly at the wavelength of 575 nm. The colored product is believed to be 3-amino-5-nitrosalicylic acid (Fig. 3.5) (Scherz and Bonn, 1998).

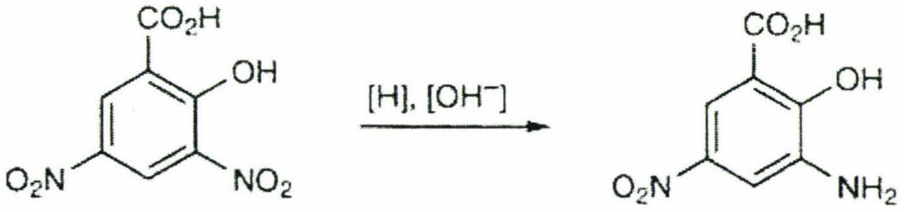


Fig. 3.3 Reaction between reducing sugars and 3,5-dinitrosalicylic acid

- **Preparation of DNS reagent**

The preparation of DNS reagent began with dissolving the following chemicals, in order, in about 600 mL distilled water. The volume was then made up to 1 liter with distilled water.

10 g	sodium hydroxide
182 g	potassium sodium tartrate (Rochelle Salt)
10 g	dinitrosalicylic acid (added slowly while stirring)
2 g	phenol
0.5 g	sodium sulfite

- **Standard curve**

The standard curve was based on the standard solutions of 0.1 to 1 mg/mL of glucose. Each 1 mL standard solution was diluted with 1 mL distilled water and mixed with 3 mL of the DNS reagent. After homogenizing on a vortex mixer, the mixtures were heated in boiling water for 15 minutes and cooled in cold water for at least 20 minutes. The absorbance of standard solutions was read against the blank at λ 575 nm from the above-described UV/VIS spectrophotometer. A plot of glucose concentration (mg/mL) versus absorbance was constructed in the linear range between 0.1 and 1.0 mg/mL of glucose.

- **Determination of total reducing sugars**

The broth samples were diluted with distilled water to 0.1 to 1 mg/mL glucose concentrations. The procedure for determining total reducing sugar concentrations was the same as that of the standard solution. The final result was based on the absorbance and comparing it against the standard curves and dilution factors. All determinations were performed in duplicate.

3-8-5 Determination of EPS/IPS

3-8-5-1 Phenol/Sulphuric acid method

The procedure used for quantitative determination of EPS and IPS using Phenol/Sulphuric acid method (Dubois *et al.*, 1956) was illustrated in Fig. 3.4.

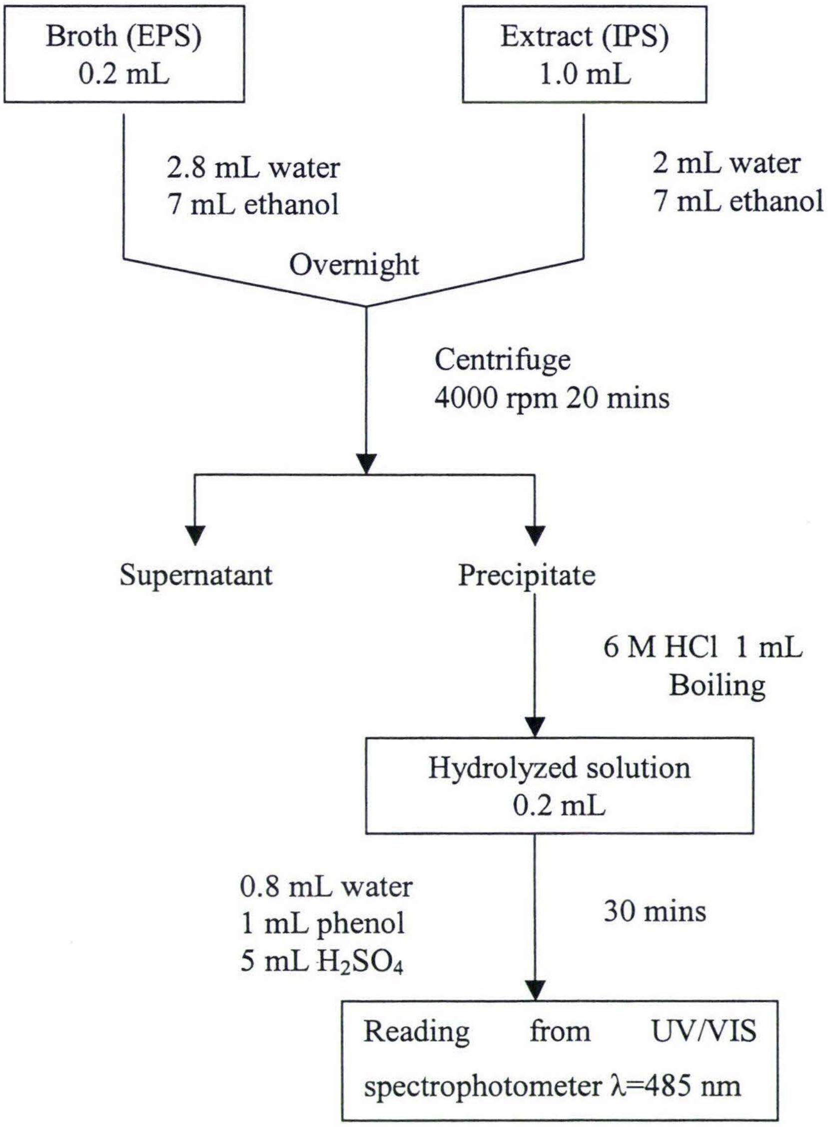


Fig 3.4 The procedure for the quantitative determination of EPS and IPS using Phenol/Sulphuric acid method

After precipitation, the EPS/IPS samples were hydrolysed with 6 M hydrochloric acid at 100 °C for 15 minutes. The hydrolysed solutions were diluted to the concentrations ranging between 10 to 100 μg/mL of glucose equivalent. After treating the samples with Phenol/Sulphuric acid, EPS/IPS levels were determined at λ 485 nm on the UV/VIS spectrophotometer. The final result was based on the absorbance and comparing it against the standard curves and dilution factors. All determinations were performed in duplicate.

3-8-5-2 DNS method and combined DNS and Phenol/Sulphuric acid method

In addition to the Phenol/Sulphuric acid method described in section 3-8-5-1, the DNS method and combined DNS and Phenol/Sulphuric acid method were also used for the determination of EPS/IPS. The key steps involved are deigned as shown in Fig. 3.5.

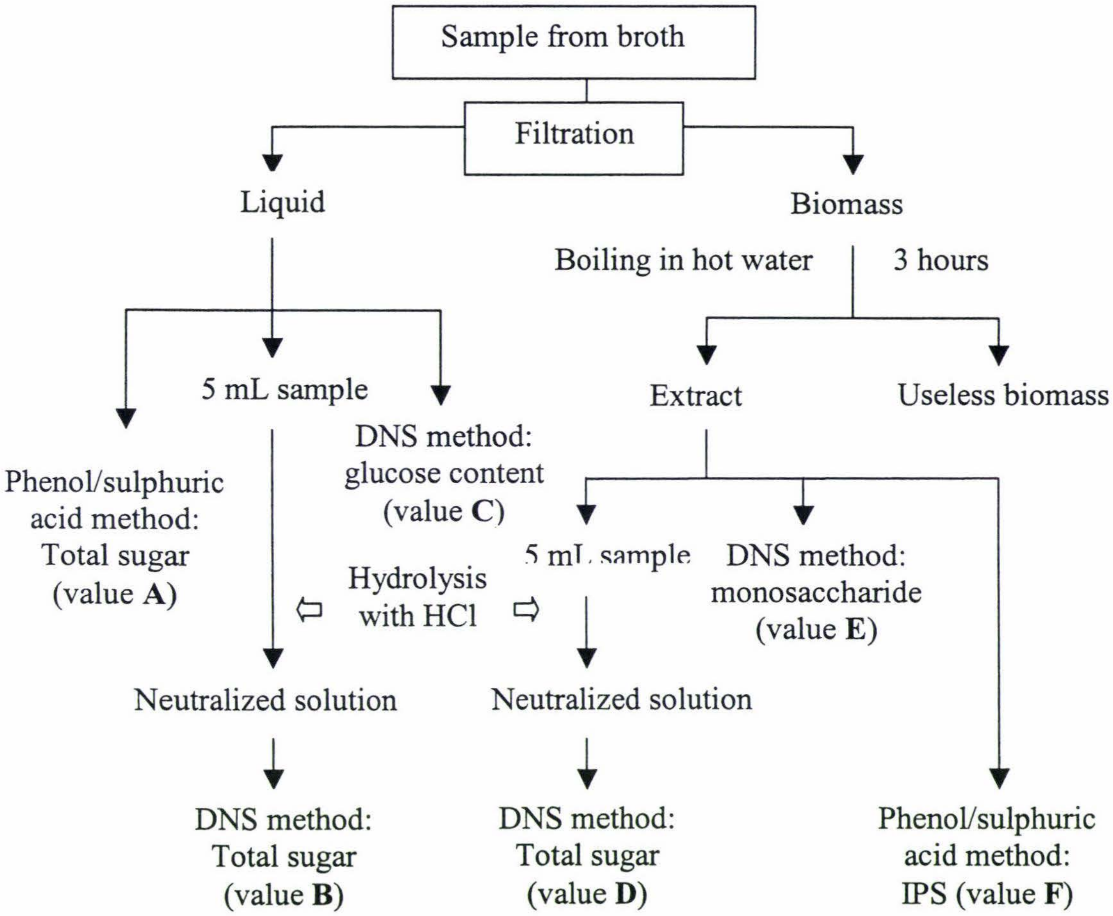


Fig.3.5 Procedures for quantifying EPS and IPS from fermentation broth. The polysaccharide levels were calculated according to following equations: EPS (mg/mL) = A-C or B-C; IPS (mg/mL) = F-E or D- E.

The DNS method is specific for determining reducing sugar levels while the Phenol/Sulphuric acid method is specific for total sugar determination. Therefore, EPS/IPS concentrations can be also determined by combining these two methods (You, et al., 2001). Based on the method, the EPS levels could be obtained by the value A minus C and the IPS levels can be obtained by the value F minus E. Another method of determining EPS/IPS concentrations was employed by Cui (2000) based on DNS method. Reducing sugar level in the sample and total reducing sugar level after EPS or IPS is hydrolysed are determined by NDS method. The levels of EPS or IPS can be obtained by comparing the values of the two. Therefore, according to Fig. 3.5, EPS level can be obtained by the value B minus C and IPS level can be obtained by the value D minus E. However, differences in results from these methods exist as shown in Appendix II.

3-8-6 Preparation of IPS sample prior to determination

IPS was extracted from broth samples before quantitative determination. A defined volume (50-100 mL) of the broth sample from shake flasks or fermenter was filtered through a Buchner funnel. The biomass cake was washed with ten-time volumes of distilled water and then suspended in about 80 mL distilled water or 0.2 M aqueous sodium hydroxide solution. The slurry was heated in a hot water bath of 85-95 °C for 3 hours. The extract was neutralized with hydrochloric acid to pH 6-7 after cooling and the exact volume was recorded for the dilution calculation. After filtration of the slurry, the filtrate was used for the level determination of IPS.

3-9 Characterization of CPS

3-9-1 Sample preparation

All the samples for the physical characterization were prepared based on the procedures described in section 3-5 and 3-6. All samples were diluted in distilled water to required concentrations and sterilized either by filtering the samples through 0.02-μ sterile filters or by autoclaving.

3-9-2 Determination of weight-average molecular weight using SEC-MALLS

Weight-average molecular weight (Mw) determination of CPS was carried out by size exclusion chromatography coupled to a multi-angle laser light scattering (SEC-MALLS). In the SEC-MALLS method, the laser beam passes through the diluted solutions of the polysaccharide samples. The intensity of scattered light is measured as a function of the scattering angle. The Mw can be obtained according to the Zimm's equation (Kennedy and White, 1983).

The set-up consisted of Shodex OHpak SB-806 HQ column (Japan), LC 1200 UV detector (GBC Scientific Equipment Pty Ltd, Dandenong, Victoria, Australia), laser light scattering detector (Dawn®DSp Laser Photometer, Wyatt Technology Corporation, USA) and RI 2000 RI detector (Schambeck SFD GmbH Bad Honnef, Germany) in series. The HPLC system was equipped with LC 1150 HPLC pump (GBC Scientific Equipment Pty Ltd, Dandenong, and Victoria, Australia). Flow rate was controlled at 0.5 mL/min after 20 µl sample was injected. Mili-Q water containing 0.02 % w/v sodium azide was used as the mobile phase. The helium-neon laser had a wavelength of 632.8 nm. The Debye plot was used for the light scattering data. A dn/dc value of 0.14 was assumed based on the average value of most polysaccharide samples. The Aux calibration constant of 1.059E-3 was determined prior to the experiment.

The solvent/mobile phase was filtered through a 0.2 µm followed by 0.02 µm filter to remove dust particles and degassed. A 1 % (w/v) dextran (molecular weight: 9,300 Da, from DIFCO Laboratory, Detroit, Michigan, U.S.A.) solution was used for normalization before introducing the CPS sample.

3-9-3 Total nitrogen contents of EPS

Total nitrogen contents were determined by Leco FP-2000 nitrogen/protein detector (Leco Corporation, St. Joseph, Michigan, U.S.A.). Each crude EPS sample (100 mg) from ATCC-20545 and Wr-74 was used for the determination. The protein content was calculated based on the conversion factor of 6.25

3-9-4 Amino acid analysis

Quantitative analysis of amino acids was conducted by the amino acid analyser using Waters® HPLC system (Waters Corporation, Milford, U.S.A.), which include Waters® 715 Ultra Wisp Sample Processor and Waters® UV/VIS 490 E detector. Analysis of protein hydrolysates from both standard and sample was performed on an ion-exchange column.

3-9-5 HPLC analysis of monosaccharides

Quantitative analysis of monosaccharide samples was performed using Waters® 2690 Separation module HPLC platform (Waters Corporation, Milford, U.S.A.). The Aminex® HPX-87H column (catalog number 125-0140, Bio-Red Laboratories, Hercules, California, U.S.A.) was used for all separations.

The detectors were Waters® 2410 differential refractometer and Water® 2487 Dual λ UV/Vis absorbance detector (Waters Corporation, Milford, U.S.A.). Analysis was conducted at ambient temperature. The mobile phase was 0.018 M sulphuric acid solution and was filtered through 0.2 μm membrane filter and degassed prior to use. The flow rate was 0.6 mL/min. 20 μl of all unknown samples that passed 0.2 μm membrane filter was injected in duplicate into the HPLC system.

The quantitation of individual monosaccharides was conducted by measuring the peak height of the sample with reference to peak height of a standard curve. The standard curves were linear within the range of 2.5 g to 10 g/L. All programmes of HPLC were driven by "Millenium^{32}" software.

3-9-6 Preparation of hydrolysed samples for HPLC analysis

3-9-6-1 Hydrolysis of lactose in the milk permeate

Hydrolysed milk permeate (HMP) was used in this project as a main component of fermentation medium for CPS production. The HMP samples were analysed by HPLC to investigate the extent of lactose hydrolysis to glucose and galactose. The enzyme, Maxilact® L2000 (activity > 2000 NLU/g), was used for the hydrolysis of lactose in the milk permeate. After a series of experiments on the hydrolysis conditions, optimum enzyme concentrations in the milk permeate were found between 0.04 and 0.06 % (v/v). The enzymatic reaction was carried out under agitation (120 to 150 rpm) at 38 °C for 20 to 24 hours with the pH of milk permeate adjusted to 6.8 prior to hydrolysis. The obtained HMP can be thus used for the medium preparation and autoclaved at 120 °C for 15 minutes. The hydrolytes were analysed by the HPLC.

3-9-6-2 Hydrolysis of EPS/IPS

The hydrolysis of EPS/IPS was adopted from the method described by Scherz and Bonn (1998). The EPS/IPS were hydrolysed either by sulphuric acid or trifluoroacetic acid (TFA). Because TFA, a volatile acid is easily removed by heating, it is more widely used for such purpose.

- **Hydrolysis of EPS/IPS with Sulphuric acid**

Each sample (11 to 15 mg) of EPS/IPS was suspended in 1 M aqueous sulphuric acid and heated in sealed tubes at 100 to 105 °C for 6 to 8 hours until no pellet was seen. After cooling, the acidic solution was diluted to 25 mL and neutralized by 0.25 M aqueous barium hydroxide solution to pH 5 to 6. To remove sulphate ions, the neutralised slurry was centrifuged under 4000 rpm for 15 minutes. After standing for 1 hour, the supernatant was concentrated on the rotary vacuum evaporator to dryness. Ezch sample was redissolved in about 2 mL water and then passed through 0.2 μm filter. The filtrate was subjected to HPLC analysis.

- **Hydrolysis with Trifluoroacetic acid (TFA)**

Each sample (5 mg) of EPS/IPS was suspended in 2 mL of 2 M aqueous TFA and heated in a sealed tube at 100 °C for 20 hours. After cooling, each of the hydrolysed sample was diluted with water and evaporated on the rotary evaporator under vacuo to dryness. The sample was redissolved in about 2 mL water and then passed through a 0.2 μm filter. The filtrate was subjected to HPLC analysis.

CHAPTER IV STRAIN SCREENING

4-1 Introduction

Since CPS were developed in the late 1970s, a variety of *Coriolus versicolor sp.* has been investigated and screened for CPS production. From the American Type Culture Collection (ATCC) organization, 39 species of *Coriolus versicolor* were recorded. Of the 16 strains deposited in ATCC by Kureha Chemical Industry Ltd, CM-101 was described as a high CPS producing strain (Hotta, *et al.*, 1981). However, CM-103 that also produced high CPS level was more widely reported (Sugiura *et al.,* 1980; Ueno *et al.,* 1980a,b; Yoshikumi, *et al.,* 1978a,b; Hotta, *et al.*, 1981). For many other CPS products available on the markets such as PSP, the strains used in the production of CPS were different from those deposited in ATCC.

The objective of the work described in this chapter was to select a high EPS/IPS producing strain among 12 strains provided by the New Zealand Forest Research Institute. The selected strain would be compared to ATCC-20545 strain (CM-103) on the basis of EPS/IPS level, weight-average molecular weight (Mw) and physical appearance of the fruit bodies. Milk permeate (MP) was used as the base medium in the latter part of the strain screening process which is discussed in detail in Chapter V.

4-2 Results and discussion

4-2-1 Preliminary screening of the strains

The first two batches of the preliminary screening were carried out based on eight strains grown in shake flasks containing MEG medium (Table 3.5) and cultured at 27 °C for 6 days. The culture broth from the flasks was harvested at the end of fermentation and the samples were analyzed for the levels of EPS produced. Other measurements included pH, biomass dry weight, and relative viscosity. One of the experimental results is summarized in Table 4.1.

The eight strains were listed in increasing order of EPS levels. The results indicated that all eight strains grew well and produced relatively high levels of EPS in

Table 4.1 Preliminary screening results based on the MEG medium

Strains for screening	Wr-61	Cv-15D	Wr-35	Wr-45	Cv-75	Wr-74	ATCC-20545	Wr-87
EPS levels (mg/mL)	1.31	2.11	2.19	2.30	2.35	2.92	3.25	3.38
EPS yield on biomass ($Y_{p/x}$)	0.114	0.184	0.185	0.245	0.211	0.320	0.258	0.275
pH at end of fermentation	2.55	2.80	3.10	3.69	4.00	3.20	2.91	2.63
Biomass dry weight (mg/mL)	11.52	11.44	11.84	9.39	11.16	9.13	12.60	12.29
Relative viscosity	1.06	6.27	8.35	4.02	1.82	14.01	24.71	69.15

the MEG medium. The average biomass dry weight was approximately 11 mg/mL and the final broth pH values ranged between 2.5 and 4. Generally, relative viscosity of broth increased with the EPS concentration, with the exception of strains Wr-75 and Wr-45. The highest EPS concentration (3.38 mg/mL) produced by strain Wr-87 was accompanied by a high biomass dry weight (12.29 mg/mL) and an extra-ordinarily high relative viscosity (69.15). The ATCC-20545 and Wr-74 strains also appeared to be good producers of EPS. In terms of the EPS yield on biomass ($Y_{p/x}$), strain Wr-74 presented a highest $Y_{p/x}$ value (0.320), suggesting its character of a highly productive strain. All pH values decreased at the end of fermentation. The dry weight of biomass had no strong correlation with EPS production, which is well in accord with the report by Cui (2002).

Four more fermentation batches were carried out during the preliminary stage to screen ten strains of *C. versicolor* using 50%MP-MEG medium (Table 3.5). Strains Wr-75B and Wr-75C were excluded in the screening simply because of their extremely slow growth on PDA agar slant. The results are shown in Fig. 4.1 to 4.5 and the error bar on each column is calculated based on the standard deviation of data from the four batches. The results were rather constant with those obtained in the previous experiments given in Table 4.1. Strain Wr-74 consistently had the highest $Y_{p/x}$ (0.309, Fig. 4.2). The relative viscosities of 11 strains generally followed the trend of EPS production with the exception of strains Wr-35, Wr-45, Cv-75E and Cv-75D (Fig. 4.1 and Fig. 4.5).

However, neither pH values (Fig. 4.3) nor dry weights of biomass (Fig. 4.4) correspond with EPS levels.

ATCC-20545 and Wr-74 produced the highest EPS levels (3.19 and 2.82 mg/mL, Fig. 4.1) in 50%MP-MEG medium. Wr-87 ranked in the fourth place of EPS production in the 50%MP-MEG medium (Fig. 4.1) compared to being in the first place in MEG medium. This indicated that the same strain could behave differently in different media. However strains Wr-74 and ATCC-20545 were consistent in producing high levels of EPS in both MEG medium and 50%MP-MEG medium (Table 4.1 and Fig. 4.1), suggesting that the milk permeate could be used as a base medium for the production of EPS.

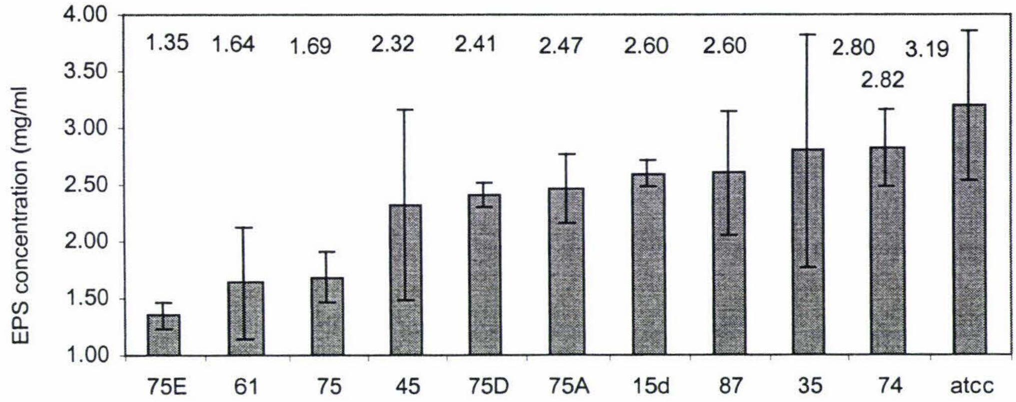


Fig. 4.1 EPS production profile in 50%MP-MEG broth

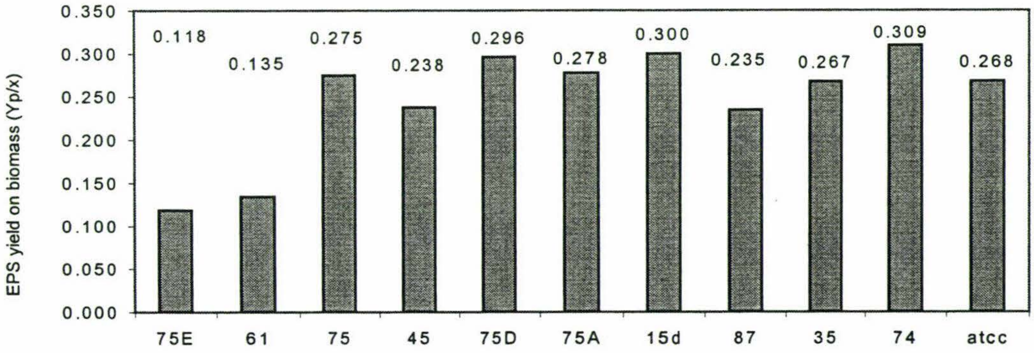


Fig.4.2 Profile of EPS yields on biomass ($Y_{p/x}$) in 50%MP-MEG broth

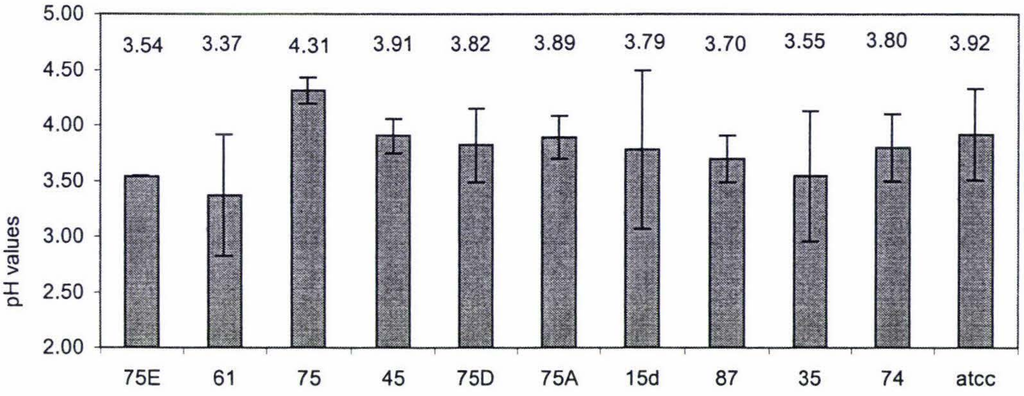


Fig. 4.3 pH values of 50%MP-MEG broth at the end of fermentation

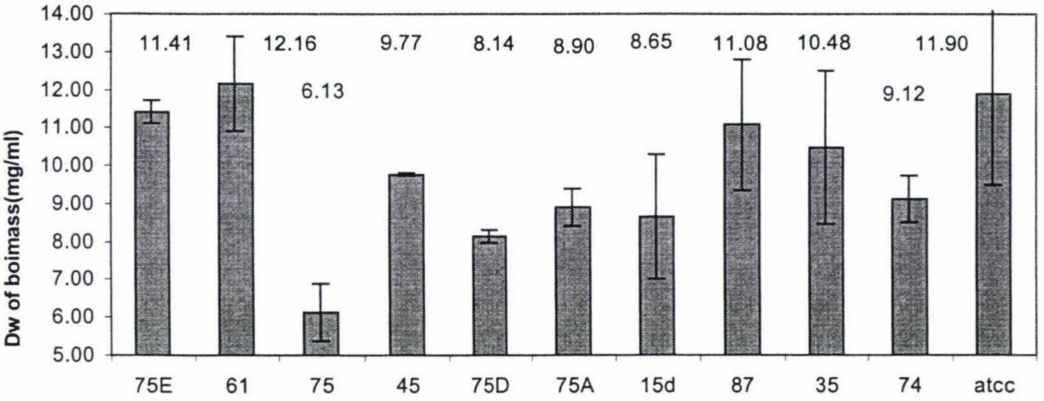


Fig. 4.4 Biomass dry weight profile in 50%MP-MEG broth

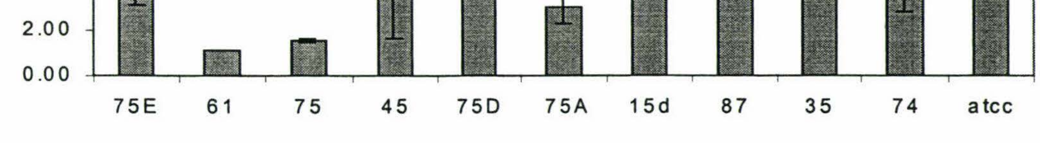


Fig. 4.5 Relative viscosity profile of 50%MP-MEG culture

4-2-2 Secondary screening of the selected strains

In the secondary screening stage, five strains were selected according to the results of the preliminary screening. Two fermentation batches were carried out in 50%MP-MEG medium with ATCC-20545 as control. The results are shown in the Fig. 4.6 to Fig. 4.11. The error bar on each column is calculated based on the standard deviation of data from the two fermentation batches.

