# EXTRACTION OF GLUCAN FROM *Acremonium diospyri* AND ITS APPLICATION IN *Macrobrachium rosenbergii* LARVAL REARING SYSTEM ALONG WITH BACTERINS AS MICROSPHERES

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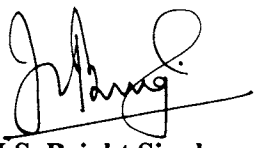
# CERTIFICATE

This is to certify that the research work presented in this thesis entitled "Extraction of glucan from *Acremonium diospyri* and its application in *Macrobrachium rosenbergii* larval rearing system along with bacterins as microspheres" is based on the original work done by Mr. A. Anas under my supervision at Centre for Fish Disease Diagnosis and Management, School of Environmental Studies, Cochin University of Science and Technology, Kochi 682 016, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.



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# CHAPTER – 1

# GENERAL INTRODUCTION

The fresh water prawn *Macrobrachium rosenbergii* is the largest of the family palaemonidae, indigenous to tropical fresh and brackish water habitats of the Indo – Pacific region. India is the third largest producer of *M. rosenbergii*, with an annual production of 24,230 mt during 2001 and 30,450 mt during 2002 – 2003 -periods. This is expected to increase to 50,000mt by 2010 (Nambudiri 2003). One of the major impediments in the growth of freshwater prawn industry in India is the lack of adequate supply of seed at the right time. In India though 71 fresh water prawn hatcheries with a built in production capacity of 1.83 billion post larvae are under operation (Bojan 2003), the larval production technology has not yet been perfected. One of the reasons pointed out is the recurrence of vibriosis (Sindermann 1977) which reduces larval survival rate to a level of economic non viability.

Sindermann (1977) implicated Vibriosis as a major mortality factor in juvenile penaeid shrimp, whereas song and coworkers (Song et al. 1993) classified it as secondary infection transpire after the deterioration in water quality. Pillai and Jayabalan (Pillai & Jayabalan 1993) suggested good environmental management for control of vibriosis. Singh et al. (1985) reported gradual increase in the population of vibriosis from eggs to post larvae of *Fenneropenaeus indicus* and along with which mass mortality of the larvae occurred especially during mysis and post larval stages. Singh (1990) reported high incidence of *Aeromonas* in eggs, which failed to hatch out and during instances of larval mortality in *Macrobrachium rosenbergii*. Sahul Hameed and coworkers (Sahul Hameed et al. 2003) recorded *Vibrio* species as major taxon in the larvae and post larvae of *M. rosenbergii*. According to Miyamoto et al. (1983) and Anderson et al. (1989), the total number of bacteria gradually increase from eggs to post larvae in *M. rosenbergii*. In a review of *M. rosenbergii*, Johnson (1980) reported heavy mortality in hatchery systems due to vibriosis. Colorni (1985) studied the bacterial flora associated with *M. rosenbergii* and Yolanda & Vega (1992) could isolate *Aeromonas*, *Pseudomonas*, and *Bacillus* from the haemolymph during morbidity.

There are four methods for the control of pathogenic invasions in prawn larval rearing system, among which the most widely practiced one, is the use of chemotherapeutic substances. Baticadose & Paelibare (1992) observed that chemotherapeutants like chloramphenicol, erythromycin, oxytetracyclin, nitrofurans, formalin, malachite green and $KMnO_4$ are widely used in prawn hatcheries to prevent diseases. They even observed mortalities and morphological deformities of larvae due to the indiscriminate use of these chemicals. Use of oxytetracyclin has been reported to have caused increased bacterial resistance in shrimp farms (Nash et al. 1992) and similar enhanced resistance to chloramphenicol has also emerged through misuse of the antibiotics in shrimp hatcheries in Ecuadore and Philippines (Baticadose & Paelibare 1992). Abraham et al. (1997) have isolated *Vibrio harveyi* strains from diseased shrimp, resistant to most of the antibiotics used in aquaculture systems. Tendencia & delapena (2001) have compared antibiotic resistance in bacterial isolates from pond water, pond sediment and cultured shrimp, and reported that the incidence of resistance to oxytetracyclin was the highest followed by furazolidone, oxolinic acid and chloramphenicol. Sahul Hameed et al. (2003) reported that more than 90% of their bacterial isolates from larvae and post larvae of fresh water prawn were resistant to erythromycin, oxytetracyclin and furazolidone.