Among the five strains screened, Wr-74 produced the highest EPS level (3.48 mg/mL, Fig. 4.6) although its IPS concentration was a bit lower than the ATCC-20545 and Cv-75D (Fig. 4.7). Unlike the preliminary screening, the biomass dry weights

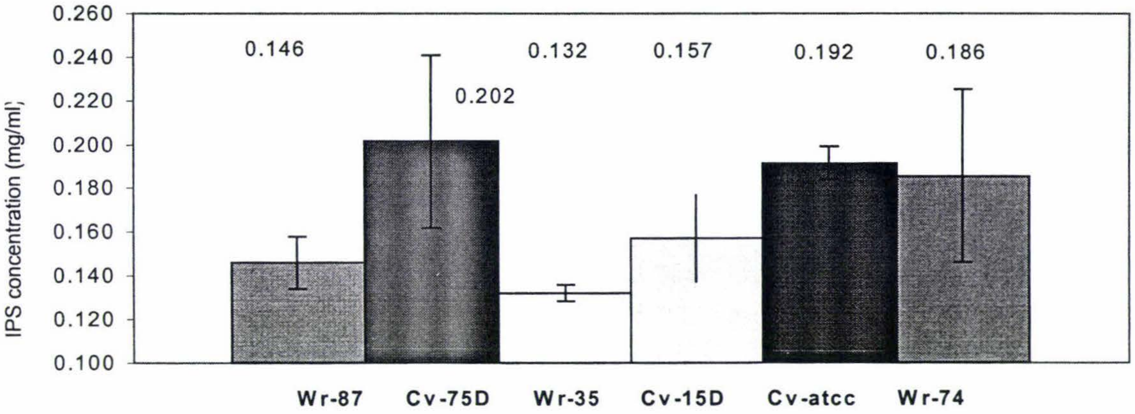


Fig. 4.6 IPS production profile in 50%MP-MEG broth

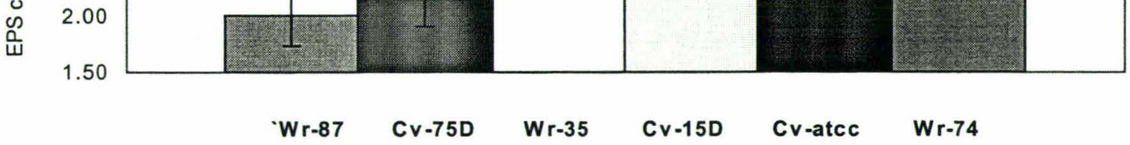


Fig. 4.7 EPS production profile in 50%MP-MEG broth

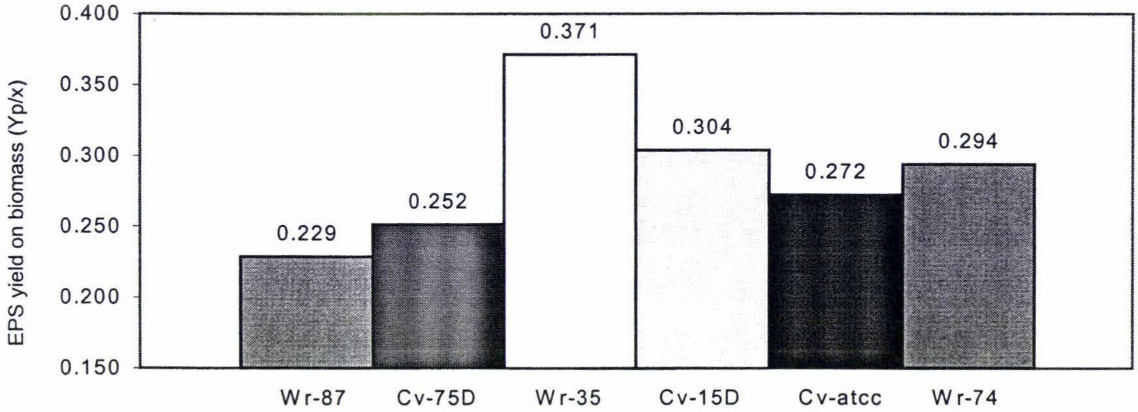


Fig. 4.8 Profile of EPS yields on biomass ($Y_{p/x}$) in 50%MP-MEG broth

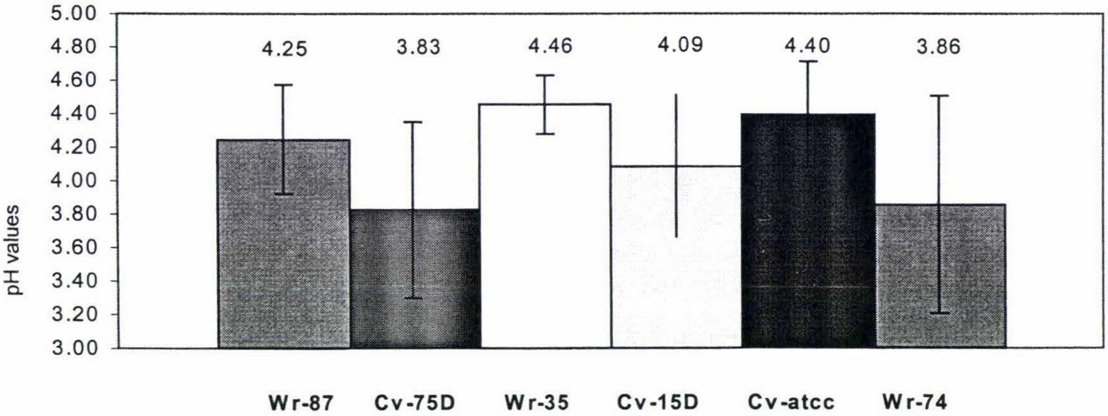


Fig. 4.9 pH values of 50%MP-MEG broth at the end of fermentation

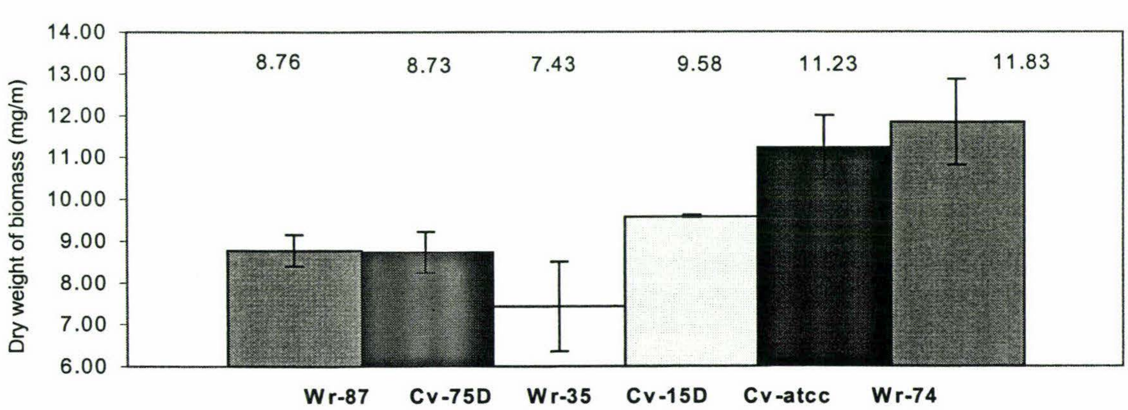


Fig. 4.10 Biomass dry weight profile in 50%MP-MEG broth

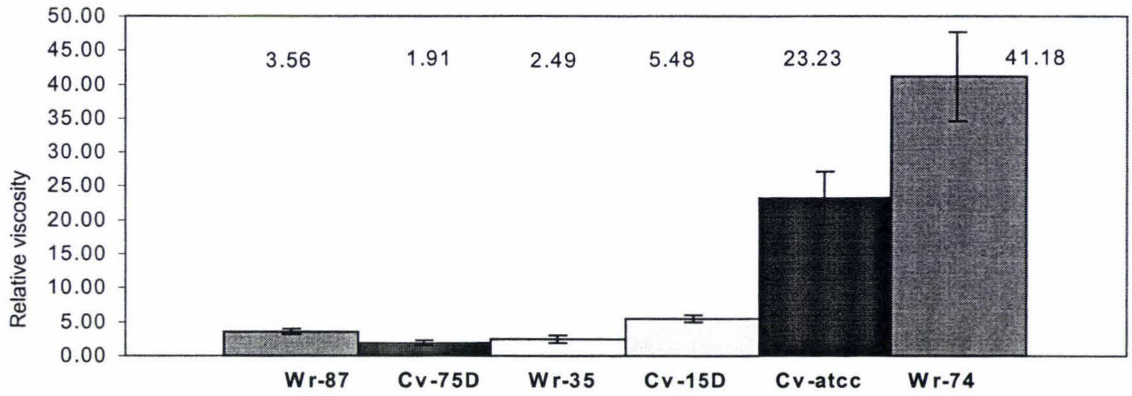


Fig. 4.11 Relative viscosity profile of 50%MP-MEG culture

and relative viscosities of the six strains had very similar trend with the levels of EPS produced (Fig. 4.10 and Fig. 4.11). However, the unusually low biomass dry weight of Wr-35 led to an exceptionally high EPS yield on biomass ($Y_{p/x}$) (Fig. 4.8 and 4.10) possibly due to an error in the determination of dry weight of biomass. The $Y_{p/x}$ value of Wr-74 was consistently amongst the highest. Though the Cv-15D presented a higher $Y_{p/x}$ (0.304, Fig. 4.8) than the rest of strains, its EPS and IPS production were considered too low. Interestingly, Wr-87 that once produced high levels of EPS turned out to produce the lowest level of EPS in these two fermentation batches. This could be ascribed to its genetic instability over several generations, leading to changes in genetic background. The final pH values of the six strains (Fig. 4.9) constantly showed no correlation with other parameters.

4-2-3 Comparison of the strains, ATCC-20545 and Wr-74

4-2-3-1 EPS/IPS production and metabolic profile of *C.versicolor*

By comparison and analysis of data obtained from the six batches of preliminary screening and two re-screening experiments, both Wr-74 and ATCC-20545 strains presented close similarities in terms of EPS and IPS production and their metabolic patterns (Fig. 4.12). Error bar on each column is calculated based on the standard deviation of data from the six screening batches.

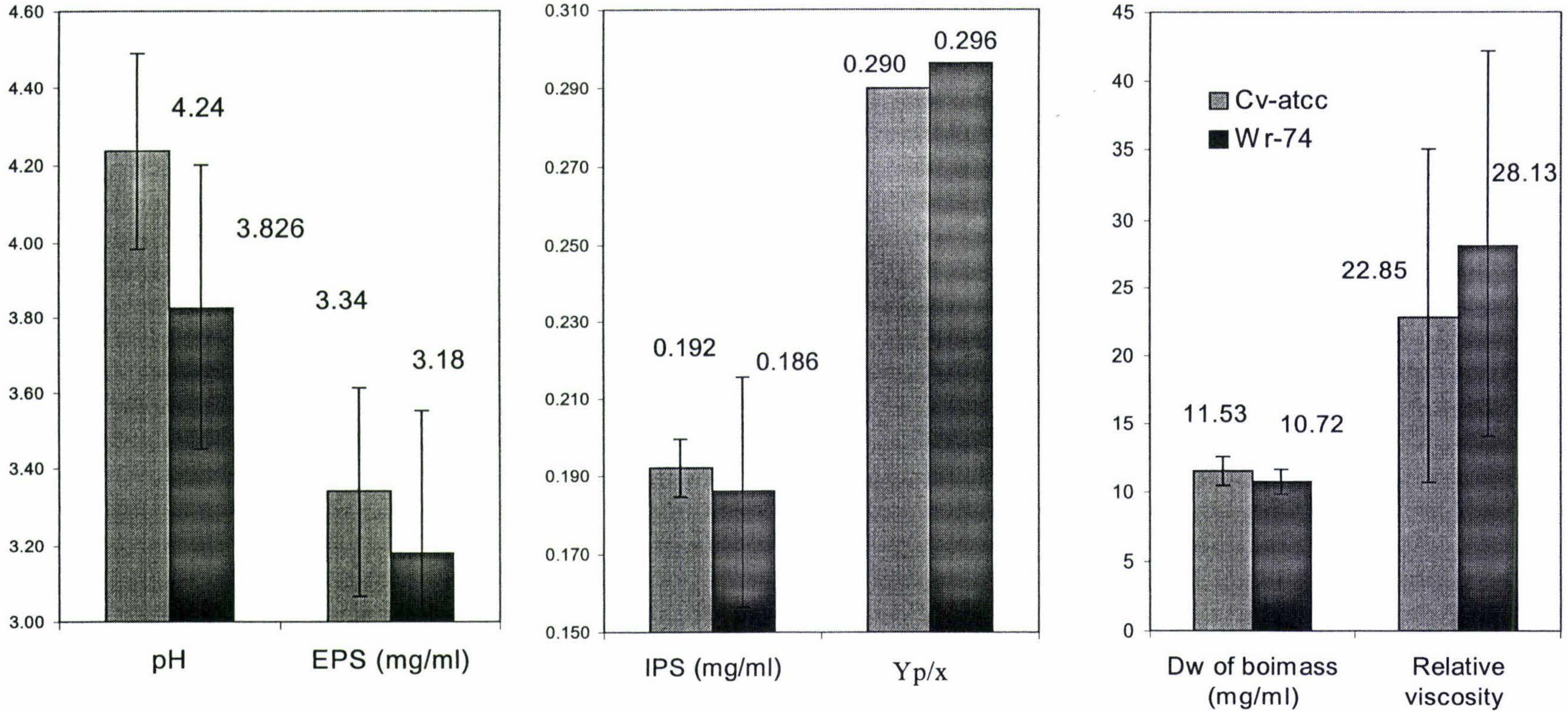


Fig. 4.12 Comparison of the metabolic, EPS and IPS production profiles of strains ATCC-20545 and Wr-74 The average values shown were obtained from six batches of shake flask fermentation

ATCC-20545 produced slightly higher levels of EPS and IPS than Wr-74. However, the slightly lower biomass dry weight of Wr-74 led to a higher $Y_{p/x}$ value than ATCC-20545. The largest difference between the two strains was observed in their relative viscosities, which was approximately 18.8 %. However, the large deviation error bar indicated that the viscosities varied widely among the batches and the average values should not be regarded as absolute. Overall, Wr-74 was considered to be most similar to ATCC-20545 in terms of EPS/IPS production and metabolic patterns even though other small differences were noted in the appearance (less yellowish) and odor (less sweet) of the fermented broth.

4-2-3-2 Weight-average Molecular weight of EPS/IPS

The weight-average molecular weights of CPS from ATCC-20545 and Wr-74 were determined by a Size-Exclusion Chromatography coupled to a Multi-Angle Laser Light Scattering (SEC-MALLS). Characterization of the EPS and IPS by SEC-MALLS provided the absolute weight-average molecular weight (Mw) of the macromolecules in dilute solution. The amount of light scattered is directly proportional to the product of the weight-average molar mass and the concentration of the macromolecule ($LS \propto M_w \times c$). The angular variation of scattered light is directly related to the size of the molecule. The Rayleigh Debye-Gans light scattering model for dilute polymer solutions can be expressed as the following equation (Zimm's formalism):

$$\frac{K^*c}{R(\Theta)} = \frac{1}{MwP(\Theta)} + 2A_2c$$

$R(\Theta)$ is the excess intensity of scattered light at DAWN angle Θ

c is the sample concentration

Mw is the weight-average molecular weight (molar mass)

A_2 is a second viral coefficient

K^* is an optical parameter equal to $4\pi^2 n^2 (dn/dc)^2 / (\lambda_0^4 N_A)$

dn/dc is the value used for mass calculation using the RI detector signal

n is the solvent refractive index and dn/dc is the refractive index increment

N_2 is Avogadro's number

λ_0 is the wavelength of the scattered light in vacuum.

$P(\Theta)$ is the function that describes the angular dependence of scattered light.

The setup of the static light scattering experiments consists of three detectors: UV absorbance (UV), light scattering (LS) and refractive index (RI), connected in series. The Debye method was used to fit the data. In the experiment, a dn/dc value of 0.14 was used based on the average values of most known polysaccharides. The Mw of the purified EPS/IPS samples were obtained by the ASTRA® software.

The SEC-MALLS chromatograms generally showed two poorly separated peaks seen in all EPS and IPS samples. It appeared that the EPS and IPS each contained two fractions of polysaccharides, each having different Mw. The Mw values of both the EPS and IPS were based on the two prominent peaks of elution volumes of 8.2 – 8.7 mL and 9.8-10.3 mL, respectively (see Fig. 4.13 and Fig. 4.14). Among all the samples, only

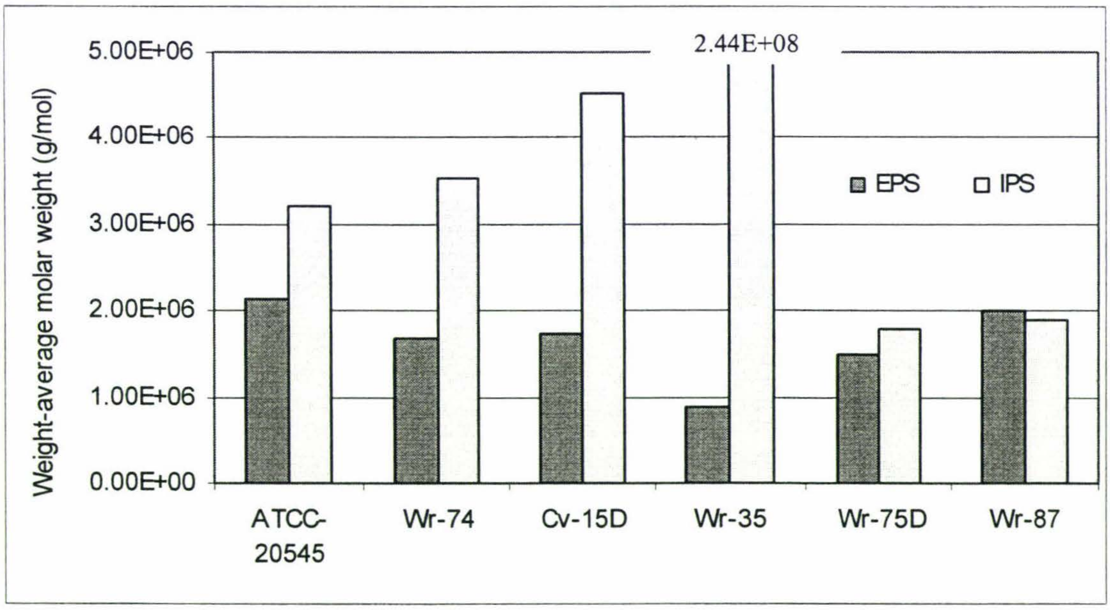


Fig. 4.13 Weight-average molar weights of EPS and IPS from six strains in the elution volume range of 8.2 – 8.7 mL of SEC-MALLS chromatograms

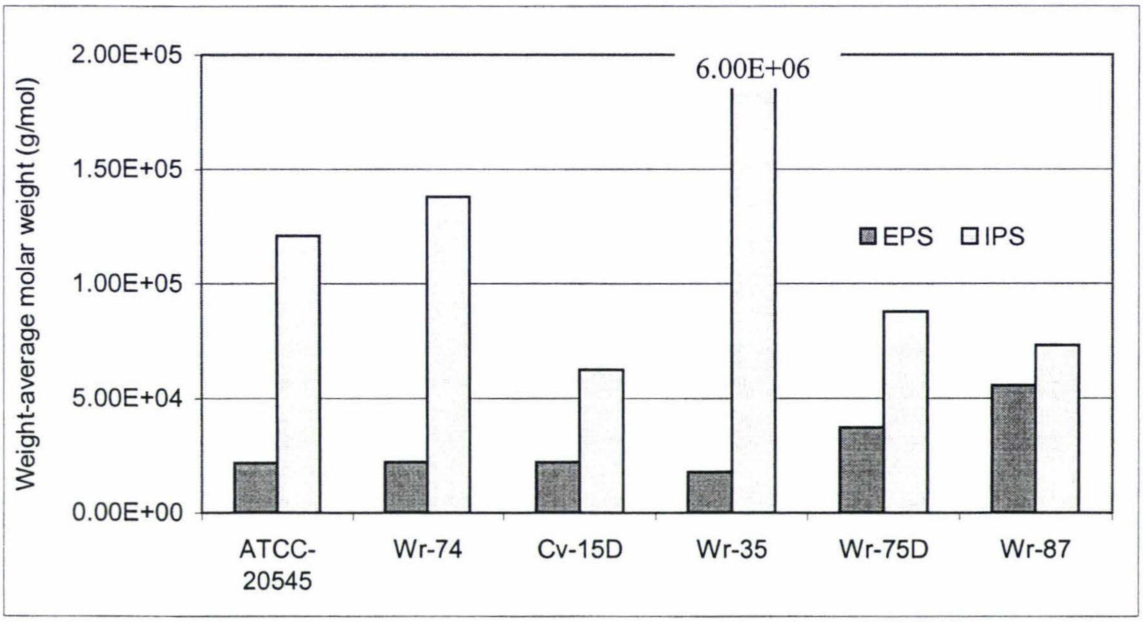


Fig. 4.14 Weight-average molar masses of EPS and IPS from six strains in the elution volume range of 9.8- 10.3 mL of SEC-MALLS chromatograms

the Mw values of the EPS and IPS samples from the ATCC-20545 and Wr-74 strains were most similar in these two elution volume ranges as shown in Table 4.2. The chromatograms in Fig. 4.15 to Fig. 4.18 present the UV (AUX1), RI (AUX2) and LS signals of EPS and IPS from the two strains. It was noted that that the EPS from both

Table 4.2 Weight-average molar weights of EPS and IPS from the both strains in two elution volume ranges of SEC-MALLS chromatograms

Samples	8.2 - 8.7 mL	9.8 - 10.3 mL
EPS from ATCC-20545	(2.138 ± 0.479)e+6	(2.184 ± 0.038)e+4
EPS from Wr-74	(1.676 ± 0.292)e+6	(2.224 ± 0.165)e+4
IPS from ATCC-20545	(3.203 ± 0.097)e+6	(1.208 ± 0.100)e+5
EPS from Wr-74	(3.526 ± 1.197)e+6	(1.379 ± 0.089)e+5

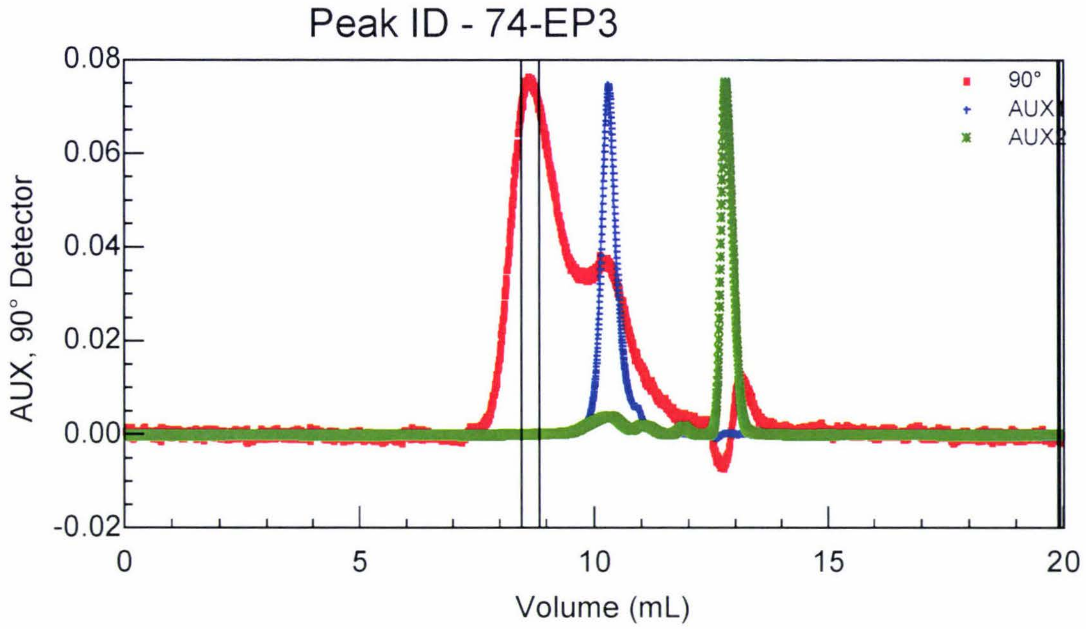


Fig. 4.15 **EPS from Wr-74: UV (AUX1), RI (AUX2) and LS traces for the detector at 90 ° angle (the highest light scattering signal)**

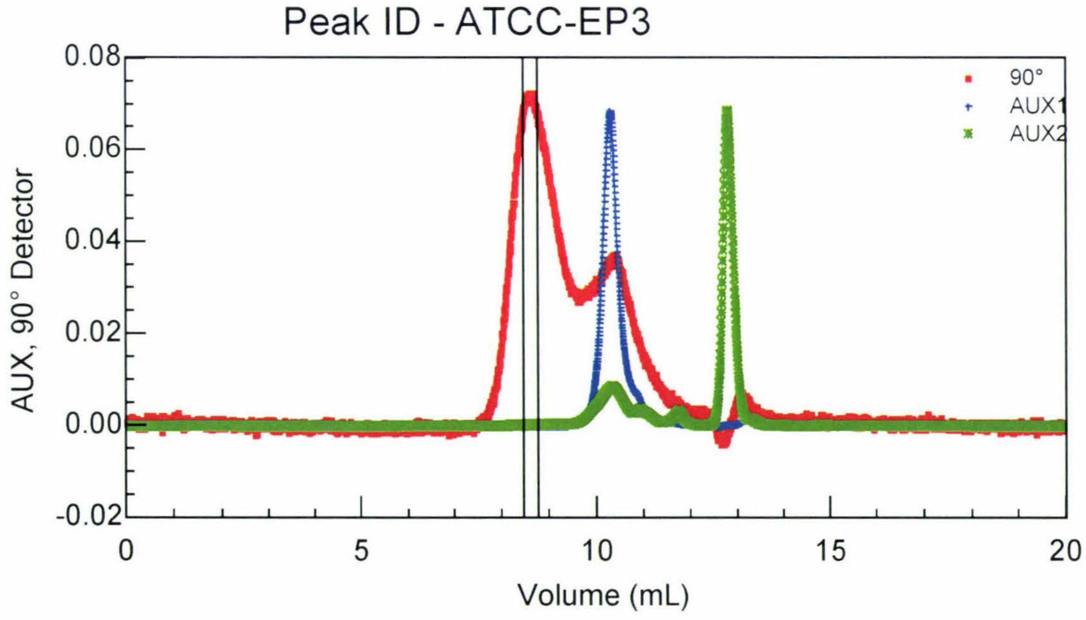


Fig. 4.16 **EPS from ATCC-20545: UV (AUX1), RI (AUX2) and LS traces for the detector at 90 ° angle (the highest light scattering signal)**

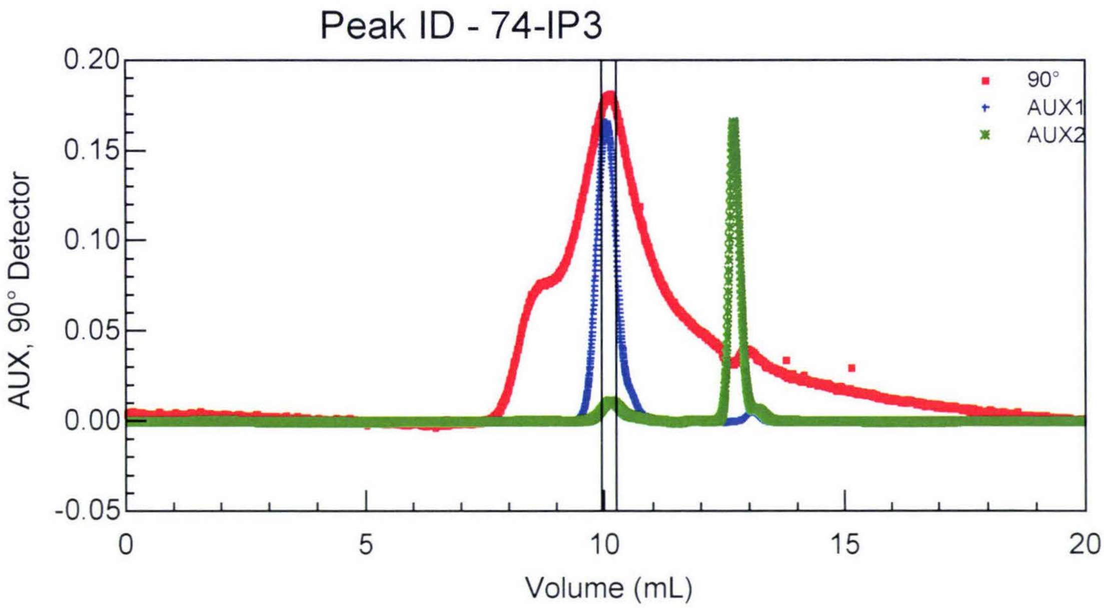


Fig.4.17 IPS from Wr-74: UV (AUX1), RI (AUX2) and LS traces for the detector at 90 ° angle (the highest light scattering signal)

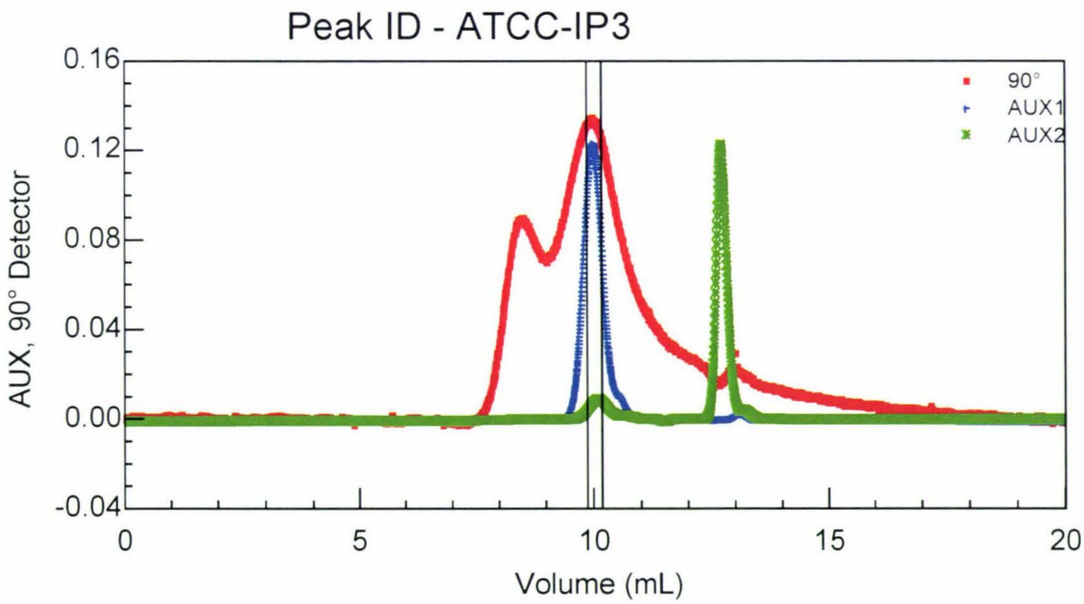


Fig. 4.18 IPS from ATCC-20545: UV (AUX1), RI (AUX2) and LS traces for the detector at 90 ° angle (the highest light scattering signal)

strains contained more of the larger Mw fraction (~ 10^6 g/mol) in the elution range of 8.2 - 8.7 mL. This fraction contained only polysaccharides compared to the smaller Mw fraction (~10^5 g/mol) that also contained protein in the elution range of 9.8 - 10.3 mL. On the other hand, the IPS from both strains contained more of the smaller Mw fraction (~ 10^5 g/mol) in the elution range of 9.8 - 10.3 mL than the larger Mw fraction (~ 10^6 g/mol) in the elution range of 8.2 - 8.7 mL. The presence of the UV-absorbing material in the smaller Mw fraction in the elution range of 9.8 - 10.3 mL also showed the presence of protein in addition to polysaccharides. This agreed with the widely reported protein-bound polysaccharides of *C. versicolor*. The larger Mw fraction in IPS could be a carry-over from the EPS. Comparing with chromatograms of the polysaccharides from the six strains, only Wr-74 and ATCC-20545 presented very similar Mw distributions. Therefore, Wr-74 was more likely to produce EPS/IPS with Mw of greater similarity to ATCC-20545 than other strains.

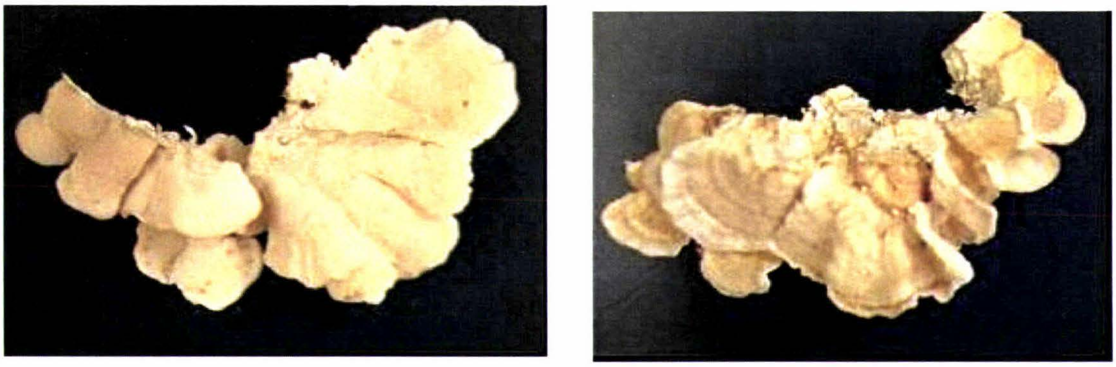
4-2-3-3 Fruit bodies

Further comparative study of both ATCC-20545 and Wr-74 strains was conducted by morphological examination of their fruit bodies. In this experiment, ATCC-20545 and Wr-74 were cultured on the artificial logs as described in section 3-5-5 to form fruit bodies. The photographs of the fruit bodies of the ATCC-20545 and Wr-74 strains shown in Fig 4.19 (e) and (f) were taken after 33 days and 55 days of cultivation respectively, both under similar conditions. The fruit bodies of Wr-74 strain appeared 20 days later than the ATCC-20545 strain.

C. versicolor is commonly known as “turkey tail” mushroom in North America, which aptly describes it. By examining the physical appearance (see Table 4.3), the two fruit bodies were very similar in the shapes and zoned patterns of the bracket’s top and reverse side. Since the fruit bodies of Wr-74 were still in their early stages of growth, the appearance of the densely overlapping brackets on the logs was not fully formed as in ATCC-20545 strain (see Fig. 19 (a) to (d)). However, the fine structures on both side of brackets looked almost the same, which possessed the densely hairy upper-surfaces and homogenous polyporous structures on the reverse side of the brackets.

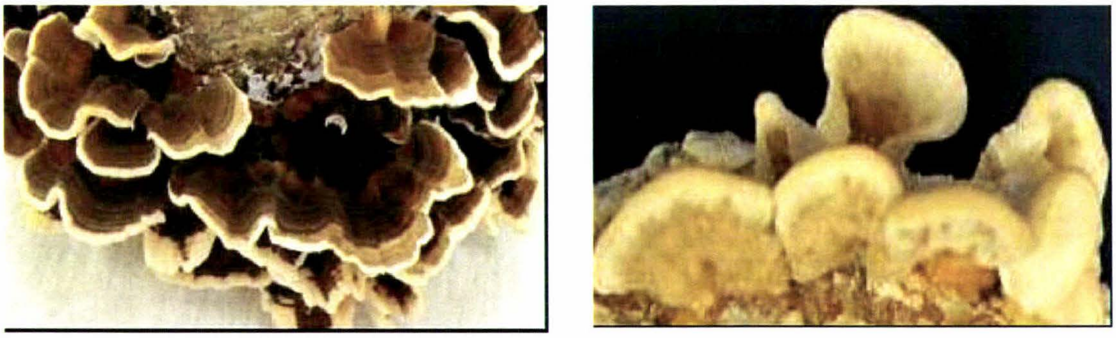
(a) Reverse side of ATCC-20545 mushroom

(b) Top side of ATCC-20545 mushroom



(c) Reverse side of Wr-74 mushroom

(d) Top side of Wr-74 mushroom



(e) ATCC-20545 mushroom on log

(f) Wr-74 mushroom on log

Fig. 4.19 Morphological examination of *Coriolus versicolor* fruit bodies from the strains of ATCC-20545 and Wr-74.

Table 4.3 Comparison of the main morphological features of the fruit bodies of ATCC-20545 and Wr-74 cultivated in artificial environment

Morphological features	ATCC-20545	Wr-74
Mushrooms on logs	Dense overlapping brackets with semicircular, flattened, thin and tough pileus	Dense overlapping brackets with semicircular, flattened, thin and tough pileus
Broadness of pileus	2.5 – 3.5 (mature)	1 –1.5 (not fully mature)
Colorfully zoned surface of pileus	Concentric dark brown and yellowish brown zones with wavy margins.	Concentric yellow zones with light brown wavy margins
Pileus surface under microscopic*examination	Densely hairy	Densely hairy
Reverse side surface of pileus under microscopic*examination	Delicately polyporous with no zoned colours	Delicately polyporous with no zoned colours
Stalk	absent	absent
Edibility	Too leathery to be eaten	Too leathery to be eaten

* OLYMPUS VMZ Inverted Microscope

Based on the description by Soothill and Fairhurst (1977), both ATCC-20545 and Wr-74 strains are *Coriolus versicolor*. Although the colour of Wr-74 fruit bodies was yellowish while that of ATCC-20545 was brown, both colors were included in the description of the *C. versicolor* fruit bodies by Soothill and Fairhurst (1977).

4-3 Conclusions

The results of the strain screening experiment demonstrated that Wr-74 was the most productive strain in terms of EPS/IPS production among the ten selected strains in 50%MP-MEG medium. Based on the metabolic patterns and molecular weights of EPS/IPS, Wr-74 appeared to be the most comparable to ATCC-20545. In addition, morphological examination of the fruit bodies of ATCC-20545 and Wr-74 by the means of visual assessment and microscope examination confirmed that the two strains belonged to *Coriolus versicolor*. All experimental results provided strong evidences that Wr-74 was the most suitable strain for the further work besides ATCC-20545.

CHAPTER V MEDIUM OPTIMISATION

5-1 Introduction

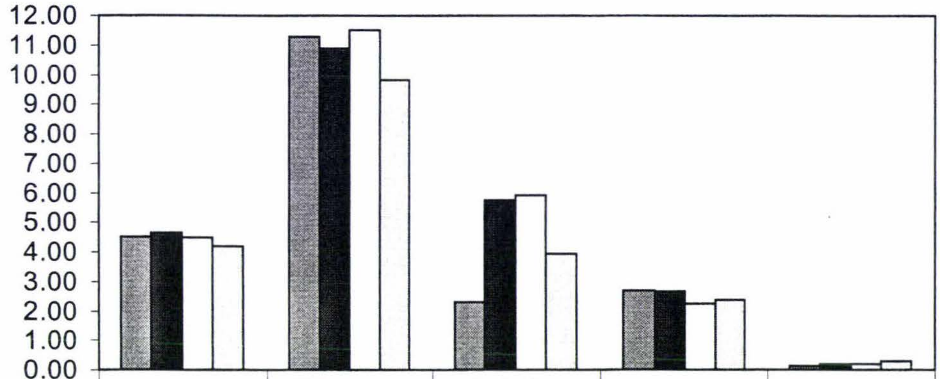
When considering medium optimisation, two distinct biological requirements are essential for the medium design. Firstly, nutrients have to be supplied to establish the growth of microorganisms. Secondly, proper nutritional conditions have to be provided to maximize the product (Demain et al. 1999). Since CPS is a primary metabolic product of *C. versicolor,* the growth of *C. versicolor* and the production of CPS are determined by the medium components. With respect to industrial fermentation, it is of interest to select nutrient components that are cost-effective and readily available. Therefore, the milk permeate (MP), a by-product of the dairy industry was investigated as a base medium in the project for CPS production. In addition, the effects of different nitrogenous sources and salts on the growth of *C. versicolor* and the EPS/IPS production were also investigated.

5-2 Results and discussion

5-2-1 Using MP as a base medium

In this study, MP was used as a base medium for the fermentation of *C. versicolor.* The various metabolic profiles were compared to the fermentation batch using MEG medium (Table 3.5).

The results in Fig. 5.1 indicated that the MEG media supplemented with 70% (v/v) and 50% (v/v) MP were comparable to MEG medium in terms of EPS/IPS levels for strain Wr-74. Although the highest IPS yield was found using MEG medium, both 70%MP-MEG medium and 50%MP-MEG medium (Table 3.5) produced more EPS than MEG medium. It was also noted that a higher IPS yield on biomass ($Y_{p/x}$=0.04) was obtained in the 50%MP-MEG medium than in 70%MP-MEG medium ($Y_{p/x}$=0.01). In terms of total EPS/IPS production, similar concentrations were found in both media. Therefore, 50%MP-MEG was considered as a cost effective medium for the fungal growth and EPS/IPS production. On the other hand, Wr-87 in the 30%MP- MEG medium did not achieve a level of EPS as high as other media. The relative viscosity and dry weight of biomass were found to have no strong correlation with EPS/IPS levels in this case.



	pH	Biomass (mg/ml)	Relative viscosity	EPS (mg/ml)	IPS (mg/ml)
▨ 70% MP-MEG	4.53	11.3	2.3	2.71	0.116
■ 50% MP-MEG	4.65	10.88	5.74	2.66	0.186
□ 30% MP-MEG	4.49	11.5	5.91	2.24	0.194
□ MEG	4.21	9.83	3.94	2.37	0.289

Fig. 5.1 Profiles of metabolism and EPS/IPS production of strain Wr-87 in the MEG media with different percentages of MP.

5-2-2 Homogenous experimental design for medium optimisation

Homogenous experimental design is a statistical design method developed by a Chinese mathematician in the 1980s. It has proven to be a very efficient and popular method in the chemical engineering and biopharmaceutical industries. Based on the design, experimental data are homogenously distributed in the pre-designed form. The number of experimental runs was equal to the number of the levels. Therefore, relatively optimum results could be achieved through a minimum number of experimental runs (Xiong, 2001).

In the experiment, the simplest homogenous design format U_5 (5^4) (4 factors and 5 levels) was used and the 3 components (factors) and 5 levels of MP-based YEG media were selected (Appendix III a, c). Then, the five media with three factors were designed as shown in Table 5.1 according to ' Usage of U5 (5^4) format' (1, 2, and 4 column indicated in Appendix III b). The average EP/IPS levels obtained from shake flask experiments are illustrated in Fig. 5.2. The error bar on each column is calculated based on the standard deviation of data from the two batches.

Table 5.1 MP-based YEG media designed based on $U_5(5^4)$ format

Factors / Levels	MP %, v/v	Yeast extract %, w/v	Glucose %, w/v	Other Components*
Medium 1	100	0.4	2	same
Medium 2	80	0.8	1.5	same
Medium 3	60	0.2	1.0	same
Medium 4	40	0.6	0.5	same
Medium 5	20	1.0	2.5	same

*other components contained FS solution (Table 3.3) only

The results showed that the highest EPS/IPS levels were found in the medium-2 composition (2.63 and 0.26 mg/mL, respectively). Compared with the EPS/IPS levels in the control 50%MP-YEG medium (2.18 and 0.171 mg/mL, not shown in Fig.5.2), EPS/IPS levels were increased by 20.6 % and 52.0 %, respectively. The EPS/IPS production appeared to be highly dependent on the medium compositions and correlated with the relative viscosity of the culture broth and the dry weight of biomass.

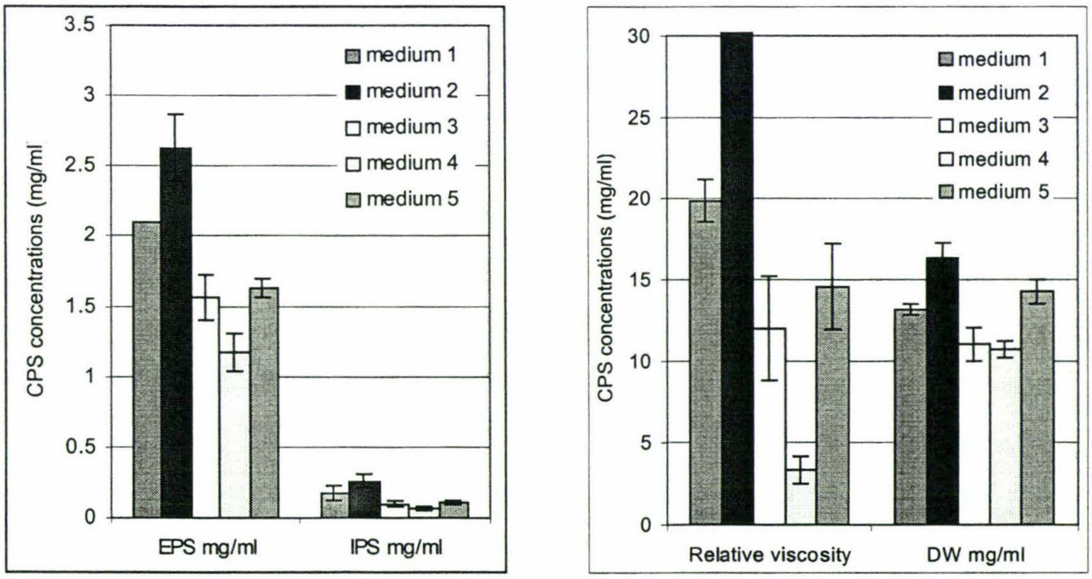


Fig. 5.2 Profiles of EPS, IPS, biomass and relative viscosity of Wr-74 in MP-based YEG media designed based on $U_5(5^4)$ format

Table 5.2 Optimum levels of medium components and EPS/IPS produced calculated using Homogenous Design software package

	Contents	Minimum values	Maximum values	Optimal values for IPS	Optimal values for EPS
X1	MP	20	100	100	81.07
X2	Yeast extract	0.2	1.0	1.0	1.0
X3	Glucose	0.5	2.5	0.5	2.5
Y1	DW biomass	9.98	17.3	19.61	18.36
Y2	EPS	1.03	2.87	2.58	3.57
Y3	IPS	0.05	0.31	0.35	0.34
Y4	Relative viscosity	2.49	342	185.7	512.8

Further analysis of the data using the Homogenous Design software package that was based on multiple regressions was carried out and the results are presented in Table 5.2. The results showed that MP, yeast extract and glucose were positive factors in the MP-based YEG media for EPS production (detailed statistical data are omitted). However, high amounts of glucose could inhibit the IPS production. As shown in Table 5.2, the optimum composition of the growth medium included 80 % (v/v) MP, 1% (w/v) yeast extract and 2.5% (w/v) glucose, which achieved the maximum calculated level of EPS (3.57 mg/mL). The fermentation of Wr-74 in the medium with 100 % (v/v) MP, 1% (w/v) yeast extract and 0.5% (w/v) glucose could produce a maximum level of IPS (0.35 mg/mL).

Table 5.3 MP-based MEG media designed based on U_5 (5^4) format

Factors Levels	MP %, v/v	Glucose %, w/v	Malt extract %, w/v	Other Components*
Medium 1	20	1.5	0.5	same
Medium 2	30	2.5	0.4	same
Medium 3	40	1	0.3	same
Medium 4	50	2	0.2	same
Medium 5	60	3	0.6	same

*Other components included constant 0.4% (w/v) yeast extract and FS solution.

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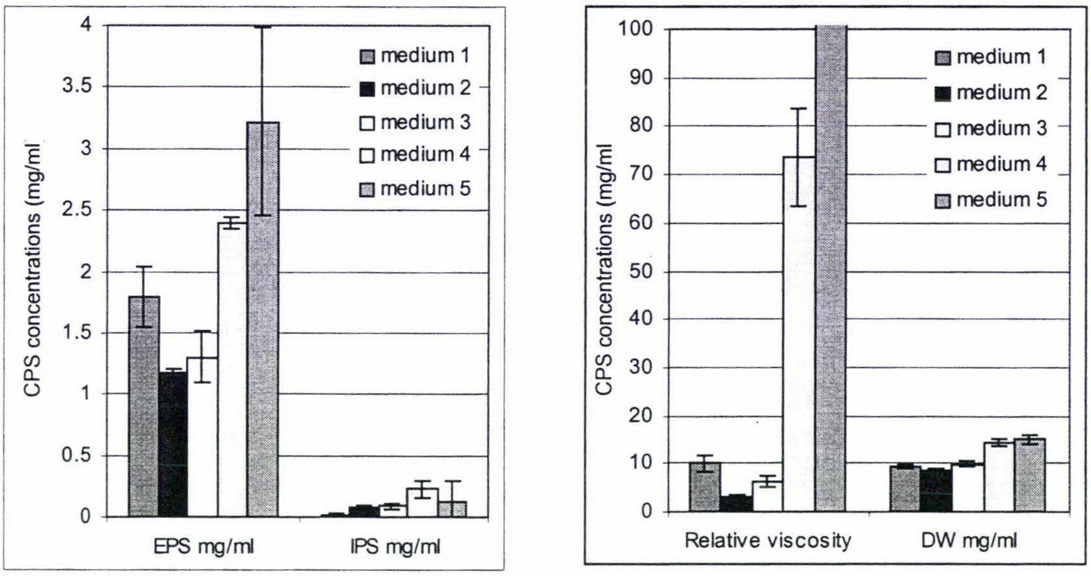


Fig. 5.3 Profiles of EPS, IPS, biomass and relative viscosity of Wr-74 in MP-based MEG media designed based on $U_5\,(5^4)$ format

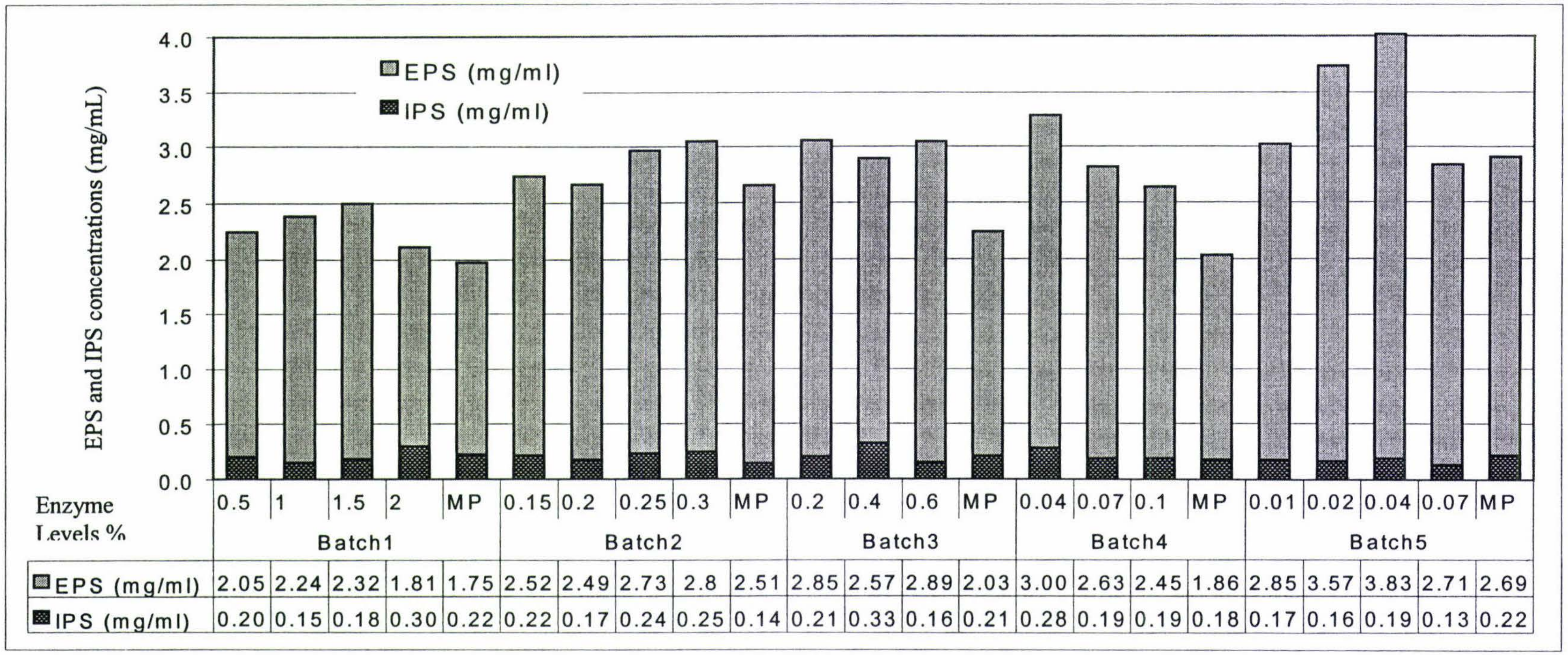
Similarly, MP-based MEG media were also designed according to U_5 (5^4) format shown in appendix III a,b,d. Compositions of these media are shown in Table 5.3. The results in Fig 5.3 showed the highest EPS level (3.22 mg/mL) in medium-5 contained maximum levels of MP, glucose and malt extract, suggesting that these three components were all positive factors for EPS production. The highest IPS concentration and the relative high EPS concentration were achieved using medium-4 among. The positive effect of malt extract on EPS production was less obvious than MP and glucose because the dramatic decrease in the malt extract level (0.2 %, w/v) in the medium-4 showed no huge decrease in the EPS production. However, it could not be explained why medium-1 with the lowest MP and glucose content resulted in a higher EPS and higher relative viscosity than medium-2 and medium-3. The medium could be further refined by selecting the optimum range for each variable.