It is believed that the indiscriminate use of antibiotics resulted in the development of drug resistant bacteria and the extreme stage is the transfer of this drug resistant gene to the human pathogens. Moreover, the increased use of antibiotics leads to their accumulation in shrimp/prawn meat making the consignment unacceptable in the international market. Therefore it is highly essential to find an alternative to antibiotics, and the concept of immunostimulant is one of the promising areas for research and development. The basic idea is in fact to exploit the immune system of the animal by administering compounds of yeast or bacteria. The organism misjudge that it is attacked by these cells and will try to fight of them by raising its defense mechanism to a higher level. In a previous study (Anas & Singh 2003) it was demonstrated that a preparation of glucan cum bacterin could increase the survival rate of the larvae of *Macrobrachium rosenbergii*, but the problem faced was the lack of proper delivery system. Considering all above, the present study was undertaken with three objectives. The first objective was

to develop an aquaculture grade glucan from the fungus *Acremonium diospyri*. This was achieved by extracting the glucan by employing different extraction protocols modified to suit to the requirements. The glucan thus prepared were compared for its structure and immunostimulatory property using *Fenneropenaeus indicus* as the animal model. Aiming at a large-scale production of the same and the requirement of waste management as part of the production process, a consortium of microorganisms to be used in a treatment system was also developed. Finally microcapsules using chitosan as the wall material were developed as drug delivery system for the larvae of *Macrobrachium rosenbergii* though with glucans and bacterins could be delivered.

## 1.1. Glucan

### 1.1.1. Glucans – Sources, structure and extraction

Polysaccharides are a very important class of biopolymers, which consist of long chains of repeating sugar units. Mainly the type of monomer units, the chain length, the kind of glycosidic linkage and the degree of branching structurally characterize them. Nature continuously synthesizes huge amounts of polysaccharides that serve particularly as structural scaffolds like cellulose in plants and chitin in animals or as storage carbohydrates like starch and glycogen. A special group of β-1,3-linked poly glucose usually named as "glucan" is wide spread in many bacteria, fungi, mushroom, algae and higher plants and has attracted attention of researchers because of bioactive and medicinal properties such as immunostimulating, anti-inflammatory, antimicrobial, anti-infective, antiviral, anti-tumoral, cholestrol lowering, radio protective and wound healing properties (Stone & Clarke 1992, Bohn & BeMiller 1995, Kogan 2000, Freimund et al. 2003).

β-Glucans have a common structure, a main chain consisting of (1-3)-linked β-D-glucopyranosyl units along which are randomly dispersed single β-D-glucopyranosyl units attached by 1-6 linkages giving a comb like structure (Figure 1.1), but the fine structures and conformations of these polymers vary as do their activities (Wagner et al. 1988, Jamas et al. 1991). The most active forms of β-glucans come from the mycelia, fruiting bodies and culture fluids of fungi (Table 1.1). The mechanism of the effect of

1,3-β glucan is not yet fully understood and probably depends on the specific molecular structure, which is influenced by the molecular weight, branching, the presence of charged residues and conformational features like the formation of helices (Kelly 1996, Kim et al. 2000, Ishibashi et al. 2002).

Salkowski (1894) prepared an insoluble polysaccharide from yeast, which was termed "yeast cellulose". Zeichmeister & Toth (1934, 1936) reinvestigated this substance and showed by methylation analysis that it contained a preponderance of 1-3 linkage. All other polysaccharides including cellulose, which had been investigated up to that time, contained 1- 4 linkages between glucose units. The product also differs from cellulose by not being soluble in ammoniacal copper oxide solution (Schweitzers reagent) and by not giving a characteristic blue colouration with a solution of iodine in potassium iodide and treatment with strong acid. In order to avoid confusion with cellulose Zechmeister and Toth referred to their material as "Yeast Polyose"(Zeichmeister & Toth 1936). Hassid et al. (1941) confirmed the presence of 1-3 linkages in bakers yeast glucan by methylation analysis. They suggested that the molecule be probably of the closed chain type. Low specific rotation of acetylated and methylated derivatives and upward rotation during hydrolysis suggested a predominance of β linkages in the glucan. Very similar result was obtained by Barry & Dillon (1943) who worked with bakers yeast (Harrison & Rose 1971).