Comparing Fig. 5.2 and Fig. 5.3, MP-based MEG medium seemed better than the MP-based YEG medium in terms of EPS and IPS level. However, it was noted that the high carbohydrate content (97.1 %, w/v) of the malt extract, determined by the Phenol/Sulphuric acid assay, could lead to error in the analysis of total carbohydrates and could be mistaken as either EPS or IPS. Therefore, MP-based YEG medium was preferred as a base medium instead of MP-based MEG medium in subsequent experiments.

5-2-3 Hydrolysed MP (HMP) used as a based medium component

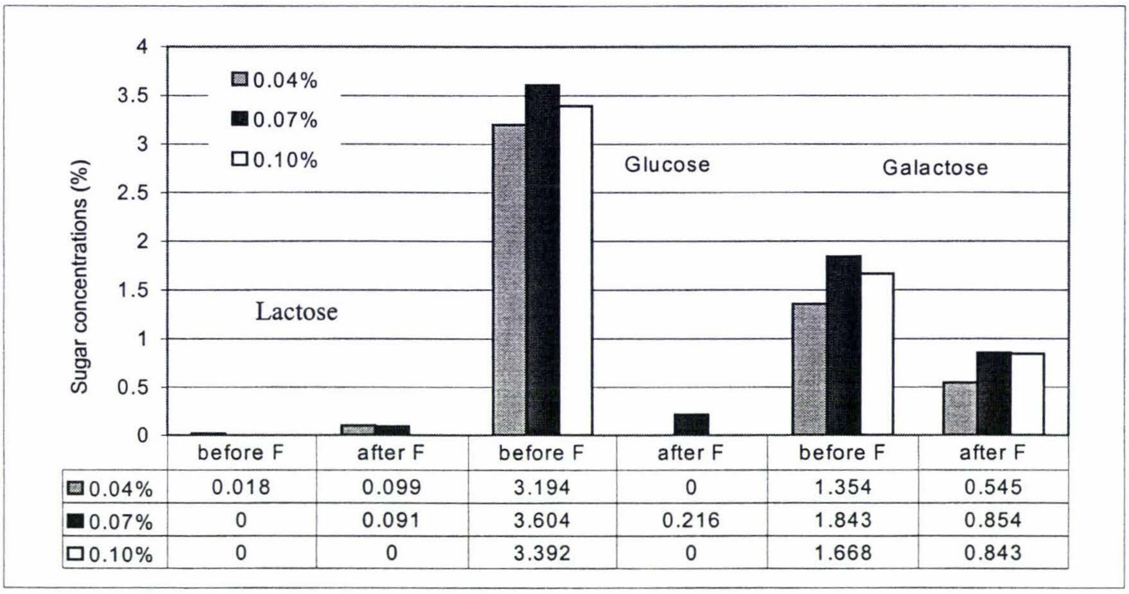
Lactose, a main component in the MP (5%, w/v) can be hydrolysed by the enzyme Maxilact® L2000 into glucose and galactose, which are good carbon sources for *Coriolus versicolor* in EPS production. Therefore, the hydrolysed MP (HMP) could provide a more readily available carbon source than the unhydrolysed MP. The HMP was prepared according to the procedure described in section 3-9-6-1 and the enzyme concentrations ranged from 0.02 to 0.2 % (v/v). Five batches of experiments, covering different levels of the enzyme were carried out and all media were prepared based on 50%HMP-YEG. The 50%MP-YEG medium (Table 3.5) was used as a control. The results are shown in Fig. 5.4

The HMP in the first three batches had been treated with relatively higher enzyme concentrations than batch 4 and 5. The results showed the trend that EPS levels within each batch generally increased with an increase in enzyme concentrations with the exception of batch 4. The highest EPS levels within batch 1 to 5 were 28 %, 12%, 45 %, 61 % and 41 % higher than the corresponding control samples. In batch 4 and 5, the highest EPS levels (3.0 mg/mL and 3.8 mg/mL) were found in the medium with the enzyme concentration of 0.04 % (v/v). The results suggested that enzyme concentration indirectly affect the amount of EPS produced. The optimum enzyme concentrations which ranged from 0.02 % to 0.04 % (v/v) could achieve significantly high EPS levels ($p \leq 0.05$). However, no significant difference ($p \leq 0.05$) in IPS levels was observed in all the batches of fermentation. Based on the results of the HPLC analysis of batch 4 fermentation (Fig. 5.5), *C. versicolor* used up all of the glucose and about 50% of the galactose in the medium hydrolysed by 0.04 % (v/v) enzyme, and produced the highest both EPS and IPS levels (Fig. 5.4). The enzyme level of 0.04 % (v/v) was considered as the critical concentration required for sufficient hydrolysis of lactose in the MP, which produced optimum carbohydrate concentrations for EPS/IPS production. Enzyme concentrations higher than the critical concentration could have some inhibitory effects on EPS/IPS production as shown in batch 4 and 5. However, enzyme concentration lower than 0.04 % (v/v) also resulted in lower EPS/IPS levels probably due to incomplete hydrolysis of lactose.



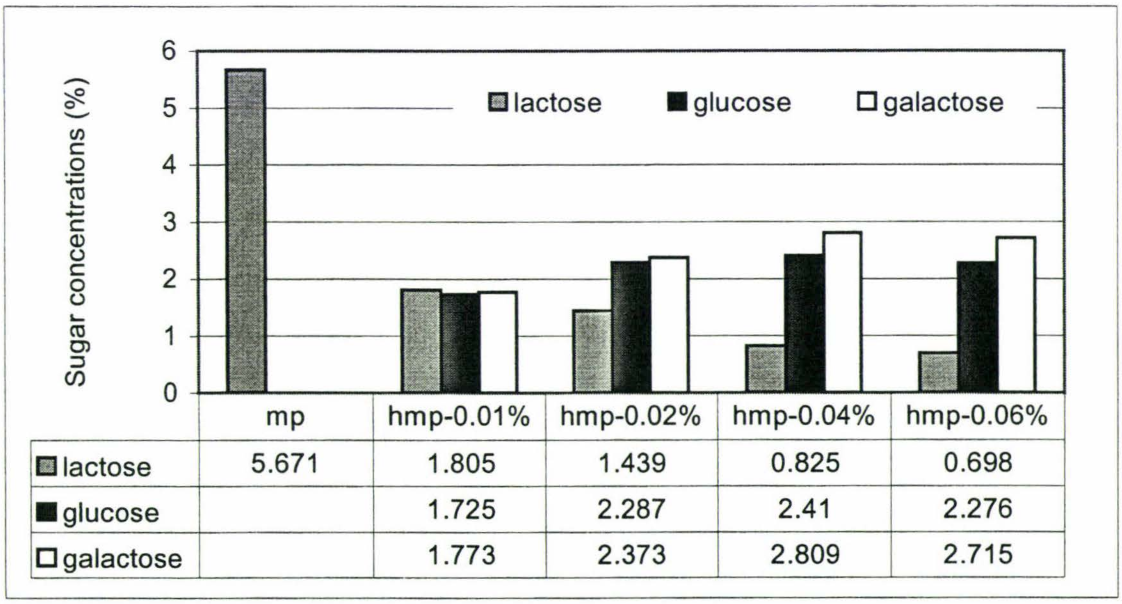
Enzyme Levels %	0.5	1	1.5	2	MP	0.15	0.2	0.25	0.3	MP	0.2	0.4	0.6	MP	0.04	0.07	0.1	MP	0.01	0.02	0.04	0.07	MP
	Batch1					Batch2					Batch3				Batch4				Batch5				
▨EPS (mg/ml)	2.05	2.24	2.32	1.81	1.75	2.52	2.49	2.73	2.8	2.51	2.85	2.57	2.89	2.03	3.00	2.63	2.45	1.86	2.85	3.57	3.83	2.71	2.69
▩IPS (mg/ml)	0.20	0.15	0.18	0.30	0.22	0.22	0.17	0.24	0.25	0.14	0.21	0.33	0.16	0.21	0.28	0.19	0.19	0.18	0.17	0.16	0.19	0.13	0.22

Fig. 5.4 Comparison of the EPS and IPS levels produced in 50%HMP-YEG media with 50%MP-YEG medium as control in five batches of shake flask fermentation HMP was prepared by hydrolysing lactose in the MP with the enzyme Maxlact®L2000 at different enzyme concentrations (%, v/v); MP represents 50%MP-YEG medium.



	before F	after F	before F	after F	before F	after F
▨0.04%	0.018	0.099	3.194	0	1.354	0.545
■0.07%	0	0.091	3.604	0.216	1.843	0.854
☐0.10%	0	0	3.392	0	1.668	0.843

Fig. 5.5 Concentrations of lactose, glucose and galactose in the batch-4 media before and after fermentation (F) HMP was obtained by hydrolysing the lactose in the MP with enzymes of different concentrations (0.04%, 0.07% and 0.1%, v/v).



	mp	hmp-0.01%	hmp-0.02%	hmp-0.04%	hmp-0.06%
▨lactose	5.671	1.805	1.439	0.825	0.698
■glucose		1.725	2.287	2.41	2.276
☐galactose		1.773	2.373	2.809	2.715

Fig. 5.6 Concentrations of lactose, glucose and galactose in HMP solution compared with MP before used in batch 5 fermentation HMP was obtained by hydrolysing the lactose in the MP with enzymes of different concentrations (0.01 %, 0.02 %, 0.04 % and 0.06%, v/v).

Fig. 5.6 presents typical results of enzymatic hydrolysis of the lactose in MP. Concentrations of lactose, glucose and galactose were determined by HPLC. When the enzyme concentration increased, levels of glucose and galactose increased while lactose concentration decreased. The maximum extent of lactose hydrolysis occurred at 0.04 % (v/v) enzyme concentration. In batch 5, the trend of the glucose levels in the media corresponded with the levels of EPS produced (Fig. 5.4). The enzyme concentration of 0.04 % (v/v) achieved the highest level of glucose and galactose and resulted in high levels of EPS/IPS. The reason why 0.07 % (v/v) enzyme concentration led to lower levels of glucose and galactose still remained unclear.

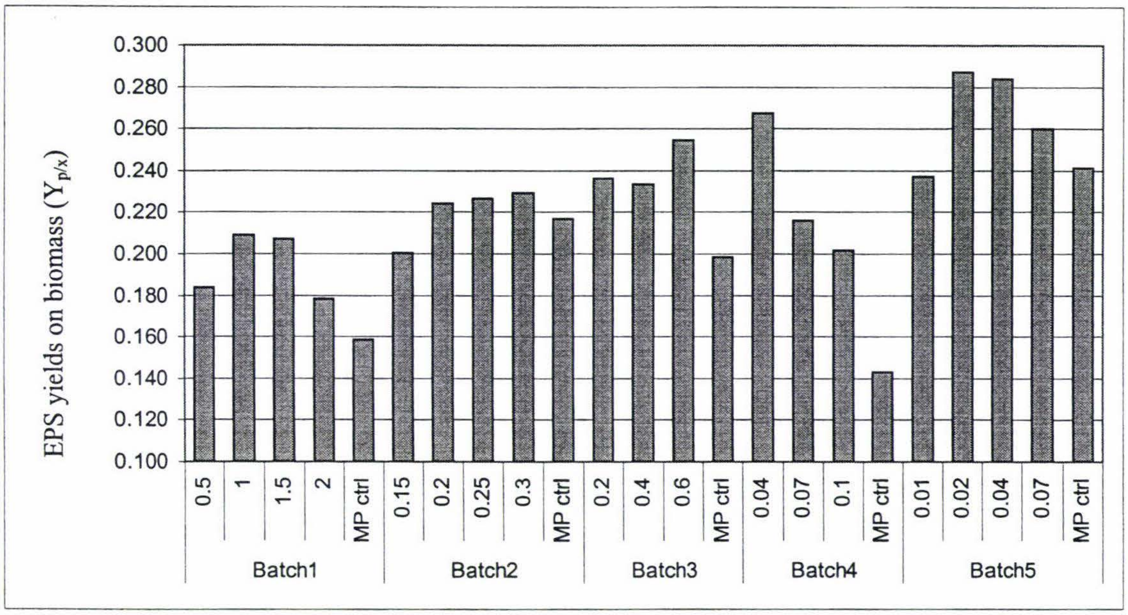


Fig. 5.7 EPS yields on biomass ($Y_{p/x}$) in the 50%HMP–YEG media HMP was prepared under different enzyme concentrations (%, v/v).

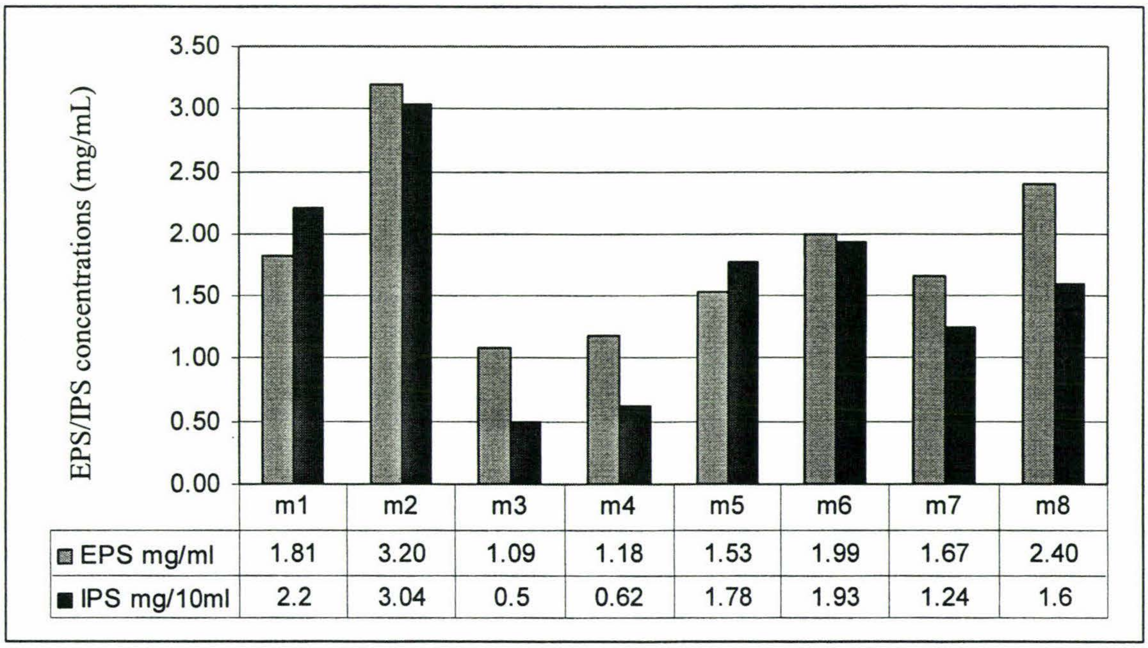
The trends of EPS yields on biomass ($Y_{p/x}$) in the 50%HMP-YEG media are shown in Fig. 5.7. The highest $Y_{p/x}$ values of 0.287 and 0.284 were obtained in batch 5 media treated with 0.02 % (v/v) and 0.04 % (v/v) enzymes, respectively. The second highest $Y_{p/x}$ value (0.268) was in the batch 4 medium treated with 0.04 % (v/v) enzyme. The Overall results suggested that the optimal enzyme concentration for hydrolysing the lactose in MP was around 0.04% (v/v). 50%HMP-YEG medium prepared under such condition would likely lead to higher EPS levels and EPS yields on biomass.

5-2-4 Utilization of 100 % (v/v) HMP as a base medium

Further research was carried out using 100 % (v/v) HMP as the base medium with 50%HMP-YEG medium as control. The HMP was prepared according to the procedure described in section of 3-9-6-1. An enzyme concentration of 0.04 % (v/v) was chosen to perform the enzymatic hydrolysis of the lactose in MP. Media were prepared as shown in table 5.4. Carbon/nitrogen (C/N) ratio was calculated based on the indication in section 3-5-4.

Table 5.4 Formulas of 100 % (v/v) HMP-based YEG media

Growth media	HMP (%,v/v)	Glucose (%, w/v)	Yeast extract (%, w/v)	C/N ratio	FS solution
m1	100	1.5	0.4	110	added
m2	100	1.5	0.6	73	added
m3	100	2.5	0	∞	added
m4	100	2.5	0.2	248	added
m5	100	2.5	0.4	124	added
m6	100	2.5	0.6	88	added
m7 (ctrl)	50	2.5	0.6	59	added
m8	100	2.5	0.6	88	no



	m1	m2	m3	m4	m5	m6	m7	m8
▨ EPS mg/ml	1.81	3.20	1.09	1.18	1.53	1.99	1.67	2.40
■ IPS mg/10ml	2.2	3.04	0.5	0.62	1.78	1.93	1.24	1.6

Fig. 5.8 EPS/IPS levels of Wr-74 in 100 % (v/v) HMP-based YEG media

The EPS and IPS levels of Wr-74 in the 100% (v/v) HMP-based YEG media and a control using 50%HMP-YEG mediun (m7, see Table 3.5) are shown in Fig. 5.8. The results demonstrated that the highest levels of EPS and IPS were produced in 100%HMP-YEG medium (m2, see Table 3.5) with a C/N ratio of 73, approximately twice those of 50%HMP-YEG medium. The lowest EPS and IPS levels were found in m3 that contained no yeast extract. Increase the levels of yeast extract in the media appeared to lead to increasing levels of EPS/IPS as seen in m4, m5 and m6, indicating that yeast extract was a crucial component for EPS and IPS production. Further optimisation of the medium could be achieved by increasing yeast extract content beyond 0.6 % (w/v). However, this was further investigated. It was also noted that the profiles of EPS/IPS concentrations, biomass dry weights and amounts of glucose utilized (Fig, 5.9) followed a similar trend, suggesting a particularly good correlation between EPS/IPS levels and amount of glucose utilized and biomass dry weight in different 100% (v/v) HMP-based YEG media.

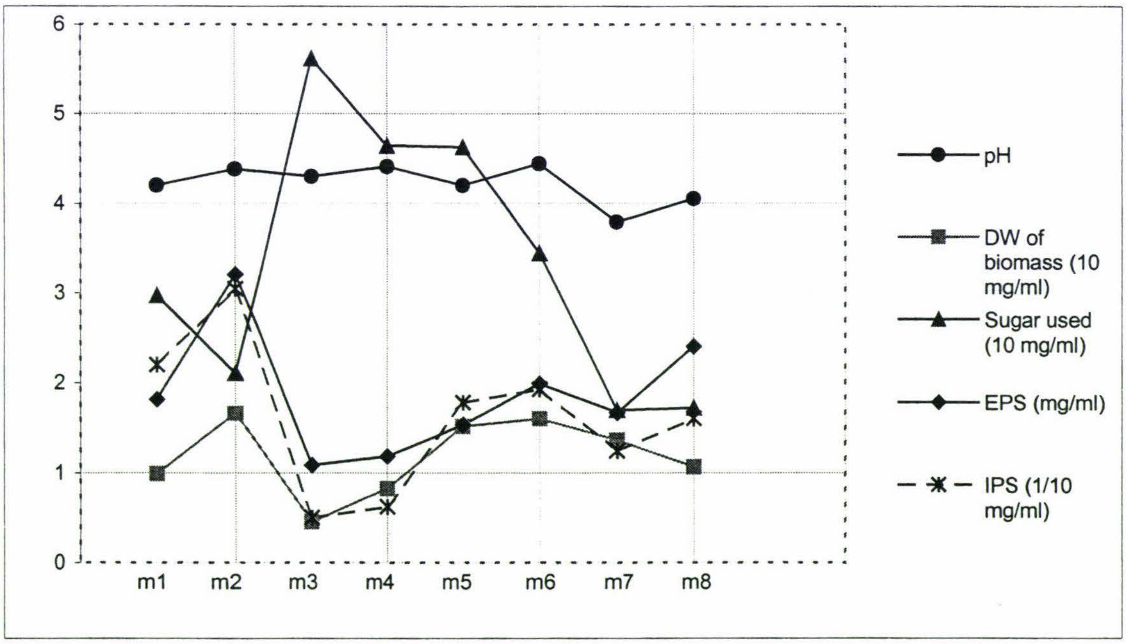
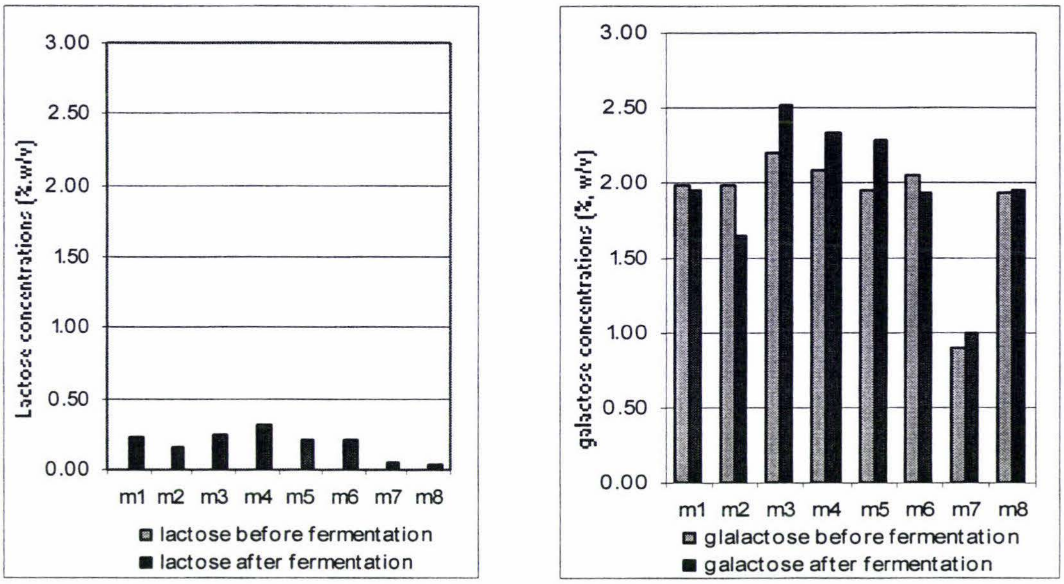
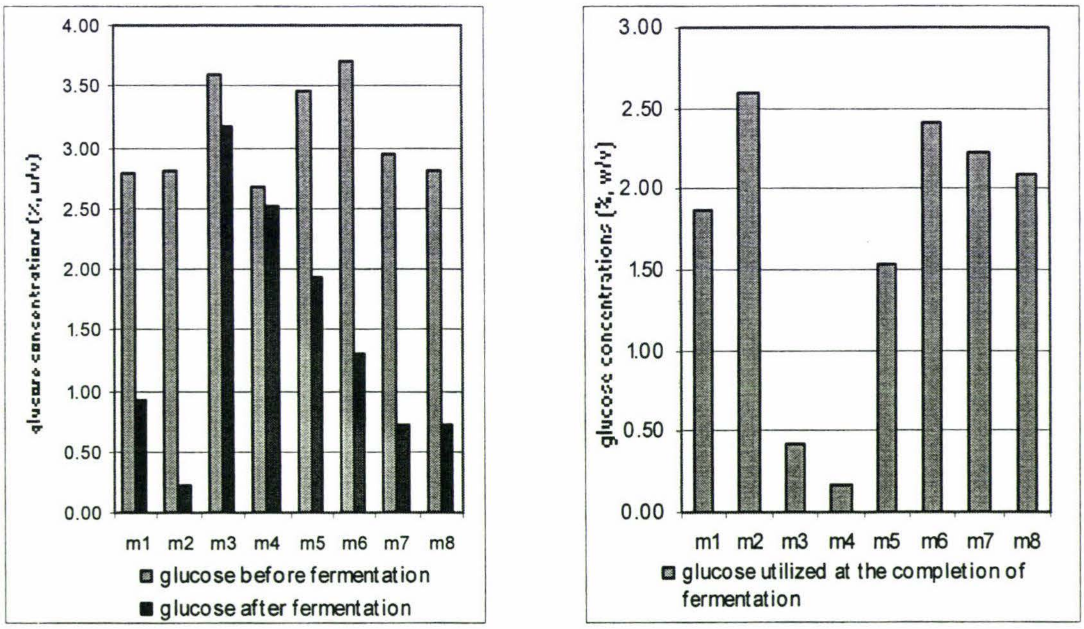


Fig. 5.9 Profiles of pH, dry weights of biomass, sugar used, EPS and IPS levels of Wr-74 in 100 % (v/v) HMP-based YEG media



(a) Lactose levels before and after fermentation

(b) Galactose levels before and after fermentation



(c) Glucose levels before and after fermentation

(d) Amount of glucose utilized at the completion of fermentation

Fig. 5.10 Concentrations of lactose, glucose and galactose before and after fermentation of Wr-74 in 100 % (v/v) HMP-based YEG media

Figure 5.10 exhibits the metabolic profiles of lactose, glucose and galactose before and after the fermentation of Wr-74 in the 100% (v/v) HMP-based YEG media. Before fermentation, all lactose was hydrolysed as shown in Fig. 5.10 (a). However, lactose appeared to be synthesized during fermentation by enzymes secreted by the mycelia in the presence of excess glucose and galactose. In the case of galactose, it was utilized only to a small extent in media m1, m2 and m6 (Fig.5.10b). The amounts of galactose in m3, m4 and m5 increased slightly possibly via the enzymatic conversion from glucose to galactose. At the same time, the amounts of glucose utilised in m3, m4 and m5 were among the lowest. The inability of *C. versicolor* to utilize glucose in these media was reflected in low EPS/IPS levels. The reason for this could be due to an overly high C/N ratio, so much so that the production of EPS/IPS was retarded. In contrast, the control 50%HMP-YEG medium (m7) with low C/N ratio produced lower levels of EPS/IPS than m6, suggesting the biosynthesis of EPS/IPS needed a balance of glucose and nitrogen concentration. Comparing the profile of glucose utilization (Fig. 5.10c and 5.10d) and EPS/IPS production (Fig. 5.8) in m1, m2, m6, m7 and m8 (m8 will be discussed in section 5-2-6), higher glucose levels were utilised in an environment with lower C/N ratios, resulting in a higher EPS/IPS yield.

5-2-5 Effect of nitrogenous sources

The types and levels of nitrogen sources in the media were investigated to maximise EPS/IPS production. Nitrogenous sources such as yeast extract (DIFCO), malt extract, bacto™ casitone peptone (BCP), Ypp3, YM broth (Table 3.2), tryptic soy broth, tryptone and urea were screened.

Table 5.5 Media containing different nitrogenous sources

Media	Main components (%, w/v) in the media					
	New components	Glucose	Yeast extract	Malt extract	MP	Salts
m1,50%MP-YEG		2	0.6		50	added
m2, 50%MP-YEG		2	0.4	0.6	50	added
m3		2	0.2		50	added
m4	BCP 0.6	2			50	added
m5	BCP 0.6	4				added
m6	YM 2.1	4				added

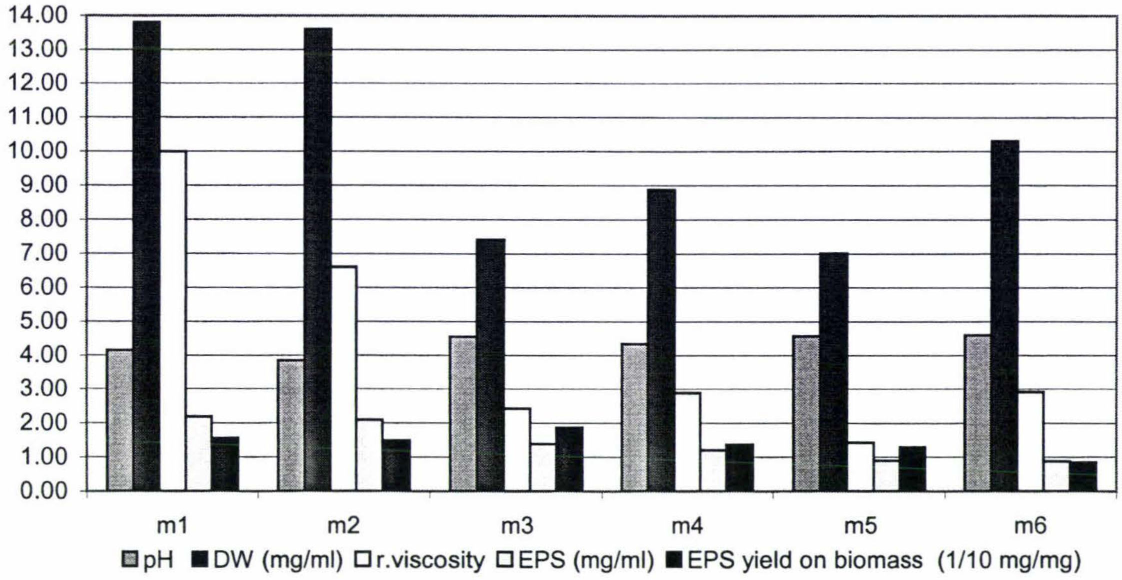


Fig. 5.11 Effects of different nitrogenous sources on the EPS production

Six media containing different nitrogenous sources (Table 5.5) were investigated. The results are showed in Fig. 5.11, which indicated that yeast extract was an important nitrogenous source, contributing the most to the EPS production in m1 and m2. Malt extract in m2 was not considered as a medium component due to the reason discussed in section 5-2-2. Use of Bacto^{TM} casitone peptone as the nitrogenous source in m4 and m5 resulted in a low EPS level compared to m1 and m2. Though YM broth in m6 contains similar components used in MEG medium, the lowest EPS level was obtained when the culture was grown in the medium. Other nitrogenous sources such as tryptic soy broth, casein hydrolysate (tryptone) and urea were also investigated in the experiment. However, they were found to provide no improvement in EPS production (the results are not shown). These suggested that nitrogenous sources and their proportions were specifically required by the fungi for the EPS/IPS production.

An alternative source of yeast extract (Ypp3) was also investigated. Ypp3 is a low-cost yeast extract provided by Fonterra Co-operative Group Ltd, New Zealand. The results were shown in Fig. 5.12 and the error bar on each column is calculated based on the standard deviation of data from the four batches of shake flask fermentations.

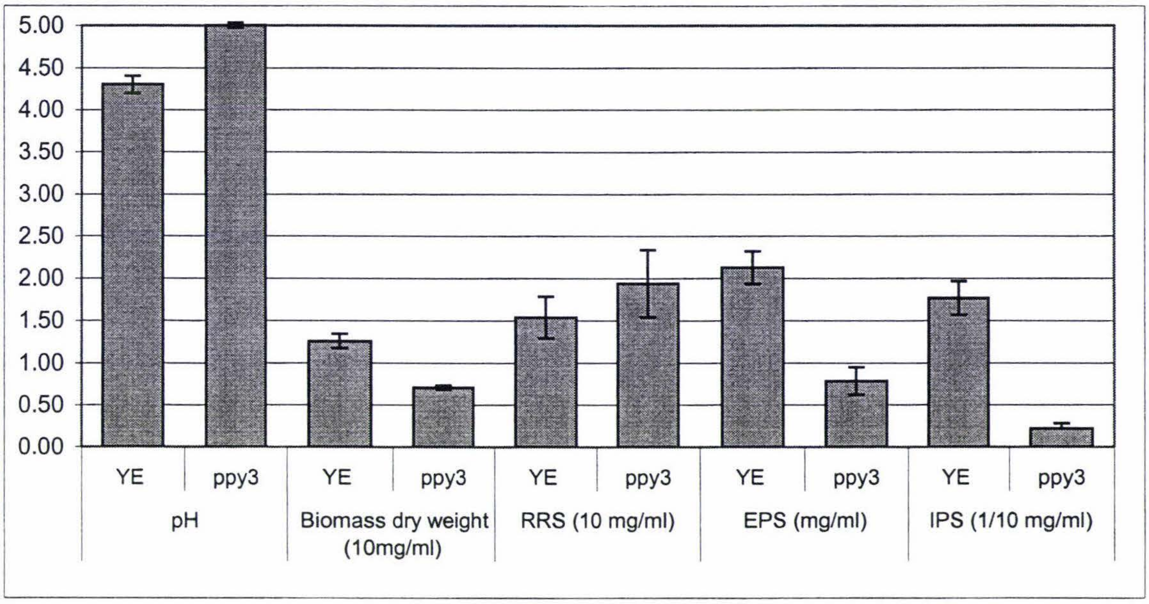


Fig. 5.12 Comparison of yeast extracts (YE) from DIFCO and yeast extract (Ypp3) from Fonterra in terms of the EPS/IPS production, pH, biomass dry weight and residual reducing sugar (RRS)

The study indicated that Ypp3 was not a good replacement for yeast extract from DIFCO. In the growth medium containing Ypp3, the fungi utilized less reducing sugar and produce lower biomass, EPS and IPS than control. Therefore the yeast extract from DIFCO was regarded as the best nitrogen source among the screened. However, further investigation of a cheap alternative to replace the DIFCO yeast extract would be warranted for economical industrial production of EPS/IPS.

5-2-6 Effect of mineral salts

The ammonium sulphate and potassium dihydrogen phosphate were reported to be essential for fungal growth (Garraway *et al.,* 1984). Three media containing ammonium sulphate and potassium dihydrogen phosphate and one without salt addition were compared to observe their effects on EPS/IPS production. The results are shown in Fig. 5.13 and the error bar on each column is calculated based on the standard deviation of data from the three batches of shake flask experiments.

The study shows that IPS levels were enhanced in the presence of the fungal growth trace element (FGTE) solution (Table 3.4) and fungal salt (FS) solution (Table 3.3), while the Bushnell-HAAS (BH) broth (Table 3.1) affected both EPS and IPS production negatively. However, the EPS production in the media containing FS and

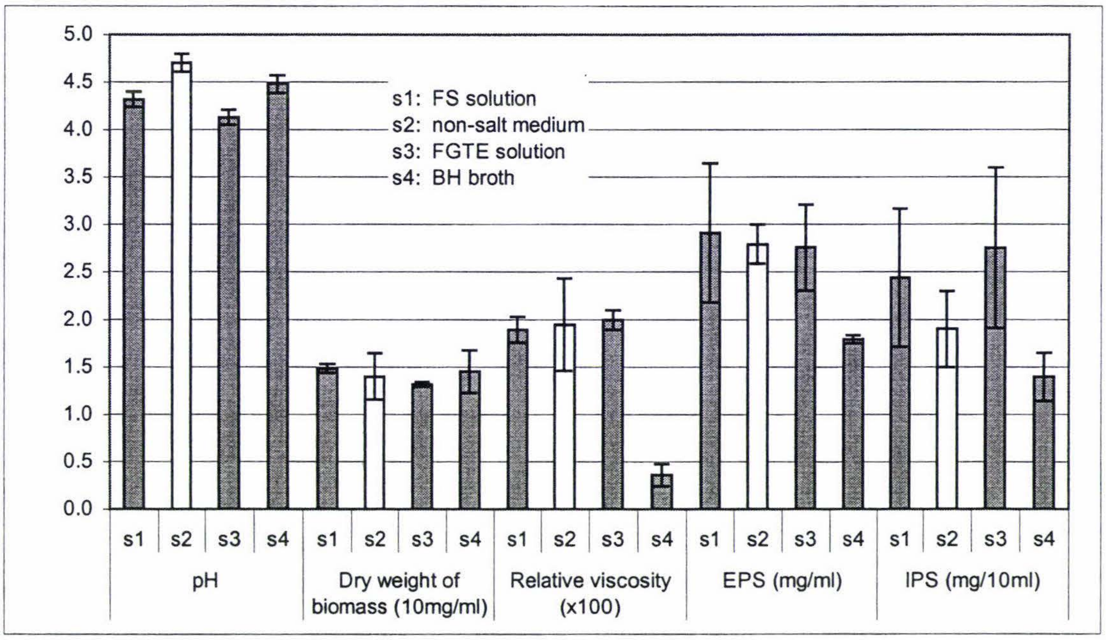


Fig. 5.13 Effects of salt solutions on the EPS/IPS production. The experiment was based on 50%MP-YEG media, supplemented with FS solution, FGTE solution and BH broth

FGTE solutions were not found to be significantly different from the growth medium without salt addition.

When compared with the results from m6 with added salt and m8 without salt addition in section 5-2-4, it was also indicated that salt addition in m6 enhanced higher biomass dry weight and IPS production. However the fungi grown in m8 without salt addition produced higher EPS level than m6 (Fig. 5.9). In spite of lacking repetition, the results shown in Fig. 5.13 and Fig. 5.8 suggest that trace elements in the MP could provide the essential minerals for the fungal growth and IPS production. Consequently, the addition of salts has little effect on EPS production. However shake flask experiments are not always a good guide to large-scale production, where the effects of complex salt solution on the fungi growth and EPS production might be more obvious.

The BH broth contained different proportions of the salts (Table 3.1) that were included in FS and FGTE solutions. The negatively effects of the salt solution indicated that salt compositions had important influence on EPS/IPS production and fungal growth.

5-3 Conclusions

Milk permeate (MP), the waste stream from dairy industry was a suitable base medium for the submerged-culture fermentation of *C. versicolor*. The utilisation of MP will not only increase the financial returns, but also alleviate disposal problem of MP in the dairy Industry.

The initial results showed that 50%MP-MEG and 50%MP-YEG media could relace MEG and YEG media for EPS/IPS production. The results of the medium optimisation using homogenous design method showed that MP, glucose, yeast extract and malt extract were all positive factors for EPS/IPST production. The calculated optimal levels of medium components for the maximum EPS level of 3.57 mg/mL contained 80 % (v/v) MP, 1% (w/v) yeast extract and 2.5% (w/v) glucose. Malt extract was not considered as one of components in the medium due to its high carbohydrate content (97 %, w/v).

Hydrolysed MP could release not only glucose and galactose that were positive carbon source for EPS/IPS production, but also other trace nutritious substances that favour the growth. The optimal Maxilact® L2000 enzyme concentration to produce the HMP, which resulted in the highest EPS yield was 0.04 % (v/v). The EPS level in 50%HMP-YEG medium has increased by more than 40 % compared to the 50%MP-YEG medium. When 100 % (v/v) HMP was used instead of the 50 % (v/v) HMP in the medium, approximately twice the amount of EPS (3.201 mg/mL) and IPS (0.304 mg/mL) was obtained in the 100%MP-YEG medium.

Levels of EPS/IPS produced was closely related to the amount of glucose utilized by the fungi based on the observation on concentrations of lactose, glucose and galactose before and after fermentation. It was also found that the ratio of carbon to nitrogen source played a critical role affecting EPS/IPS production. The optimum C/N ratio was found to be approximately 70.

Yeast extract (DIFCO) was found to be the best nitrogen source. Further study is needed to determine if further increase in yeast extract levels would increase EPS production. The addition of salts to the growth medium, particularly FGTE and FS solutions enhanced IPS production. However, salt addition did not improve EPS yield in the shake flask experiments. The effects of salts on EPS production would probably be better reflected in the large-scale fermentation because of its positive effect on the mycelium growth.

CHAPTER VI SUBMERGED-CULTURE FERMENTATION OF *CORIOLUS VERSICOLOR*

6-1 Introduction

Many CPS products on the market are produced through the extraction of artificially cultivated mushrooms, while some are produced by industrial submerged-culture fermentation. The latter is considered to be the most advanced and cost effective technique to produce CPS. However, the fungal mycelia are sensitive to the high-shear agitation of stirred fermenters. Though various low shear airlift bioreactors and bubble columns have been widely used in the fermentation of shear-sensitive microorganisms, the applications of such bioreactors in the fungal fermentation are severely limited by the high biomass density of the fungal broth. Therefore, in this project, a bench-scale fermenter was modified and used to carry out the batch fermentation.

The objective of the work was to study the submerged-culture fermentation of ATCC-20545 and Wr-74 in the modified fermenter. Data obtained from the experiment were analysed as described in section 3-5-4.

6-2 Results and discussion

6-2-1 Impeller-assisted airlift bioreactor used in the project

A bench-scale airlift bioreactor was modified by adopting the design developed by Lawford and Rousseau (1991, see section 2-6). This fermenter was termed as *impeller-assisted airlift bioreactor*. In the 7-liter airlift bioreactor (46 cm in height and 14.5 cm in diameter), a 20-cm-long draught tube with a diameter of 9 cm was installed to help broth circulation with annular liquid down-flow (Fig. 6.1). This fermenter was equipped with two impellers. The lower two-blade helical impeller mounted on a shaft, 5 cm above the vessel base, assisted the uplift of thick broth in the advance stage of fermentation. The top 6-blade Rushton impeller mounted on the same shaft, 9 cm above the helical impeller, was used for reducing the size of air bubbles to improve the distribution of dissolved oxygen in the broth.

The fermentation of *C. versicolor* in the *impeller-assisted airlift bioreactor* was investigated using 50%MP-YEG and 50%HMP-YEG media (Table 3.5).

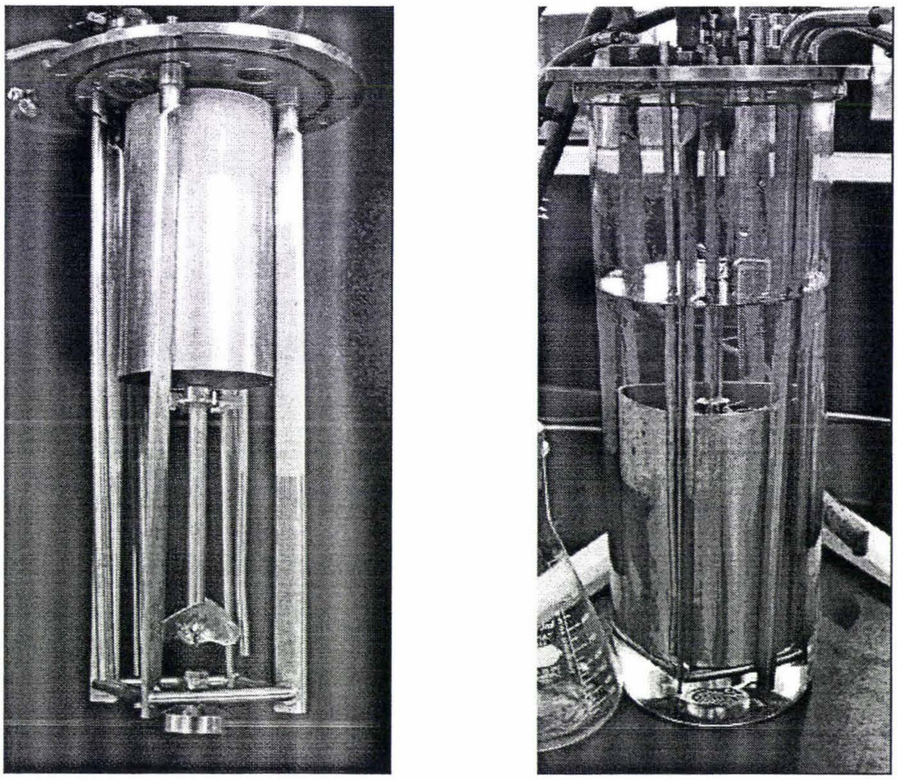


Fig 6.1 Structure of the *impeller-assisted airlift bioreactor*

During the fermentation process, mycelial pellets were homogeneously distributed in the broth and circulated annularly through the draught tube. No dead volume was observed when the broth became thick in the advance stage of the fermentation because of the additional lift powered by the helical impeller and large-diameter sparger (Fig. 6.2). The metabolic profiles obtained from six batches of fermentation are shown in Fig. 6.3 to Fig. 6.9. The fermentation was carried out either under agitation or non-agitation condition. The two media used were supplemented either with salts or without salts. The metabolic profiles appeared to be similar to the shake flask fermentation, suggesting that the designed fermenter was good in facilitating the conditions necessary for *Coriolus versicolor* fermentation. Detailed effects of the fermenter on the growth and EPS/IPS production will be described in the following sections.

6-2-2 Fermentation performance of ATCC-20545 and Wr-74

Comparison of the metabolic profiles was made between ATCC-20545 and Wr-74 in the 7-L *impeller-assisted airlift bioreactor*. The results are summarized in

Fig. 6.2 Broth appearances and pellet flow images in the period of 2-day (left), 3-day (middle) and 5-day (right) of the fermentation

Table 6.1. The data of first two columns in Table 6.1 are based on those from the 'ATCC-agitn' and '74-agitn' curves in left graph of Fig. 6.3 to 6.9. The data of last two columns in Table 6.1 are based on those from the 'ATCC-no salts' and '74-no salts' curves in right graph of Fig. 6.3 to 6.9. These four batches of fermentations were carried out under the agitation speed of 145-rpm in 50%MP-YEG medium supplemented with FS solution (Table 3.3) and 50%HMP-YEG medium without salt addition.

During the early stages of fermentation, the pH profiles as shown in Fig. 6.3 correlated well with the biomass and EPS/IPS production. Generally, ATCC-20545 fermentation resulted in lower pH values than Wr-74 in both media. After around 72-hour fermentation, the broth pH decreased to around 4.0. The low pH which appeared to trigger the onset of exponential growth and EPS/IPS production remained constant until the end of fermentation (~114 hours). After this time, increase in pH values was observed, which was contributed by the lysed mycelia. In the advanced stage of the fermentation, pH values of the broth were generally higher (pH 4.0-4.5) than those

Table 6.1 Comparison of the fermentation performance of ATCC-20545 and Wr-74 in 50%MP-YEG medium and 50%HMP-YEG medium

	50%MP-YEG medium		50%HMP-YEG medium	
	ATCC-20545	Wr-74	ATCC-20545	Wr-74
pH value	6.0/4.0*	5.7/4.3	5.8/4.7	6.2/4.9
Biomass dry weight (mg/mL)	10.60**	8.90	10.20	9.06
EPS concentration (mg/mL)	1.15	1.32	2.01	2.30
IPS concentration (mg/mL)	0.084	0.096	0.135	0.12
Sugar used (mg/mL)	14. 30	17.88	19.8	17.71
EPS yield on biomass ($Y_{p/x,}$)	0.108	0.148	0.197	0.254
EPS yield on substrate ($Y_{p/s}$)	0.080	0.074	0.102	0.130
Maximum specific growth rate (, h^{-1})	0.0097	0.0118	0.0123	0.0213

*6.0/4.0 means initial 'pH value/final pH' values of the broth.

**10.6 means all the values in the table were obtained at the end of fermentation except the maximum specific growth rates.

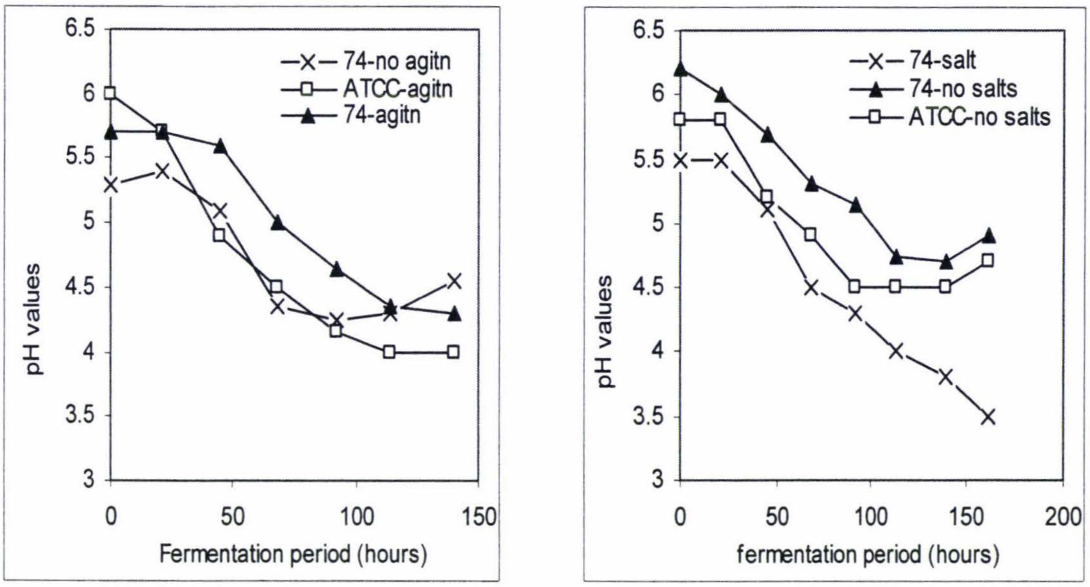


Fig. 6.3 pH profiles of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph). '74no agitn' means the fermentation of Wr-74 was carried out under non agitation condition, while 'ATCCagtin' means the fermentation of ATCC-20545 was carried out under 145 rpm agitation condition. '74salts' means the medium for culturing Wr-74 contains FS solution, while 'ATCCno salts' means the medium for culturing ATCC-20545 was not supplemented with salts.