During the period 1950-1960 differences of opinion developed on the structure of the alkali insoluble glucan from bakers yeast was on the basis of methylation analysis. Bell & Northcote (1950) suggested that it had a highly branched structure with relatively short chains of 1-3 linked glucose residue inter linked by about 11% 1-2 glucosidic linkage. Peat & Whelan (1958) using partial acid hydrolysis concluded that the glucan was linear and contained certain sequence of 1-3 and 1-6 linked β glucose residues. The presence of about 10-20% of 1-6 linkages was confirmed by tosylation followed by iodination of primary hydroxyl groups (Peat & Whelan 1958).

In attempts to resolve these differences, Manners & Patterson (1966) carried out further methylation, periodate oxidation and also enzymatic degradation studies and concluded that yeast glucan had a branched structure containing main chain of 1-6 linked β glucose residues, to which were attached linear side chains of 1-3 linked β glucose residues. An alternative structure was proposed by Misaki et al. (1968) which was similar in some respect to that proposed by Manners & Patterson (1966).

A key observation by Bacon & Farmer (1969) eventually led to solution of the problem. These workers showed that yeast glucan prepared as already described was heterogeneous and contained an acetic acid soluble polysaccharide, which was shown to be a β-1,6- glucan. Their suggestion that 'yeast glucan' was in fact a mixture of major β-1,3- glucan components and a minor β-1,6- glucan components. Manners & Patterson (1966) suggested that different samples of glucan differ in the degree of substitution of main chain.

In nearly all of these early experiments yeast glucan was isolated after drastic treatments of whole yeast first with hot dilute alkali followed by heating for several hours with dilute HCl. This left an insoluble residue, which was washed with water, ethanol and ether and dried. Hot sodium or potassium hydroxide has been proven to be very efficient to remove proteins and alkali soluble polysaccharides from yeast or yeast cell walls. Additionally lipids are hydrolysed under basic conditions in to glycerine and fatty acids. Thus most of the glucan isolation methods (Hassid et al. 1941, Northcote & Horne 1952, Peat & Whelan 1958, Misaki et al. 1968, Manners et al. 1973a, Jamas et al. 1989, Williams et al. 1991, Kelly 1996, Zulli & Suter 1998) are based on the treatment of yeast or yeast cell walls with hot NaOH or KOH , preferred at concentrations of 0.75 – 1.0M and temperatures of 60 - 100°C. The insoluble residue afterwards is usually washed with water or with acids serving for cleavage of the linkages between glucan, proteins and polysaccharides as well as for neutralization of the mixture. Subsequently, a lipid removal by the use of organic solvents is performed.

Bell & Northcote (1950) used a similar approach for the preparation of glucan from bakers yeast, except that they replaced mineral acid by acetic acid (0.5 N) at 75-80°C to avoid the degradation of glucan during extraction. Further work was done by Peat & Whelan (1958) who purified bakers yeast glucan as recommended by Bell & Northcote (1950) except that they found it necessary to autoclave the residue after acetic acid extraction, (0.02 M Sodium acetate at pH 7.0) and wash several times with water to remove glycogen completely.

Hydrochloric acid is a strong protic acid, which would be difficult to use in the extraction of pharmaceutical glucans due, in part, to its corrosive nature, toxicity, and waste management. To address these concerns, Muller et al. (1997) studied the comparative efficacy of acetic, formic or phosphoric acid in substituting the hydrochloric acid in the process for the extraction of β-(1,3)-D-glucan from the cell wall of Baker's yeast. The resulting microparticulate glucan were employed as the starting material for the production of (1,3)-β-D-glucan phosphate. $^{13}C$ NMR analysis of the glucan phosphate derived from the acetic, formic or phosphoric acid extracted microparticulate glucan show excellent correspondence to hydrochloric acid extracted glucan.

Williams and his coworkers have extensively studied the β-(1,3)-D-glucan isolated from the inner cell wall of *Saccharomyces cerevisiae* (Williams et al. 1991, Williams et al. 1992, Williams et al. 1994, Muller et al. 1995, Muller et al. 1997, Lowman et al. 1998, Lowman & Williams 2001, Lowman et al. 2003). They isolated the glucan by an alkaline acid hydrolysis method, originally developed by Hassid et al. (1941) and later redefined by Williams et al. (1991). They have shown that the glucan extracted by this method were chemically pure, i.e. they contained no other carbohydrates, protein or residual lipids (Ensley et al. 1994).