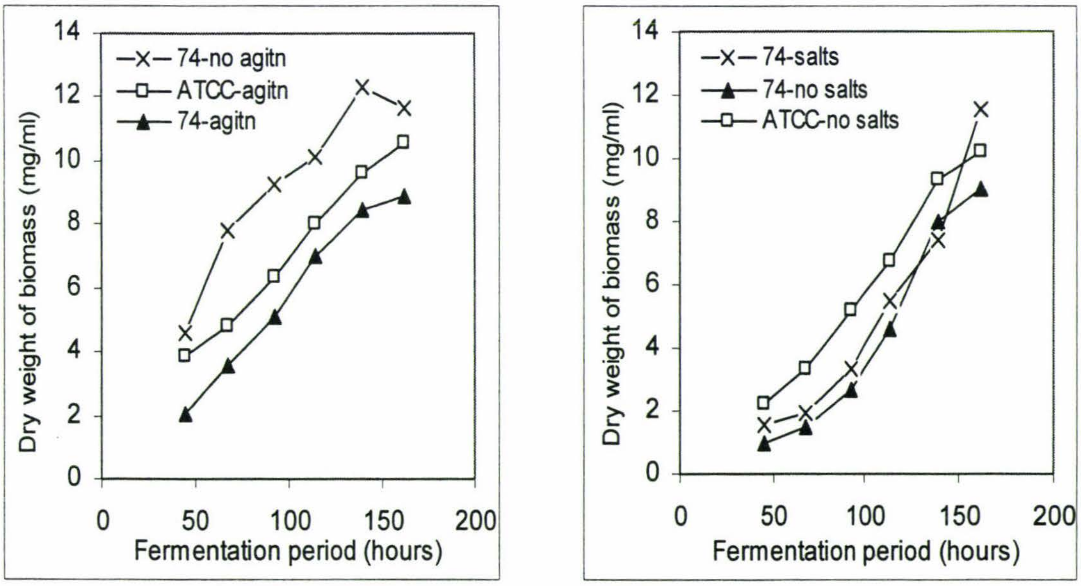


Fig. 6.4 Growth profiles of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph)

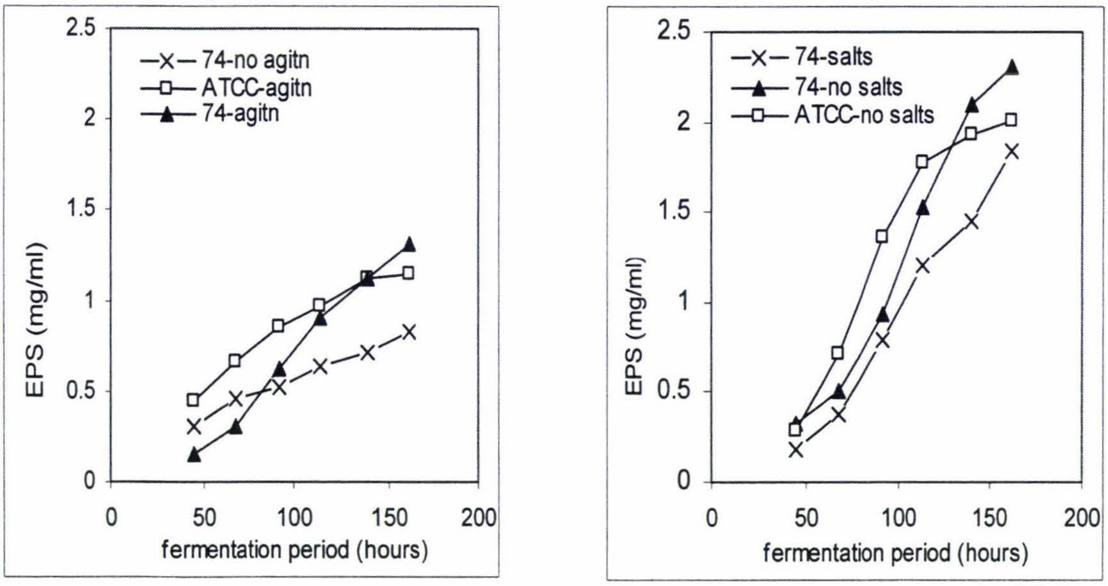


Fig. 6.5 **EPS production profiles of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph)**

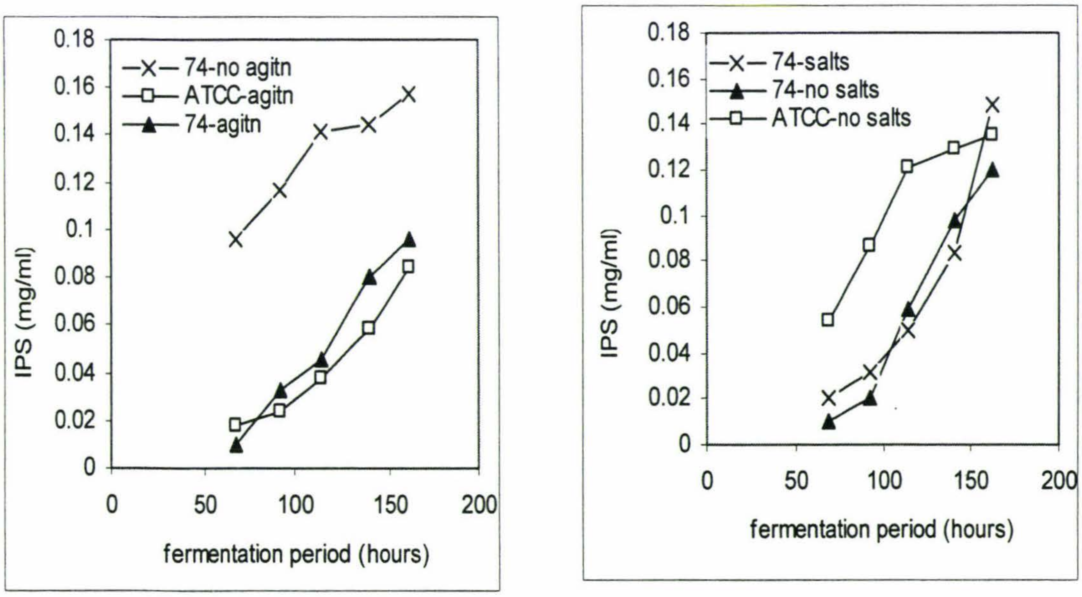


Fig. 6.6 **IPS production profiles of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph)**

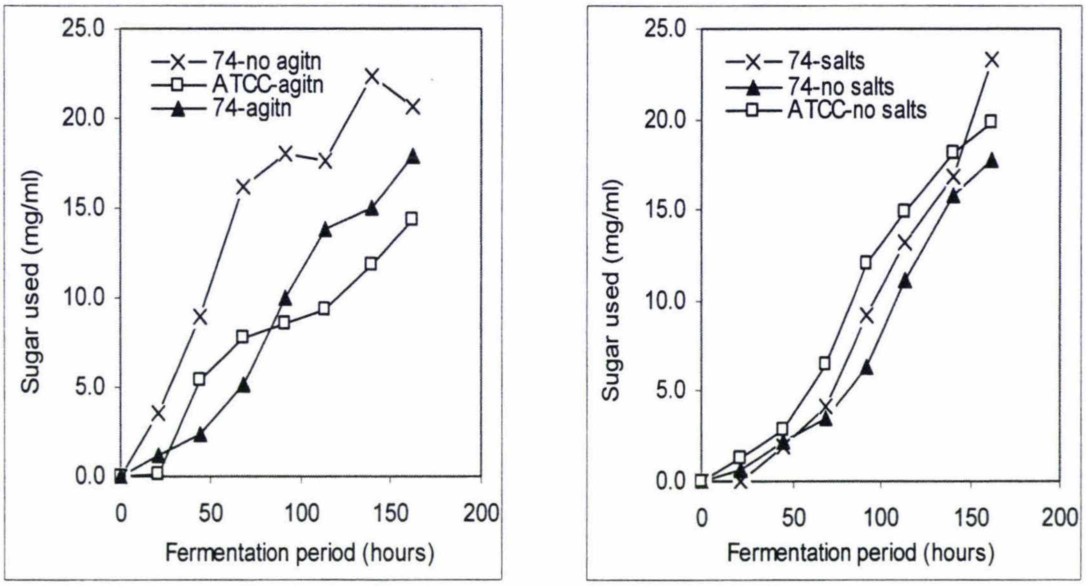


Fig. 6.7 Profiles of sugar utilization of Wr-74 and ATCC-20545 under different condition in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph)

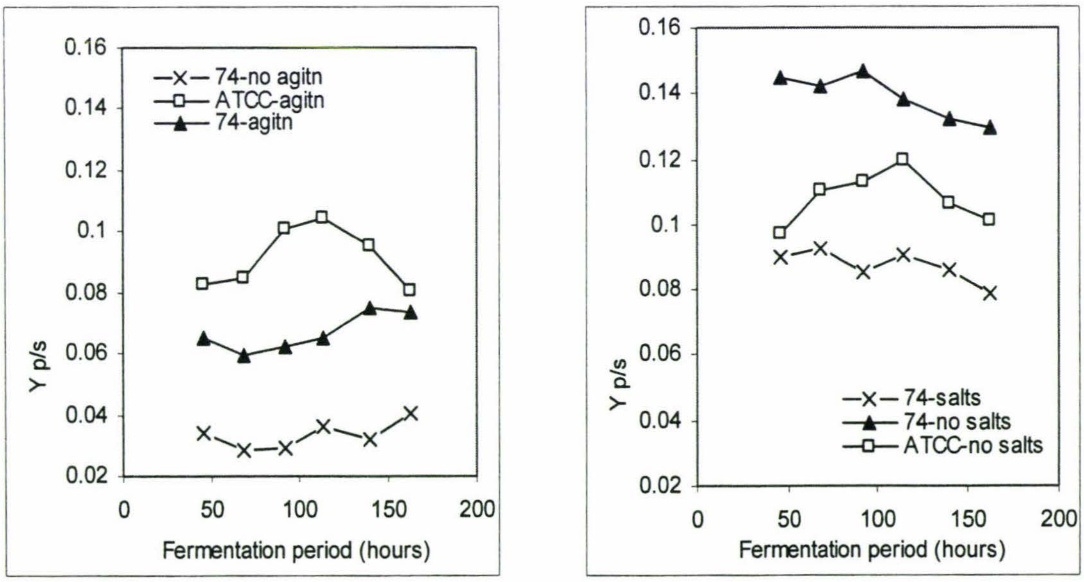


Fig. 6.8 Profiles of EPS yields on substrate ($Y_{p/s}$) of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP-YEG medium (right graph)

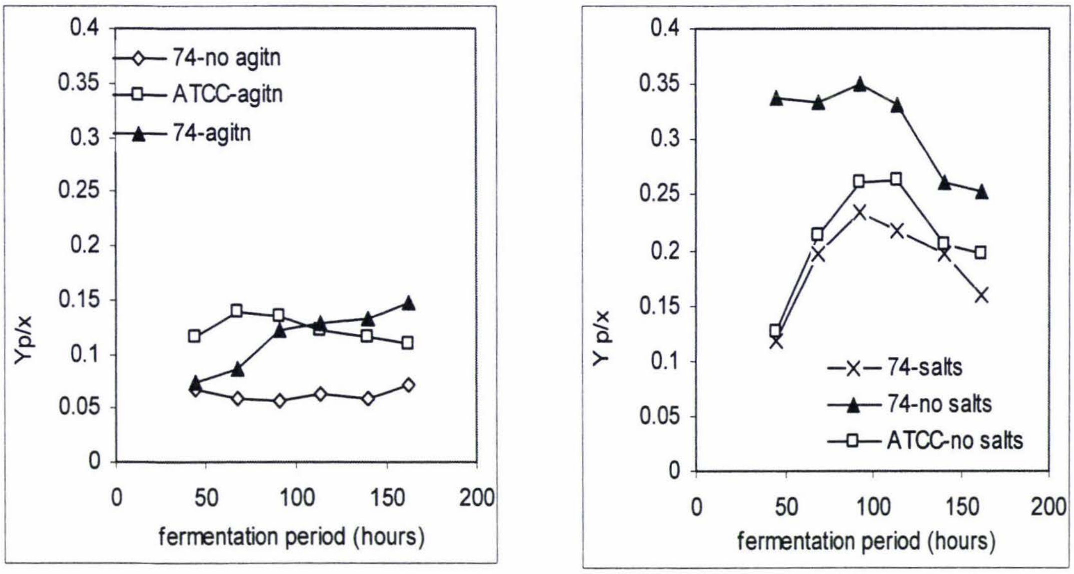


Fig. 6.9 Profiles of EPS yields on biomass ($Y_{p/x}$) of Wr-74 and ATCC-20545 under different conditions in 50%MP-YEG medium (left graph) and 50%HMP -YEG medium (right graph)

of YEG broth (pH 2.5-3.0). The reason might be ascribed to the buffering effects of the milk permeate.

Table 6.1 shows that Wr-74 produced approximately 13 % more EPS than ATCC-20545 in both media at ~150 hour of fermentation. The maximum growth rate (μ_{max}) of Wr-74 was 22 % and 74 % higher than ATCC-20545 in 50%MP-YEG and 50%HMP-YEG medium, respectively. There was no marked difference in IPS production. However, Wr-74 produced less biomass in both media. Consequently, Wr-74 presented a higher EPS yield on biomass ($Y_{p/x}$) than ATCC-20545 in both media. EPS yield on substrate ($Y_{p/s}$) for Wr-74 was close to ATCC-20545 in 50%MP-YEG medium but 26 % higher than ATCC-20545 in 50%HMP-YEG medium (Table 6.1 and Fig. 6.7). This indicated that more sugar was converted by Wr-74 to EPS than consumed for the biosynthesis of biomass. Since the biomass growth rate of Wr-74 was obviously decreased in 50%MP-YEG medium after approximately 140 hours of fermentation (Table 6.1 and Fig. 6.4), however, any further increase in EPS concentration by Wr-74 in the medium was unlikely if the fermentation period was prolonged. The overall results showed that the fermentation conditions applied were generally more favourable for Wr-74 than ATCC-20545 in terms of EPS production.

In Fig. 6.4, the biomass of ATCC-20545 continued to increase exponentially while the EPS production (Fig. 6.5) started to plateau after about 114 hours of fermentation in both media. This indicated that ATCC-20545 continued to utilise sugar mainly for cell growth rather than for EPS production. This was the main reason causing $Y_{p/x}$ and $Y_{p/s}$ (Fig 6.8 and 6.9) curves to decline after 140 hours. As a result, denser biomass was seen in the fermenter at this stage, but the EPS yield remained almost constant. In contrast, the biomass produced by Wr-74 slowed down after 114 hours, but EPS and IPS continued to increase towards the end stage of the fermentation in both media (Fig. 6.5 and Fig. 6.6). The EPS production of Wr-74 followed similar trend as the sugar-consumption curves (Fig. 6.7), resulting in relatively flat $Y_{p/s}$ curves in both culture media (Fig 6.8). Although Wr-74 appeared to utilise the sugar source for EPS production more effectively than ATCC-20545, the lower biomass level produced by Wr-74 would limit any further increase in EPS production.

6-2-3 Fermentation performance of both strains in 50%HMP-YEG medium and 50%MP-YEG medium

ATCC-20545 and Wr-74 performed differently in 50%HMP-YEG medium and 50%MP-YEG medium. The percentage differences between these two media are shown in Table 6.2 (values are derived from the data in Table 6.1).

Table 6.2 Percentage difference of the fermentation performance of Wr-74 in 50%HMP-YEG medium and 50%MP-YEG medium

	ATCC-20545	Wr-74
Dry weight of biomass	-3.8 %*	1.8 %
EPS concentration	74.8 %	74.2 %
IPS concentration	60.7 %	27.7 %
Sugar utilized	38.5 %	-1 %
EPS yield on biomass ($Y_{p/x}$)	82.4 %	71.6 %
EPS yield on substrate ($Y_{p/s}$)	27.5 %	75.7 %
Maximum specific growth rate (μ_{max})	26.8 %	94.9 %

* An example of the above calculation: $[(10.20_{50\%HMP\text{-}YEG} - 10.60_{50\%MP\text{-}YEG}) / 10.60_{50\%MP\text{-}YEG}] \times 100\ \% = -3.8\ \%$

The use of 50%HMP-YEG medium resulted in higher EPS production by both strains than the 50%MP-YEG medium. This could be due to the increase in the levels of readily available carbon sources from the hydrolysed lactose in 50%HMP-YEG medium. The dramatic increase of the EPS levels in 50%HMP-YEG medium brought about a huge increase in $Y_{p/x}$ values for both strains due to their relatively stable biomass levels. There was also a huge increase in $Y_{p/s}$ values, especially for Wr-74, with 75.7 % increase, due to its lower sugar utilization. The $Y_{p/s}$ value of ATCC-20545 only increased by 27.5 % due to the higher sugar utilization. The extra amount of sugar consumed could have contributed to the 60.7 % increase in IPS level.

6-2-4 Effect of agitation and salt addition on fermentation performance

The effects of agitation on fermentation performance of Wr-74 was investigated in the 50%MP-YEG medium (Table 6.3). One batch was under continuous agitation (145-rpm) and the other without agitation throughout the entire fermentation process as shown in '74-no agitn' and '74-agitn' curves in left graph of Fig. 6.3 to 6.9. Results in Table 6.3 show that the EPS level was 58 % higher with agitation than without agitation. Similarly, the EPS yield on biomass ($Y_{p/x}$) under agitation condition was higher than under non-agitation condition. However, fermentation without agitation seemed to be more favourable for IPS production (63.5 % higher than with agitation) and biomass production (23.7 % higher than without agitation). The higher biomass and IPS levels were due to the higher level of sugar utilization in the non-agitation condition. Consequently, EPS yield on substrate ($Y_{p/s}$) in non-agitation condition was lower than that with agitation. In terms of maximum specific growth rate (μ_{max}), fermentation with agitation was approximately twice the value observed under non-agitation condition. It was concluded that the impeller installed in the bioreactor was essential to achieve high EPS production. The lower biomass and IPS levels could be ascribed to mycelium damage caused by shear created during agitation, though the helical impeller was designed to incur the minimal shear damage on the mycelia.

The effects of salt addition on the metabolism of Wr-74 was investigated in the 50%HMP-YEG medium under agitation condition as shown in '74-salts' and '74-no salts' curves in right graph of Fig. 6.3 to 6.9. Results shown in Table 6.3 indicate that salt addition was essential for increasing biomass level, sugar utilization and IPS

Table 6.3 Comparison of the fermentation performance of Wr-74 in 50%MP-YEG medium and 50%HMP-YEG medium under different conditions

	50%MP-YEG medium		50%HMP-YEGmedium	
	No agitation (salt)	Agitation (salt)	Salt (agitated)	No salt (agitated)
pH value	5.3/4.2*	5.7/4.3	5.5/3.5	6.2/4.9
Biomass dry weight (mg/mL)	11.67**	8.9	11.53	9.06
EPS concentration (mg/mL)	0.835	1.32	1.84	2.30
IPS concentration (mg/mL)	0.157	0.096	0.149	0.12
Sugar utilized (mg/mL)	20.61	17.88	23.38	17.71
EPS yield on biomass ($Y_{p/x}$)	0.072	0.148	0.16	0.254
EPS yield on substrate ($Y_{p/s}$)	0.040	0.074	0.079	0.130
Maximum specific growth rate (μ_{max}, h^{-1})	0.0059	0.0118	0.0202	0.0213

*5.3/4.2 means initial pH value/final pH values of the broth.
**11.67 means all the values in the table were obtained at the end of fermentation except the maximum specific growth rates..

production. However, the levels of EPS, $Y_{p/x}$ and $Y_{p/s}$ increased by 25 %, 58.8% and 64.6 % in the same medium without salt addition, respectively. Obviously, the lower biomass and lower sugar utilization also contributed to the increase in $Y_{p/x}$ and $Y_{p/s}$. Similar results were observed in the shake flask experiments, as discussed in section 5-2-3. When considering downstream processing, it would be a better choice to perform the fermentation in a medium without added salts to reduce the cost of purification.

6-2-5 Effect of antifoaming corn oil on the fermentation performance

The use of antifoaming agents is always inevitable for the industrial-scale fermentation. The effects of antifoaming agents on fermentation have not been fully elustrated. For example, n-Hexadecane, besides its antifoam properties, has been reported to cause changes to the morphology of *Penicillium chrysogenum*, which in turn affects productivity (Peng and Chen, 1994) and oxygen transfer (Ho *et al.*, 1990).

Two antifoaming agents (Corn oil and Silicone-based antifoam agent) were used in the fermentation. However, only the effect of corn oil on the metabolism of Wr-74 was investigated in the shake flask-scale fermentation. The preliminary results are shown in Table 6.4.

Table 6.4 Effects of corn oil as an antifoam agent on the metabolism of Wr-74 in 50%MP-YEG medium

	Control no antifoam in shake flask	Corn oil antifoam in shake flask	Corn oil as antifoam in fermenter
pH	4.07	3.83	4.3
Biomass dry weight (mg/mL)	16.05	15.6	8.9
Sugar utilized (mg/mL)	23.01	22.55	17.88
EPS concentration (mg/mL)	2.30	1.97	1.32
IPS concentration (mg/mL)	0.141	0.122	0.089
EPS yield on substrate ($Y_{p/s}$)	0.100	0.087	0.074
Relative viscosity	64.86	4.14	2.95

Corn oil had negative effects on the EPS/IPS production by Wr-74. In the shake-flask experiment, biomass dry weight and the sugar utilization show little difference. However, EPS and IPS concentrations in the control shake flask were 16.8 % and 15.6 % higher than those in the shake flask with corn oil. The EPS yield on substrate ($Y_{p/s}$) in the control was approximately 15 % higher than that in the shake flask containing corn oil. The influence of corn oil was also reflected in the relative viscosity of the broth, with higher viscosity in the control shake flask than the shake flask containing corn oil. All these indicated that corn oil exhibited a greater negative effect in the fermenter than in the shake flask. Comparing the shake flask and the fermenter with corn oil, the EPS and IPS concentrations in the shake flask were 49.2 % and 37.1 % higher than those in the fermenter. The EPS yield on substrate ($Y_{p/s}$) in the shake flask was 17.6% higher than that in the fermenter. It is suggested that the corn oil cause changes to broth characters, which indirectly affected nutrient uptake and oxygen absorption of the mycelia.

6-3 Conclusions

An *impeller-assistant airlift bioreactor* was designed and used in the fermentation of ATCC-20545 and Wr-74. Two different media were used and conditions including those with/without agitation during fermentation, with/without salt addition and with/without antifoaming agents were investigated. The results generally indicated that the design of the fermenter was suitable for the submerged-culture fermentation of *C. versicolor*.

In both 50%MP-YEG and 50%HMP-YEG media, Wr-74 produced higher levels of EPS than ATCC-20545. About 74 % increase in EPS level was obtained when 50%MP-YEG medium was replaced by 50%HMP-YEG medium for both strains. However, there was no great difference in the biomass produced in both media. The agitation by helical impellers during fermentation had positive effects on EPS production. However, agitation also resulted in lower biomass and IPS levels due to shear damage incurred on the mycelia. This could lead to the release of IPS from the damaged mycelia and the IPS could be included in EPS quantification.

Levels of biomass and sugar uptake increased in the medium with the addition of salts. However, higher EPS level was observed in the medium without salt addition, which was consistent with the results obtained from the shake flask experiments.

Corn oil used as an antifoaming agent had negative effects on the EPS/IPS production and changed the broth viscosity. It was suggested that corn oil could have affected the nutrient uptake by the fungi and decreased the amount of dissolved oxygen in the broth.

CHAPTER VII CHARACTERIZATIONS OF CPS

7-1 Introduction

The research work on the characterization of PSK or PSP has been widely reported (Hotta et al., 1981, Sugiura et al, 1980 Yang et al., 1992a and Ng, 1998). In this study, the physical characteristics of EPS/IPS from Wr-74 were compared with ATCC 20545. This included determining the composition of the polysaccharides and protein, ratio of insoluble to soluble EPS/IPS fractions and ratio of the polysaccharide to protein fractions of EPS and IPS.

7-2 Results and discussion

7-2-1 Composition of EPS and IPS

The polysaccharide fraction of IPS has been reported to consist of glucan as the main skeleton of the polysaccharide molecule. D-glucose is the major monosaccharide present, which accounts for 99% of the total saccharides (Hotta et al., 1981). Arabinose and rhamnose are reported in PSP (Yang et al., 1992a), while mannose, xylose, galactose and fructose have been reported in PSK (Wang et al., 1996; Cheng et al., 1998).

In this experiment, the monosaccharide compositions of EPS and IPS from ATCC-20545 and Wr-74 were determined using HPLC based on the procedure in section 3-9-5. Seven standard solutions including 0.75% (w/v) glucose, arabinose, galactose, rhamnose, mannose, fructose and xylose were prepared. A standard solution containing all the seven monosaccharides with 0.75 % (w/v) concentration was also prepared.

Table 7.1 Retention time for each standard monosaccharide

Peaks of a mixture	Peak I	Peak II					Peak III
Peaks of Mono-saccharides	glucose	galactose	mannose	xylose	fructose	rhamnose	arabinose
Retention time (min)	8.687	9.309	9.348	9.37	9.534	9.642	10.234

Upon introducing the standard solution containing seven monosaccharides into the HPLC, only three peaks appeared (see Fig. 7.1). They were located in the main three retention time ranges of 8.658 min - 8.686 min, 9.308min - 9.331min and 10.211min -10.221min (the middle peak height was approximately twice as higher as the other two peaks). By comparing these to the retention time of the individual standard monosaccharide solution, shown in table 7.1, glucose appeared as the first peak and arabinose appeared as the third peak. However, rhamnose, galactose, mannose, fructose and xylose were merged as a single large peak located in the middle. This was due to the limitation of the HPLC column used that could not efficiently separate the different sugar standards.

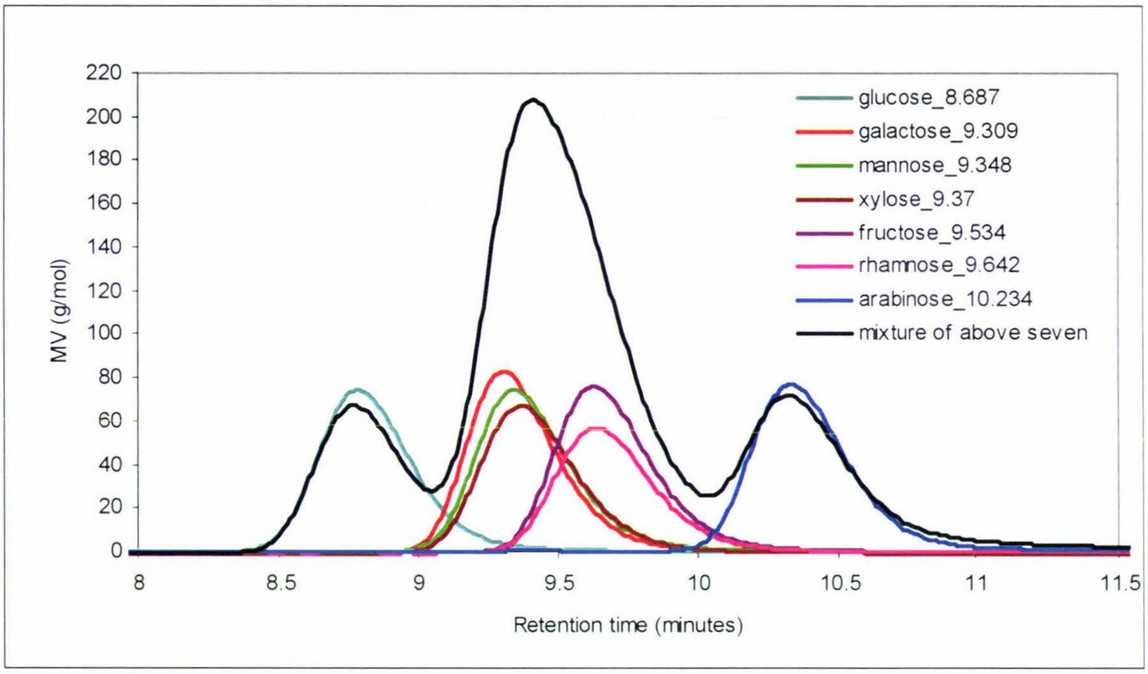


Fig. 7.1 Chromatograms of seven standard monosaccharides obtained from RI absorbance as a function of retention time

Samples of EPS and IPS from both strains were prepared according to section 3-6 and hydrolysed with TFA according to the procedure described in section 3-9-6-2. A standard solution containing glucose, galactose and arabinose each at 0.25% (w/v) was used as a control. As shown in Fig. 7.2, the EPS samples from both strains, each produced a prominent peak located within the range of the glucose retention period. The results demonstrated that EPS from ATCC-20545 and Wr-74 contained only glucose.

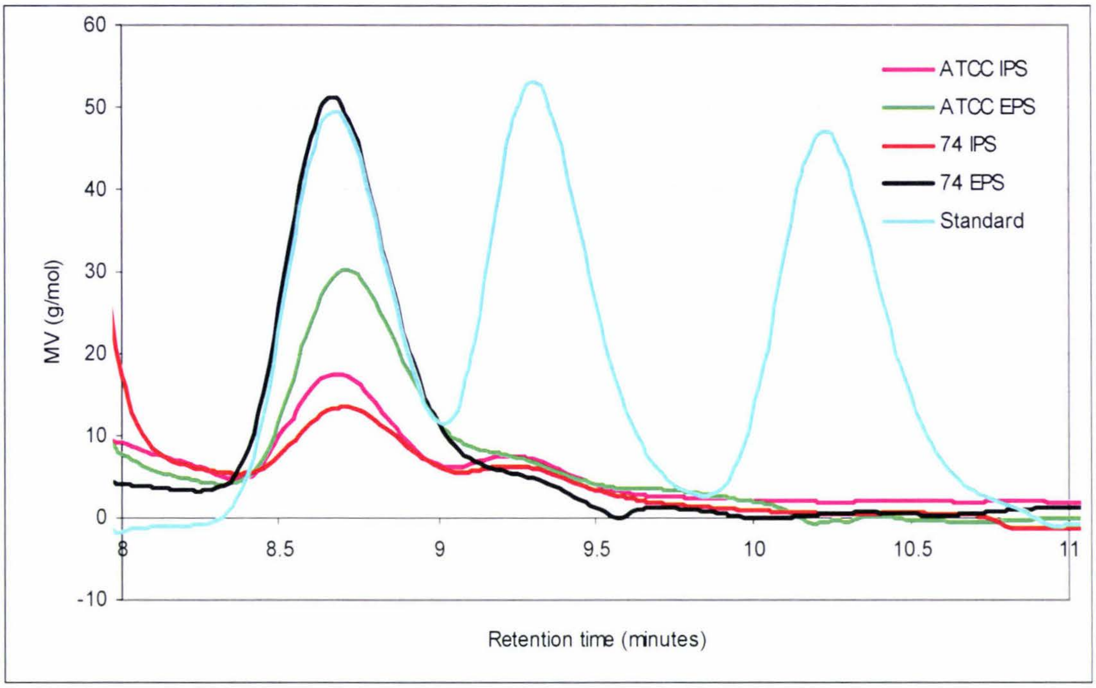


Fig. 7.2 Chromatograms of hydrolysed IPS and EPS samples based on RI absorbance as a function of retention time

The IPS samples from both strains, each formed two prominent peaks located within the range of glucose and galactose retention period. Both the EPS and IPS showed no peak appearing within the range of arabinose retention period, suggesting that no arabinose was present. However, IPS contained glucose and some or all the other monosaccharides, including mannose, galactose, xylose, fructose or rhamnose since all these five monosaccharides have merged as a peak within the range of the galactose retention time. By comparison of Fig.7.1 and Fig. 7.2, the second peaks produced by IPS appeared at about 9.25 min and the correlative peak of the standard galactose sample was about 9.29 min. Therefore, besides glucose, IPS were more likely to contain galactose, mannose or xylose rather than fructose and rhamnose because the peaks produced by former three monosaccharides were much closer to the IPS peak of 9.25 min than latter two monosaccharides. However, this would require further validation.

7-2-1 Ratio of soluble and insoluble portions of EPS/IPS

Due to the large number of hydroxyl groups, carbohydrates are strongly hydrophilic. In the case of polysaccharides, their solubilities are predominately dependent on their chemical structures. Chain molecules like cellulose are totally insoluble in water due to a high number of intra- and intermolecular hydrogen bridges, which enable, to a large extent, the formation of crystalline sub-units. Polysaccharides with branched structures are partly soluble in water often yielding colloidal solutions with high viscosities (Scherz and Bonn, 1998). Branched–chain structures, which are awkward to pack in the solid state, are more soluble and could generally exit in weakly aggregated form (Kennesy and White, 1983).

PSK is an intracellular polysaccharide with a small amount of protein bound to its polysaccharide skeleton and is reported to be water-soluble (Hotta et al., 1981; Ueno et al., 1980a,b). However, there is so far no report on the solubility of EPS.

In the experiment, water solubility of EPS and IPS samples from Wr-74 were determined. The soluble and insoluble fractions of the EPS and IPS samples were separated, purified and freeze-dried by the procedure described in section 3-6. The ratio of the two fractions was calculated based on the soluble content divided by insoluble content of polysaccharides in dried EPS or IPS sample. The levels of polysaccharides were determined by the Phenol/sulphuric acid method. The results are shown in Tables 7.2 and 7.3.

Table 7.2 Ratio of soluble and insoluble EPS

Batches	1	2	3	Average
Soluble EPS dry weight (mg)	76.5	412.4	56.3	181.7
Insoluble EPS dry weight (mg)	192.6	925.5	171.2	429.8
Soluble EPS content (mg)	70.9	371.8	46.6	163.1
Insoluble EPS content (mg)	147.4	668.5	117.3	311.0
Ratio (soluble/insoluble)	1: 2.1	1: 1.8	1: 2.5	~ 1: 2

Table 7.3 Ratio a of soluble and insoluble IPS

Batches	1	2	3	Average
Soluble IPS dry weight (mg)	126.0	588.3	225.0	313.1
Insoluble IPS dry weight (mg)	36.6	217.5	59.3	104.5
Soluble IPS content (mg)	115.5	506.9	201.5	274.6
Insoluble IPS content (mg)	28.7	137.5	34.1	66.8
Ratio (soluble/Insoluble)	4.0: 1	3.7: 1	5.9: 1	~ 9: 2

Results showed that both IPS and EPS samples prepared by the current separation process contained both soluble and insoluble fractions of polysaccharides. The average ratios of the soluble to insoluble portion of EPS and IPS were approximately 1:2 and 9:2, respectively. Therefore, approximately 82 % (w/w) water-soluble IPS and 32 % (w/w) water- soluble EPS could be obtained through the purification process. The water-soluble forms of EPS and IPS would be more suitable for the commercial application as a potential nutraceutical product.

7-2-3 Amino acid composition of the protein fraction of EPS/IPS

The presence of multiple amino acids in the composition of CPS is one of the important characteristics of the protein-bound polysaccharide. Hotta et al. (1981) reported that the protein fraction of the CPS contains 18 amino acids, of which the acidic acids and neutral amino acids are predominant, while the basic amino acids are present in only a small proportion. Aspartic acid, threonine, serine, glycine, glutamic acid, alanine, valine and leucine account for more than 70 % of all kinds of amino acids found in the protein portion.

The amino acid compositions of the protein fractions of both the EPS and IPS samples were determined using the amino acid analyser described in section 3-9-4. The analysis was carried out by the Nutrition Laboratory (Institute of Food, Nutrition and Human Health, Massey University). The IPS and EPS samples from ATCC-20545 and Wr-74 were prepared according to the procedure described in section 3-6.

The results in Table 7.4 indicate that the IPS samples from ATCC-20545 and Wr-74 contained higher levels of total amino acids (15.97 %, w/w and 16.01 %, w/w, respectively) than the EPS samples (4.14 %, w/w and 1.74 %, w/w, respectively). Some of amino acids in IPS were about 10 times higher than in EPS. These included aspartic acid, glutamic acid, glycine, alanine, methionine, tyrosine, phenylalanine, histidine, lysine and arginine. It was also found that IPS contained a relatively high level of tryptophan, but was absent in EPS. One possible reason for the abundance of amino acids in IPS can be explained by the fact that part of the intracellular functional or structural proteins might be released together with IPS from the mycelia during the hot water extraction. The acidic amino acids and the neutral amino acids are predominant in IPS, consistent with the report by Hotta *et al.* (1981). The total amount of acidic and neutral amino acids in IPS obtained from ATCC-20545 and Wr-74 accounted for 71.7 % and 75.5 %, respectively (Table 7.5). The EPS samples from

Table 7.4 Results of amino acid (AA) analysis of EPS and IPS

Amino acids		ATCC-20545 IPS	ATCC-20545 EPS	Wr-74 IPS	Wr-74 EPS
Acidic AA (%, w/w)	Aspartic acid	2.130	0.469	2.535	0.228
	Threonine	0.864	0.384	0.863	0.149
	Serine	1.069	0.406	1.378	0.147
	Glutamic acid	1.882	0.313	1.906	0.157
	Proline	0.623	0.305	0.600	0.100
Neutral AA (%, w/w)	Glycine	1.266	0.360	1.119	0.147
	Alanine	0.983	0.282	0.953	0.125
	Cysteine	0.027	0.158	0.023	0.035
	Valine	0.660	0.437	0.617	0.157
	Methionine	0.132	0.016	0.132	0.013
	Isoleucine	0.378	0.259	0.367	0.101
	Leucine	0.675	0.420	0.692	0.170
	Tyrosine	0.372	0.024	0.479	0.021
	Phenylalanine	0.389	0.079	0.403	0.073
Basic AA (%, w/w)	Histidine	0.361	0.051	0.332	0.025
	Tryptophan	1.184	-	0.924	-
	Lysine	1.595	0.127	1.473	0.048
	Arginine	1.375	0.045	1.210	0.038
Total amino acids (%, w/w)		15.965	4.135	16.006	1.734

Table 7.5 Percentage contents of three types of amino acids (AA) in EPS and IPS

Amino acids	ATCC-20545 IPS	ATCC-20545 EPS	Wr-74 IPS	Wr-74 EPS
Acidic AA (%, w/w)	41.1	45.4	45.5	45.2
Neutral AA (%, w/w)	30.6	49.3	30.0	49.6
Basic AA (%, w/w)	28.3	4.3	24.5	5.2

ATCC-20545 and Wr-74 contained higher proportions of acidic and neutral amino acids (95.7 % and 94.8 %, respectively) than their IPS samples (71.7 % and 75.5 % respectively), while the basic amino acids were lower in the case of EPS (approximately 5 %).

7-2-4 Ratio of polysaccharide to protein of EPS

Ratio of the polysaccharide to protein fractions of EPS from Wr-74 and ATCC-20545 was calculated based on the polysaccharide content determined by the phenol/sulphuric acid method and total nitrogen content (see section 3-9-3).

Table 7.6 Polysaccharide and protein contents in EPS samples

EPS source	Total nitrogen content (%, w/w)	Protein content (%, w/w)	polysaccharide content (%, w/w)	Ratio of polysaccharide and protein
ATCC-20545	0.82	5.13	79.02	15: 1
Wr-74	0.49	3.06	78.77	25: 1

Results in Table 7.6 show the total nitrogen content of EPS from ATCC-20545 was higher than that from Wr-74. The ratios of the polysaccharide to protein fractions of EPS from Wr-74 and ATCC-20545 are 25:1 and 15:1, respectively. The results were consistent with the amino acid levels given in Table 7.4. A wide range of polysaccharide to protein ratios has been reported for IPS (Ueno et al., 1980a and Garuda International Inc., USA 1998), but it is noted that no report was found on the polysaccharide to protein ratio for EPS.

7-3 Conclusions

The sugar compositions of EPS from both ATCC-20545 and Wr-74 were found to contain only glucose. However, IPS probably contained galactose, mannose or/and xylose in addition to glucose. The polysaccharide to protein ratios of the EPS samples from ATCC-20545 and Wr-74 were 15: 1 and 25: 1, respectively. This indicated that the EPS and IPS from Wr-74 and ATCC-20545 were all the protein-bound polysaccharides. The EPS samples from both strains contained approximately 80 % polysaccharides. The results of amino acid analysis showed that higher levels of amino acids were obtained in IPS (about 16 %, w/w) than EPS (about 2-4 %, w/w) for both strains. The composition and levels of amino acids of IPS from ATCC-20545 and Wr-74 were rather similar and agreed with the report by Hotta *et al.* (1981) with predominately acidic and neutral amino acids.

Solubility of EPS/IPS could be an important factor if they were to be used as nutraceuticals. The EPS and IPS samples obtained from ATCC-20545 and Wr-74 contained both soluble and insoluble fractions. The ratio of soluble to insoluble IPS fractions was 9:2, while the ratio of soluble to insoluble EPS fractions was 1:2. Therefore, approximately 82 % (w/w) IPS and 32 % (w/w) EPS were water soluble.

CHAPTER VIII PHYSIOLOGICAL ACTIVITIES OF CPS

8-1 Introduction

When a "foreign" substance (an antigen) such as bacteria, virus and other macromolecules including abnormal cells enters the bloodstream, the human immune system responds with the production of cytokine and antibodies. These immune molecules and cells are capable of recognizing and terminating the antigen. B and T lymphocytes, the main components of leucocytes, are capable of gene rearrangement to produce immunoglobulin proteins (antibodies) and cytokine molecules as an immune response. CPS has been shown to activate cell functions by stimulating overall immune function when an antigen invades the body (Di et al., 1991). Several studies have also reported the ability of CPS to enhance *in vitro* proliferation of T- and B-lymphocytes, and enhance the cytotoxic activity of NK cells to inhibit the growth of various cancers and even kill the cancer cells (Tsukagoshi et al., 1984, Wang *et al.,* 1996; Qian *et al.,* 1997; Ooi and Liu, 1999; Chu *et al.,* 2002)

In this study, the physiological activities of EPS, IPS and mushroom extracts on cytokine production from mouse spleen cells were investigated *in vitro* and the effects of EPS/IPS against proliferation of YAC-1 and K562 cancer cells were tested.

8-1 Results and discussion

8-2-1 Direct bioactivities of EPS/IPS on cancer cell lines

The cancer cell lines used in the experiment were YAC-1, which is a mouse lymphoma cell line and K562, which is a human myelogenous leukemic cell line. The levels of EPS and IPS prpared from the fermentation broth and biomass of ATCC-25454 and Wr-74 are shown in Table 8.1. The experiment was carried out in two 24-well plates in triplicate with a total of 48 samples for two cell lines (including water plus cells as control). The procedures of the experiment were described in section 3-7-1 and the results are shown in Fig. 8.1 and Fig. 8.2.

Based on the analysis using ANOVA and Tukey's pairwise comparison ($p \leq 0.05$), there was no significant difference found in the proliferation numbers of YAC-1 and K562 cancer cells among the samples treated with EPS/IPS and the

Table 8.1 The levels of EPS/IPS prepared for the test on cancer cell lines

Samples	Concentrations (µg/mL)	Sample volume (µL)	concentrations in each well (µg/mL)
74 EPS	3300	50	165
ATCC EPS	1770	100	177
74 EPS	3300	100	330
74 IPS	3500	100	350
ATCC IPS	3600	100	360
74 IPS	9950	100	995

This indicated that EPS/IPS did not have significant cytotoxic effects on these cancer cell lines. Tha cytotoxic effects of CPS on cancer cell lines have been reported to be caused by an indirect route through enhancing the activities of immune cells like NK cells and macrophages (Chen et al., 1986; Sakagami et al., 1991; Yang et al., 1992a; Liu et al., 1993; Wang et al., 1996; Qian et al., 1997; Ooi and Liu, 1999; Chu et al., 2002). However, the trend in Fig 8.1 and 8.2 appeared to show slightly decreasing cell number at higher EPS and IPS concentrations. It could also be possible that EPS/IPS could have a direct effect on the proliferation at much higher concentrations or with other cell lines such as those used by Dong et al. (1996). This would be a huge area for further research.

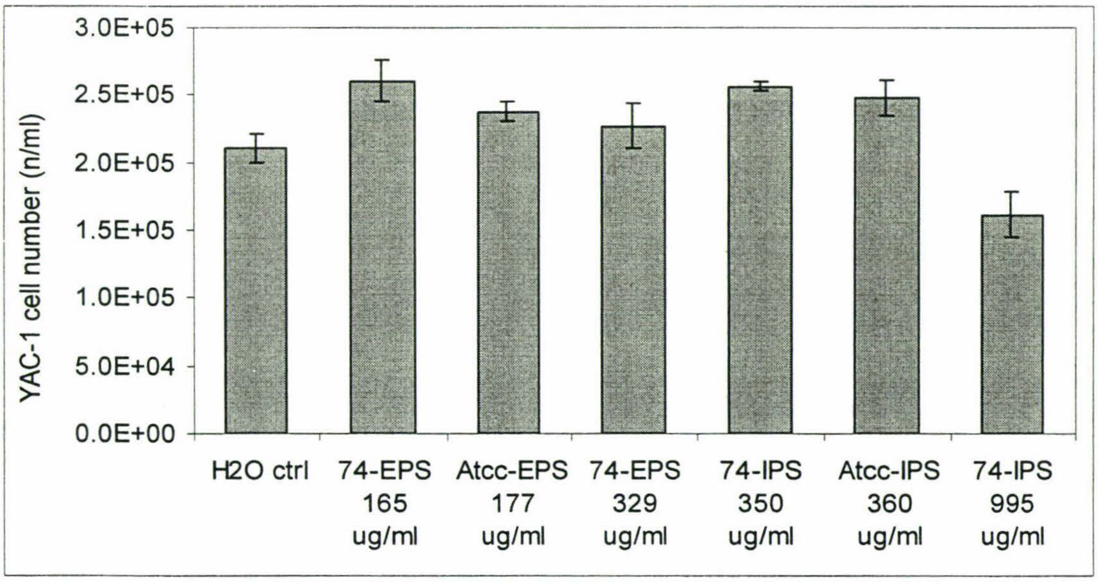


Fig. 8.1 Effect of EPS/IPS on cell numbers of YAC-1 cell lines All error bars are based on the standard deviation.

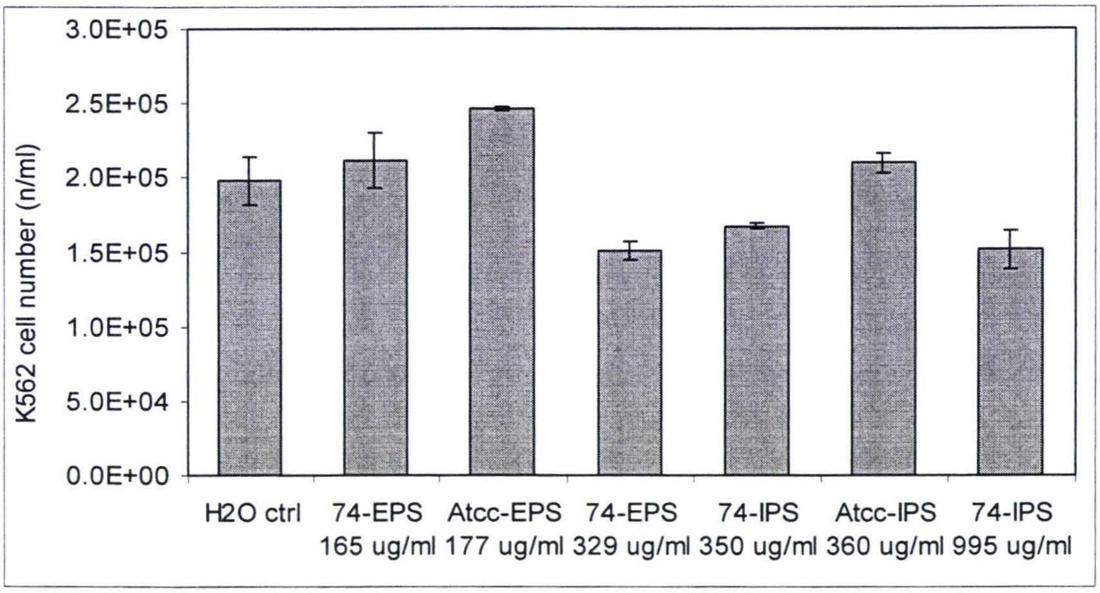


Fig. 8.2 Effect of EPS/IPS on the cell numbers of K562 cell lines All error bars are based on the standard deviation.

8-2-2 Effects of EPS, IPS and mushroom extracts on cytokine production

CPS has been widely reported to induce the production of various cytokines. Diverse physiological activities of CPS including anticancer and immuno-potentiating activities were either directly or indirectly related to cytokine production (Fung *et al.*,1996; Fisher and Yang, 2002). In the second part of this study, the effects of EPS/IPS on the cytokine productions of gamma-interferon (IFN- γ) and interlukin-12 (IL-12), from the T-cells and macrophages of murine splenocytes were investigated.

The murine splenocytes were prepared as described in section 3-7-2-1. Murine splenocytes contain mainly erythrocytes (RBC) and leucocytes (WBC). Through the cell preparation procedure described in section 3-7-2-1, the RBC were removed. Leucocytes are immune cells containing B- and T-lymphocytes (about 70 %), granulocyte (<10 %) and monocytes/macrophages (<10 %). T-lymphocytes produce IFN-γ upon stimulation by Concanavalin A (Con A) and macrophages produce IL-12 upon stimulation by *Saccharomyces cerevisiae* (SAC). Therefore, Con A and SAC were used as the stimulants in the experiment.

The aqueous solutions of EPS, IPS and mushroom extracts were prepared according to the procedure described in section 3-7-2-2. Each of the seven samples was serially diluted to obtain four different concentrations as shown in Table 8.2. Murine splenocytes were seeded at 10^5 cells per well (50 μL) into 96 well plates with

Table 8.2 Layout of sample concentrations after different dilutions

Samples	Original mg/mL	Neat μg/mL	1/10 μg/mL	1/100 μg/mL	1/1000 μg/mL
IPS from ATCC-20545	4.49	300	30	3	0.3
EPS from ATCC-20545	16.98	1130	113	11.3	1.13
Mushroom extract from ATCC-20545	23.33	1555	155	15.5	1.55
IPS from Wr-74	2.58	172	17.2	1.72	0.17
EPS from Wr-74	1.35	90	9	0.9	0.1
EPS plus IPS from Wr-74	32.64	2176	217.6	21.76	2.18
Mushroom extract from Wr-74	23.61	1574	157.4	15.74	1.58

a total volume of 150 μL per well including the sample (50 μL) and stimulant (50 μL). "Positive controls" refers to the samples containing cells and stimulant (SAC or Con A) without EPS, IPS or mushroom extracts. The sample layout in the 96-well plate and the treatment of the supernatant were described in section 3-7-2-3. The level of IL-12 and I FN-γ were determined by Enzyme Linked Immune Sorbent Assay (ELISA), which was described in section 3-7-2-4. The results of the experiment are shown in Fig. 8.3 to Fig. 8.6, demonstrating that EPS, IPS and mushroom extracts significantly improved ($p \leq 0.05$) the production of both IL-12 and IFN-γ.

Fig. 8.3 shows the levels of IL-12 produced by murine splenocytes after 48-hour incubation with the EPS, IPS and mushroom extract samples of different concentrations and in the presence of SAC as a stimulant. Based on ANOVA and Tukey's pairwise comparisons (($p \leq 0.05$), 61 % of the samples (17 concentrations of the seven samples out of total 28) induced significantly higher levels of IL-12 prod than the positive control. Higher IL-12 levels were generally induced at lower sample concentrations. This suggested a dose-dependent characteristic of a physiologically active compound.