Most fungal β-glucans exhibit immunomodulatory activity when administered intravenously or intraperitoneally. The problem is that insoluble or hardly soluble β-glucans cause significant adverse effects when administered by parenteral routes (Maeda et al. 1988). From this point of view, oral administration of water soluble glucan

preparations would have several advantages (Sandula et al. 1999). In order to improve the solubility of β-1,3-D-glucans, several derivatization procedures, e.g. carboxymethylation (Soltes et al. 1993), phosphation (Williams et al. 1991), sulfation (Williams et al. 1992) were developed. Lowering of the molecular weight of the polysaccharide may also contribute to their improved solubility and facilitate chemical derivatization. Many different methods, e.g. microwave assisted dissolution (Wang et al. 2002), enzymatic digestion and ultrasound irradiation (Sandula et al. 1999) have been applied to depolymerize glucan in to lower molecular weight fragments.

Ohno et al. (1999) have successfully developed a protocol to obtain soluble *Candida* spp. β-(1,3)-D-glucan (CSBG) by sodium hypochlorite oxidation (NaClO) and subsequent dimethyl sulphoxide extraction ($Me_2SO$) of acetone dried whole cell preparations. The β-glucan fraction was free from cell wall mannan, gives a symmetrical peak by gel filtration, and is soluble in dilute NaOH. The product is composed mainly of β-(1,3) and β-(1,6)-D-glucosidic linkages. The NaClO oxidation step is essential for this method, because β-glucan could be scarcely extracted by $Me_2SO$ without oxidation. The oxidation product was free from protein, nucleic acid, lipid, as well as mannan, and is thus useful to prepare β-glucan, especially the water-soluble form. This method has been used extensively in the recent years to extract soluble glucan from different species of yeast and fungus (Ohno et al. 1999, Tokunaka et al. 2000, Ohno et al. 2001, Ishibashi et al. 2002, Ishibashi et al. 2004).

### 1.1.2. Glucans in aquaculture

An immunostimulant may be defined as an agent, which stimulates the nonspecific immune mechanism when given alone, or the specific mechanism when given with an antigen. Many different types or groups of immunostimulants have been reported in aquaculture among which the most important one is glucan (Figueras et al. 1998). As one of the most important structural elements of fungal or bacterial cell walls (Rosenberger 1976, Duffus et al. 1982), beta glucans have been successfully used as immunostimulants for strengthening the nonspecific defense system of a wide range of animals. In mice glucans enhance nonspecific antimicrobial activity as well as anti cancer mechanism (Di

Luzio et al. 1979, Di Luzio 1983). In fish, beta glucans activate cytotoxic macrophages, lymphocytes, natural killer cells (Olivier et al. 1986), complement mediated hemolytic activity (Engstad et al. 1992), and complement system through the alternative pathway (Yano et al. 1991). In crustaceans, glucans activate haemolymph clotting in the horseshoe crab (Ohno et al. 1990) and activate the proPhenoloxidase system, causing increases in such activations as phagocytosis and encapsulation, both of which are associated with protective reactions (Smith & Soderhall 1983).

Glucan can be used on their own, to enhance the activity of non specific immune systems, indirectly increasing general disease resistance, or adjuvant with a vaccine, in order to increase the specific response against certain pathogen (Anderson 1992, Robertsen et al. 1994). Adjuvants are usually mixed and injected with antigen preparation, acting to elevate specific immune activity. Immunostimulants can be administered before with or after vaccine to amplify the specific immune response in generating elevation of humoral antibody levels and a number of antibody secreting cells (ASC).

Figueras et al. (1998) studied the significance of sequence of glucan administration when used along with vaccine. They used β-1,3 glucan extracted from *Saccharomyces cerevisiae* as adjuvant in a *Vibrio damsella* vaccine for turbot (*Scophthalmus maximus* L.). Turbot were injected with the adjuvant prior, at the same time and after the vaccine and monitored several immune parameters such as phagocytic index and rate, passive haemolytic plaque numbers and agglutinating antibody titre at different times of post inoculation. The highest activity of all the immune parameters was obtained when glucans were injected after the bacterin.