Results in Fig. 8.4a shows the mushroom extract from ATCC-20545 induced a higher level of IL-12 by approximately 100 pg/mL when compared with the mushroom extract from Wr-74. The highest IL-12 level of 286 pg/mL appeared at the lowest concentration (1.55 μg/mL) of the mushroom extract from ATCC-20545. However, in Fig. 8.4b, the IPS from Wr-74 caused a higher level of IL-12 to be

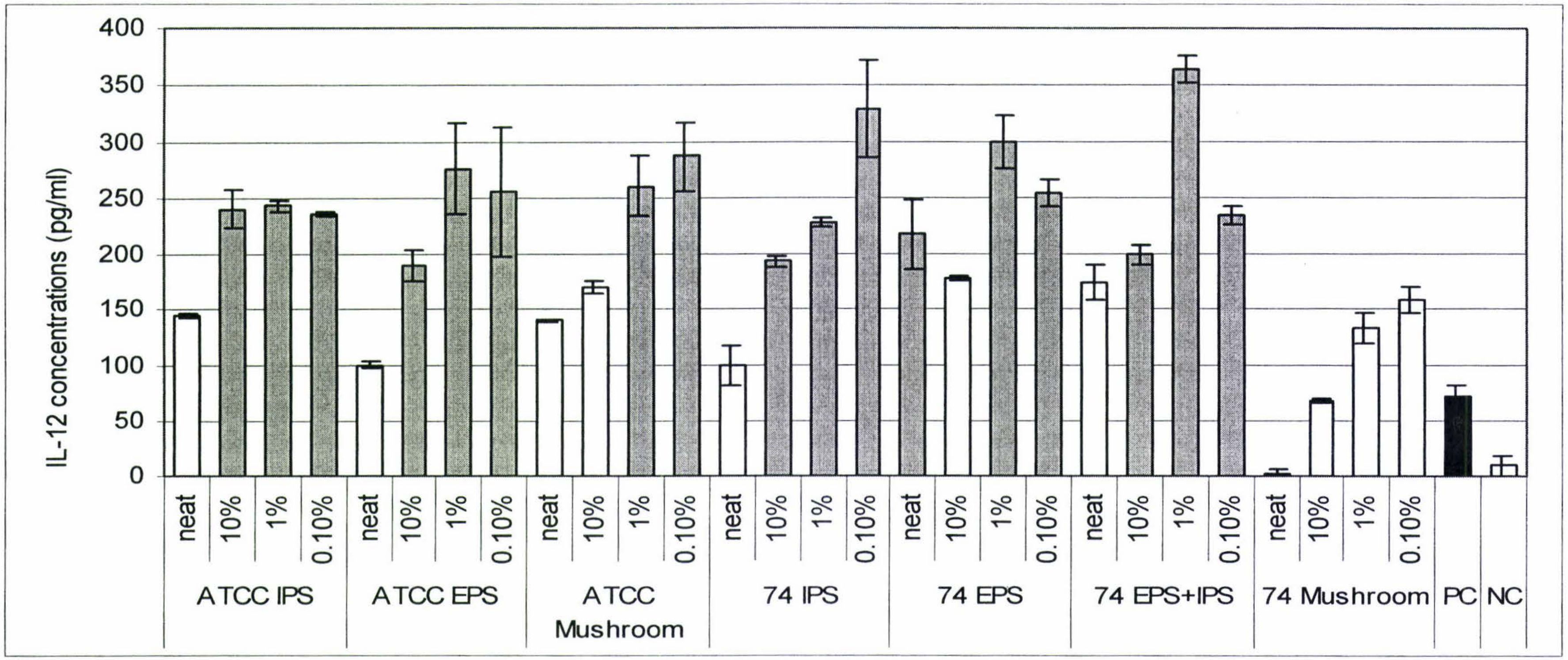
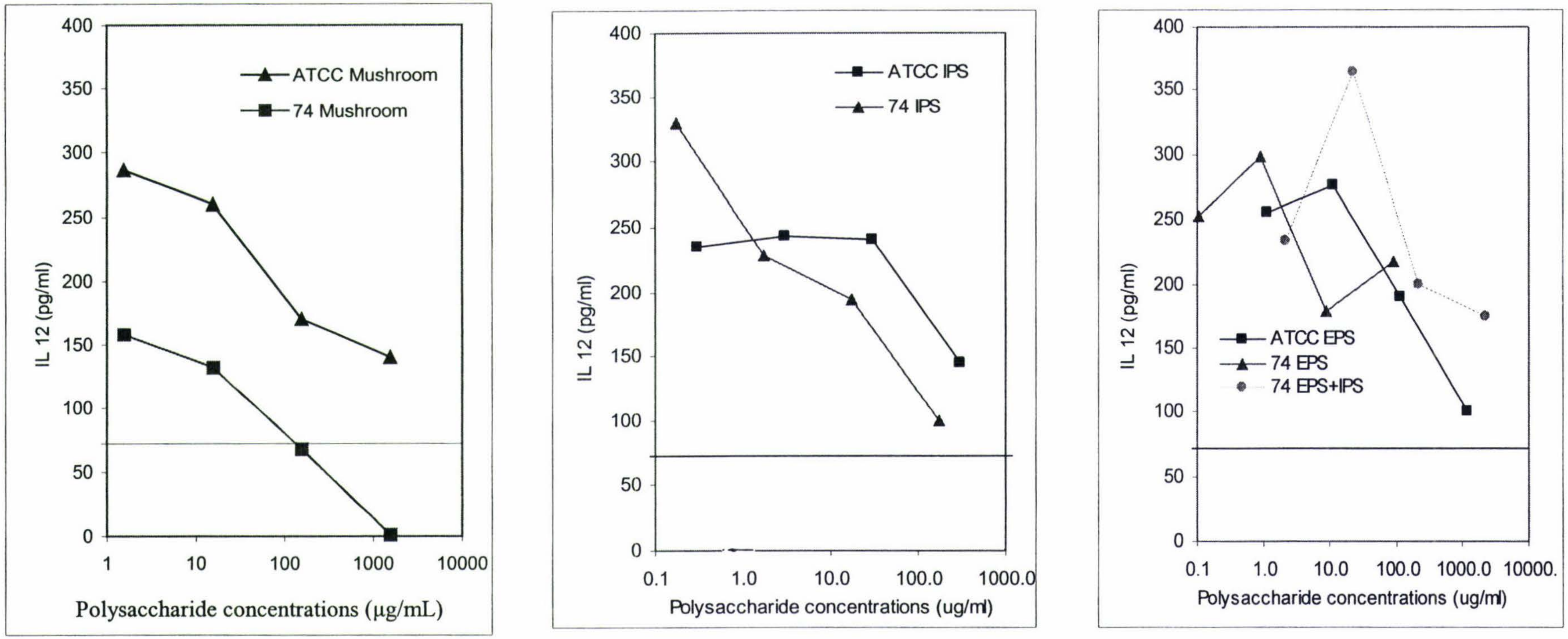


Fig. 8.3 IL-12 levels produced by murine splenocytes cocultured with EPS, IPS and mushroom extracts and stimulated by SAC PC means positive control and NC means negative control. ▨ means significant difference from PC ($p \leq 0.05$). ☐ means no significant difference from PC ($p \leq 0.05$). All error bars are based on the standard deviation. ATCC- or 74 mushroom means the mushroom extract sample from ATCC-20545 or Wr-74



(a) ATCC mushroom vs 74 mushroom **(b) ATCC IPS vs 74 IPS** **(c) ATCC EPS vs 74 EPS & 74 EPS+IPS**

Fig. 8.4 Comparisons of IL-12 levels produced by murine splenocytes cocultured with EPS, IPS and mushroom extracts of different concentrations The level of IL-12 in the positive control sample is represented by the horizontal line parallel to the X-axis (72 pg/mL) produced (329 pg/mL) than from ATCC-20545 at lowest IPS concentration. The levels of IL-12 induced by the IPS from ATCC-20545 remained almost constant (about 240 pg/mL) at the sample concentrations between 30 and 0.3 μg/mL (see Fig. 8.4 b). However, the IL-12 levels continued to increase with decreasing IPS concentrations of Wr-74. It appeared that higher levels of IL-12 could be achieved if the IPS from Wr-74 was further diluted. Fig.8.4c shows that the EPS from Wr-74 and ATCC-20545 were capable of inducing IL-12 levels of 298 and 256 pg/mL at the EPS levels of approximately 1 μg/mL. Among all the samples, mixed EPS plus IPS sample from Wr-74 induced the highest IL-12 level (364 pg/mL) at a concentration of 21.8 μg/mL, suggesting a possible synergetic effect of IPS and EPS on IL –12 production. The reason why the level dropped sharply from this point was unclear.

Similarly, Fig. 8.5 shows the levels of IFN-γ produced by murine splenocytes after incubation with the EPS, IPS and mushroom extracts of different concentrations in the presence of Con A. Based on ANOVA and Tukey's pairwise comparisons (($p \leq 0.05$), 57 % samples (16 out of total 28) induced significantly high levels of IFN-γ than the positive control.

In Fig. 8.6a, the mushroom extract from ATCC-20545 induced the highest level of IFN-γ (8612 pg/mL) at a concentration of 15.5 μg/mL. However, at its lowest concentration, the level of IFN-γ dropped sharply to 5450 pg/mL. It is suggested that the production of IFN-γ was highly sensitive to the concentration of the mushroom extract. With the mushroom extract from Wr-74, the maximum level of IFN-γ was achieved at a lower concentration (6340 pg/mL). In Fig. 8.6b, IPS from ATCC-20545 appeared to be more effective than IPS from Wr-74 in inducing IFN-γ production, although IFN-γ levels continued to increase at lower IPS concentrations. Interestingly, the EPS from ATCC-20545, EPS and EPS+IPS samples from Wr-74 (Fig. 8.6c) induced similar levels of IFN-γ. The highest levels of IFN-γ (around 4500 pg/mL) were obtained at the lowest concentrations of these samples.

It was noted in all cases that high sample concentrations could result in the reduction of cytokines produced, suggesting that the biological activities of the samples were highly dose dependent. Further work is required to determine the level of efficacy *in vivo* when CPS are administrated intraperitoneally or orally.

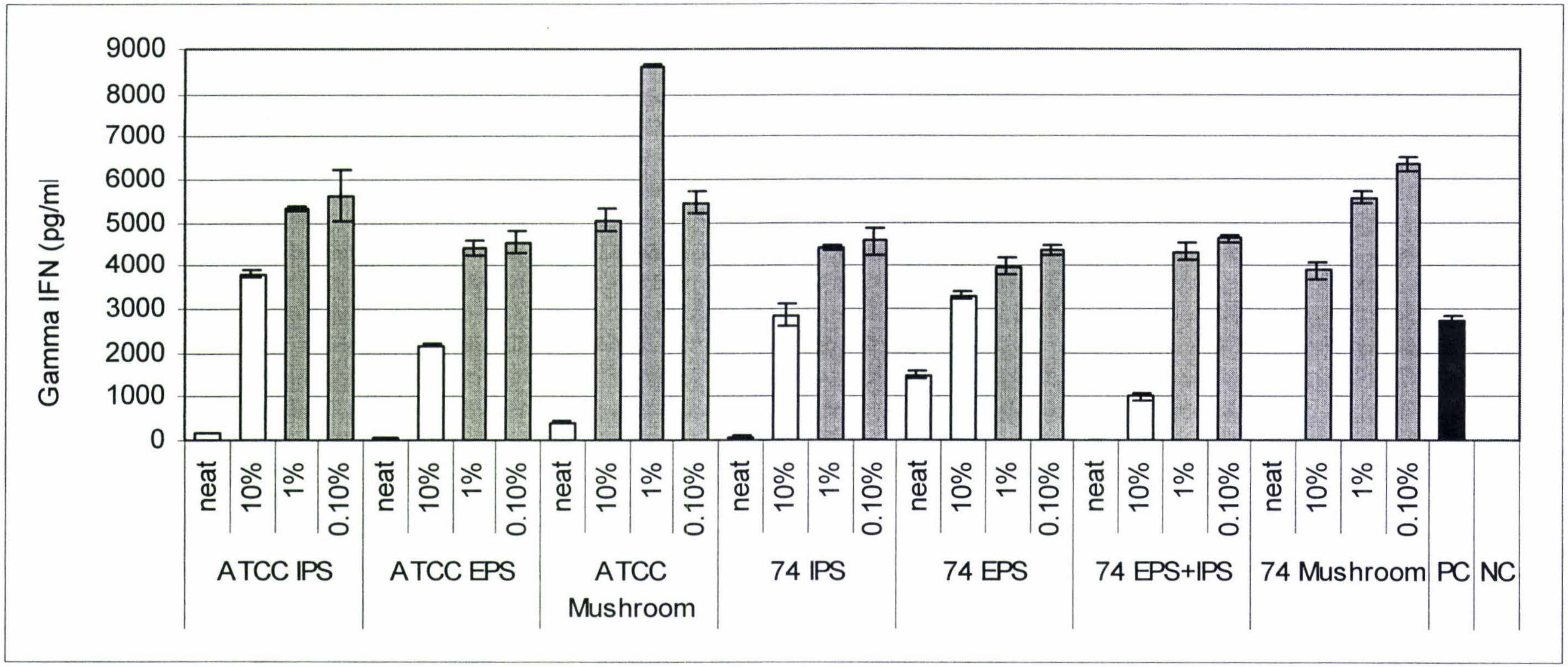
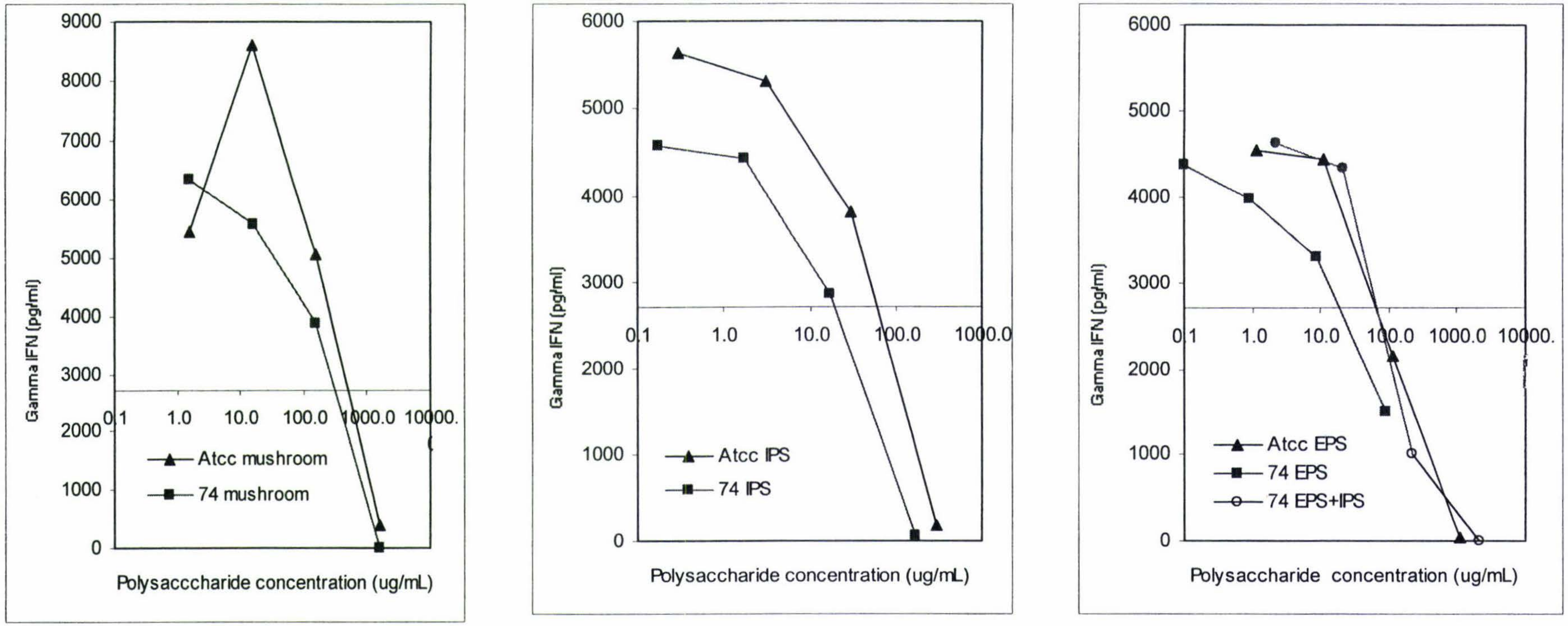


Fig. 8.5 IFN- γ levels produced by murine splenocytes co-cultured with EPS, IPS and mushroom extracts and stimulated by Con A

PC means positive control and NC means negative control. ▨ means significant difference from PC ($p \leq 0.05$); ☐ means no significant difference ($p \leq 0.05$); All error bars are based on the standard deviation.



(a) ATCC mushroom vs 74 mushroom **(b) ATCC IPS vs 74 IPS** **(c) ATCC EPS vs 74 EPS & 74 EPS+IPS**

Fig. 8.6. IFN- γ levels produced by murine splenocytes co-cultured with EPS, IPS and mushroom extracts of different concentrations

The level of IFN- γ in the positive control sample is represented by the horizontal line parallel to the X-axis (1874 pg/mL)

8-3 Conclusions

EPS/IPS had no significant cytotoxic effects ($p \leq 0.05$) on the proliferation of YAC-1 and K562 cancer cells. However, the trend appeared to show the decline in cell numbers at higher EPS/IPS concentration, suggesting that a significant decrease in cancer cells could be achieved at a higher sample concentration.

The EPS, IPS and mushroom extracts showed strong physiological activities in inducing IL-12 and IFN-γ production from murine splenocytes. The samples at lower concentrations generally induced higher levels of cytokines. However, at relatively high concentrations, samples appeared to inhibit cytokine production by murine splenocytes.

Mushroom extract from ATCC-20545 showed higher physiological activities than from Wr-74 in inducing IL-12 secretion. However, the IPS from Wr-74 at low concentration demonstrated higher physiological activity than from ATCC-20545. The EPS from both ATCC-20545 and Wr-74 induced the IL-12 at very similar levels. Among all the samples, the highest IL-12 level could be observed in the second lowest concentration of mixed EPS plus IPS sample from Wr-74, suggesting the possible synergetic effects of EPS and IPS on IL-12 production.

In terms of induction of IFN-γ, the mushroom extract from ATCC-20545 generally showed higher physiological activities than from Wr-74. In contrast to the IL-12 profile, the IPS from ATCC-20545 induced higher levels of IFN-γ than from Wr-74. The highest levels of IFN-γ induced in the presence of EPS from ATCC-20545, the EPS and EPS+IPS samples from Wr-74 were very similar.

In general, the EPS, IPS and mushroom extracts were proven to be capable of inducing the production of the cytokine molecules llike IL-12 and IFN-γ by lymphocytes and macrophages. The levels if cytokines were in fact highly dependent on the polysaccharide concentrations. This would be an important factor if they are to be used inn clinical trials.

CHAPTER IX CONCLUSIONS AND RECOMMENDATIONS

Strain Wr-74 was found to be the most comparable to strain ATCC-20545 in terms of the EPS/IPS production among the ten strains of *C. versicolor* screened. Morphological examination of the fruit bodies of ATCC-20545 and Wr-74 confirmed that both stains are *Coriolus versicolor*.

Milk permeate (MP), a waste stream of dairy industry, was found to be a suitable base medium for *C. versicolor*. The results based on the Homogenous Design experiment showed that MP, glucose and yeast extract were all positive factors for EPS/IPS production. The optimized medium calculated by Homogenous Design software package could produce a maximum EPS level of 3.57 mg/mL, which contained 80 % (v/v) MP, 1% (w/v) yeast extract and 2.5% (w/v) glucose. After the lactose in MP was hydrolyzed by 0.04 % Maxilact® lactase, a 100%HMP-YEP medium was prepared and investigated, based on which approximately twice the levels of EPS and IPS were obtained (3.2 and 0.3 mg/mL, respectively) when compared with 50%HMP-YEG medium. The EPS Levels in 50 %HMP-YEG medium were 40 to 60 % higher than in 50%MP-YEG medium.

Yeast extract (DIFCO) was crucial for EPS/IPS production and was found to be the best nitrogen source among those screened. Though glucose was the main carbon source consumed, excess glucose might retard EPS and IPS production. The optimum C/N ratio was found to be approximately 70.

A modified *impeller-assisted airlift fermenter* was used in the submerged-culture fermentation of *C. versicolor*. Results showed the fermenter deign could carry out batch fermentation with an approximately 74 % increase in EPS level obtained in the 50%HMP-YEG medium compared to 50%MP-YEG medium. The incorporation of the impeller in the airlift bioreactor was effective for EPS production and increased the EPS yield by approximately 58 %.

Salt addition did not appear to be necessary for the EPS production in these fermentations. The trace elements in the MP might be present at levels sufficient for EIP/SPS production. The corn oil used as an antifoaming agent resulted in a remarkable decrease in broth viscosity and decreased EIP/SPS production.

The polysaccharide fraction of EPS produced by both Wr-74 and ATCC 20545 contained only glucose. However, the polysaccharide fraction of IPS probably contained monosaccharides such as galactose, mannose or/and xylose in addition to glucose. The ratio of polysaccharide to protein for the EPS sample from ATCC-20545 and Wr-74 were approximately 15: 1 and 25:1, respectively.

The amino acid assay showed that the IPS from both strains contained approximately 16 % (w/w) amino acids. On the other hand, the EPS from ATCC-20545 contained 4.1 % (w/w) amino acids while EPS from Wr-74 contained 1.7 % (w/w) amino acids. Acidic and neutral amino acids accounted for over 70 % and 95 % in both IPS and EPS samples, respectively.

The polysaccharide levels in the EPS samples from both strains were found to be about 80 % (w/w). Ratio of soluble to insoluble IPS was 9:2, while the ratio of soluble to insoluble EPS was 1:2. In other words, approximately 82 % of IPS and 32 % of EPS from both strains could be obtained in the water-soluble form. The results of weight-average molecular weights (Mw) determined by SEC-MALLS showed that the IPS and EPS from both strains had very similar Mw distributions. Two distinct Mw fractions were observed. The Mw of both EPS and IPS were approximately 10^{6} Da in the elution range of 8.2 to 8.7 mL. In the second fraction, the Mw of IPS (10^5 Da) was about ten–times higher than EPS (10^4 Da) in the elution range of 9.8 to 10.3 mL.

Significant physiological activities on cytokine production were demonstrated *in vitro* by the EPS, IPS and mushroom extract samples from both strains. All the samples with lower concentrations (0.1 to 2.0 μg/mL) significantly increased the production of IL-12 (250 to 350 pg/mL) and IFN-γ (4,500 to 6,500 pg/mL) from murine splenocytes. The mushroom extracts from ATCC-20545 showed higher physiological activities on both IL-12 and IFN-γ production than that from Wr-74. The IPS from Wr-74 demonstrated higher physiological activity on IL-12 production, but lower activity on IFN-γ production than that from ATCC-20545. The EPS samples from ATCC-20545 and Wr-74 presented similar physiological activities on both IL-12 and IFN-γ production.

The effects of EPS/IPS on the proliferation of YAC-1 and K562 cancer cells were also investigated. However, no significant decrease in the cell numbers was found.

Based on this study, the following recommendations are given:

- The Wr-74 strain from Forest Research Institute of New Zealand could be used instead of the patented strain-ATCC-20545 for EPS/IPS production.
- The 100 % (v/v) HMP could be used as a base medium for EPS/IPS production. The enzyme level used to hydrolyse the lactose in MP should be approximately 0.04 %. Optimisation of the HMP based medium should be further investigated.
- The modified *impeller-assisted airlift fermenter* provided an extra option in addition to the non-agitated airlift bioreactor and stirred fermenter. Further improvements on the design of the fermenter to increase biomass production should be considered in future studies.
- Factors critical for submerged–culture fermentation of *C. versicolor*, such as the amount of dissolved oxygen supplied, speeds of the agitation provided, types and levels of antifoaming agents added and so on should be further investigated, especially when a larger scale fermenter is involved.
- Effects of EPS/IPS on the responses of animal immune systems *in vivo* should be investigated based on intraperitoneal or oral administration.

+APPENDIX I ABBREVIATIONS

AA	amino acids
ATCC	American Type Culture Collection, a global bioscience organization that provides biological products, technical services to government, industry and academic organizations.
C/N	ratio of carbon sources to nitrogen sources
Con A	concanavalin A
CPS	a general terminology referring to protein-bound polysaccharides produced by *Coriolus versicolor*
DIFCO	DIFCO Laboratory company of U.S.A
dn/dc	response of RI constant
DNS	an analysis method for determination of reducing sugars with 3,5-dinitrosalicylic acid reagent.
DW	dry weight (of biomass)
ELISA	Enzyme Linked Immune Sorbent Assay
EPS	extracellular polysaccharide(s) of *Coriolus versicolor*
FS solution	fungal salt solution;
HMP	hydrolysed milk permeate - the lactose in milk permeate was hydrolysed by the enzyme Maxilact® L2000
HPLC	High Performance Liquid Chromatography
IFN-γ	gamma interferon
IL-12	interluken-12
IPS	Intracellular polysaccharide(s) produced by *Coriolus versicolor*
K562	human myelogenous leukemic line
LS	light scattering
MP	milk permeate
Mw	weight-average molecular weight
PBP	Protein-Bound Polysaccharides
pg	pictogram (=10^{-9} gram)

PSK	Polysaccharides-Krestin, a commercial product of the intracellular polysaccharides of *Coriolus versicolor,* produced by Kureha Chemical Industry Ltd, Japan
PSP	PolysacchridoPeptides, a commercial product of the intracellular polysaccharides of *Coriolus versicolor,* reported by Yang (1992)
RI	refractive index
RS	reducing sugar
RV	relative viscosity
SAC	*Saccharomyces cerevisiae*
SEC-MALLS	Size exclusion chromatography coupled to a multi-angle laser light scattering
TFA	trifluoroacetic acid
TS	total sugar in the broth
UV	ultra-violet rays
YAC-1	mouse lymphoma line
YEG medium	The medium mainly containing yeast extract and glucose
Ypp3	yeast extract provided by Fonterra Co-operative Group
$Y_{p/s}$	EPS/IPS yield on substrate.
$Y_{p/x}$	EPS/IPS yield on biomass
μ_{max}	Maximum specific growth rate (h^{-1})

APPENDIX II: THE DIFFERENCE CAUSED BY METHODS FOR DETERMINING THE EPS AND IPS LEVELS

Phenol/sulphuric acid method is a sensitive and accurate method, and therefore widely used for quantitative determination of a wide range of mono-, oligo- and polysaccharides (Scherz and Bonn 1998). The detailed phenol–sulphuric acid method and sample preparation for EPS and IPS determination in the project were described in section 3-8-4-1 and 3-8-5-1. Besides phenol/sulphuric acid method, the EPS and IPS level can also be determined either by DNS method (Cui, 2002) or by the phenol/sulphuric acid method combined with DNS method (You et al., 2001). Detailed procedures were showed in Fig.3.5.

In the studies, the data of EPS and IPS determined by the phenol/sulphuric acid method after samples underwent a separation process were found lower than those obtained by the other two methods. In the graph below, a comparison of the

		1	2	3	4	5	6	7
◇	RS dns mg/ml	23.58	26.27	23.46	23.02	30.14	12.17	13.77
■	TS ps mg/ml	26.68	29.27	26.64	25.30	32.51	14.89	19.13
▲	EPSa (TS-RS) mg/ml	3.10	3.01	3.18	2.28	2.36	2.71	5.36
o	EPSb ps mg/ml	0.92	0.79	0.77	0.95	0.73	0.88	1.03
	Gap=(EPSa - EPSb)/EPSb	2.38	2.82	3.15	1.41	2.23	2.10	4.21

Comparison of the Phenol/Sulphuric acid method and a combined DNS and Phenol/Sulphuric acid method for level determination of seven EPS samples.

◇ **'RS dns'** means the reducing sugar levels determined by DNS method;
■ **'TS ps'** means the total sugar levels determined by Phenol/Sulphuric acid method.
▲ **'EPSa'** means the EPS levels obtained by TS minus RS.
○ **'EPSb'** means EPS level determined by Phenol/Sulphuric acid method after isolation of EPS from broth sample.

EPS levels determined using two methods was conducted based on seven broth samples, the phenol/sulphuric acid method and the phenol/sulphuric acid method combined with DNS method. It was observed that the EPSa values obtained from the combined method (TS-RS) were larger than EPSb values determined by the phenol/sulphuric acid method subsequent to isolation of EPS from broth. The average gap between the two EPS levels was 2.35 (the value of 4.21 was removed from the average according to the Q method). Therefore, the data obtained in the project using the phenol/sulfuric acid method was found to be generally lower than the data of many reports. The lower value might be ascribed to the loss of EPS to some extent during the sample preparation (section 3-8-5-1).

APPENDIX III FORMATS OF HOMOGENIOUS DESIGN

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a. $U_5(5^4)$ Format for an experiment of 4 factors and 5 levels:

Levels	Column Numbers			
	I	II	III	IV
1	1	2	3	4
2	2	4	1	3
3	3	1	4	2
4	4	3	2	1
5	5	5	5	5

b. Usage of $U_5(5^4)$ Format

Factors	Column Numbers
2	I, II
3	I, II, IV

c. Factors and levels selected for MP-based YEG media

Column	Factors Levels	1	2	3	4	5
I	MP %, v/v	100	80	60	40	20
II	Yeast extract %, w/v	0.2	0.4	0.6	0.8	1.0
IV	Glucose %, w/v	0.5	1.0	1.5	2.0	2.5

d. Factors and levels selected for MP-based MEG media

Column	Factors Levels	1	2	3	4	5
I	MP %, v/v	20	30	40	50	60
II	Glucose %, w/v	1.0	1.5	2.0	2.5	3.0
IV	Malt extract %, w/v	0.2	0.3	0.4	0.5	0.6

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