Aakre et al. (1994) showed that the presence of a glucan adjuvant in an anti-*Aeromonas* vaccine gave a high relative percent survival in Atlantic salmon after a virulent challenge. Furthermore these authors showed that the use of β-glucan as an adjuvant led to higher serum antibody level compared to vaccine given alone. Similarly Jeney & Anderson (1993) obtained enhanced immune responses in rainbow trout given a glucan adjuvanated

anti –*Yersinia ruckeri* vaccine. Rorstad et al. (1993) studied adjuvant effect of glucan along with formalin killed *Aeromonas salmonisida* vaccine against furunculosis in Atlantic salmon. In all the cases they found that vaccine supplied with glucan induced higher protection against furunculosis than vaccine without glucan.

Ogier de Baulny et al. (1996) studied the effect of glucan separately and in combination with an anti vibriosis vaccine in turbot. They observed enhanced white blood cells production in animals treated with glucan and lyzozyme activity in animals treated with adjuvanated vaccine. However, the glucan treatment doesn't give any protection against the challenge with a virulent strain of *Vibrio anguillarum*. In our previous study with *Macrobrachium rosenbergii* an increased survival rate was observed when a combination of glucan and bacterin preparation was administered orally (Anas & Singh 2003)

There are many studies which have indicated the immunostimulating and disease resistance potential of glucans. Robertson et al. (1990) studied the enhancement of non-specific resistance in Atlantic salmon (*Salmo salar*) by glucan from *Sacharomyces cerviciae*. Enhanced resistance was demonstrated against *Yersinia ruckeri*, the casual agent of enteric red mouth disease, against *Vibrio anguillarum* the casual agent of classical vibriosis and against *Vibrio salmonicida*, which caused cold-water vibriosis or Hitra disease in Salmon. They injected the glucan intraperitonially at a dose of 20mg M-glucan/ fish (20g mean weight). Maximal resistance developed in the fish 3 weeks after injection. Injection of different glucan doses and challenges one week later with *V. anguillarum*, showed that 50 -100μg glucan per fish resulted in the highest level of protection.

Brattgjerd et al. (1994) observed increased macrophage activity in Atlantitic salmon evaluated by *in-vitro* $H_2O_2$ production and phagocytosis activity on injecting yeast glucan. Same results were observed by Jeney et al. (1994) in Hybrid sturgeon when fed with 0.5% glucan.

Sung et al. (1994) suggested glucan as a short-term immunostimulant based on their studies on the disease protection and immunostimulatory potential of different concentrations of glucan in post larvae of tiger shrimp. They reported that a three-hour immersion of post larvae of tiger shrimp in 0.5 and 1.0mg/ml glucan suspension will confer the larvae better protection against *Vibrio vulnificus*, which lasted until 18 day following immersion. Song et al. (1997) obtained same results when glucan treated Tiger shrimp (*Penaeus monodon*) was challenged with *V. vulnificus* and white spot syndrome virus. The tolerance of glucan treated shrimps was slightly enhanced to stresses including catching, transport and higher ammonia content. But the growth and survival rates of treated and untreated shrimps were not significantly different.

Chang et al. (2000) evaluated the effectiveness of β-1,3 glucan derived from *Schizophyllum commune* in enhancing shrimp survival as well as haemocyte phagocytosis and superoxide anion production in brooders of *Penaeus monodon*. The brooders showed enhanced survival rate, haemocyte phagocytic activity, cell adhesion, and superoxide anion production when glucan was administered in their diets. The immunostimulatory enhancement peaked at day 24 after starting the dietary exposure and subsequently decreased to the pre-feeding level at the end of the 40 days feeding trial which supported the short term immunostimulation of glucan proposed by Sung et al. (1994).

Lopez et al. (2003) studied the efficacy of glucan and a mega dose of vitamin C in *Litopenaeus vannamei* juveniles and observed significantly higher growth rate in glucan treated animals, whereas higher blood protein, total blood cells, granular cells and proPO activity were recorded in animals fed with vitamin C. They explained the enhanced growth rate as a result of enhanced metabolisms in the animal using the energy generated via the degradation of glucan in the intestine by glucanase. The arguement of degradation of glucan in the intestine of animal is still not confirmed scientifically and the authors have not studied the presence of glucanase in the intestine. Where as beta glucan binding protein (BGBP) has been recognized in the hepatopancrease and haemolymph of many species of crustaceans, supporting the immunostimulatory property of glucan (Duvic & Soderhall 1993, Cerenius et al. 1994, Lee et al. 2000, Yepiz-Plascentia et al. 2000, Jimenez-Vega et al. 2002).

The non-effectiveness of glucans reported by many scientists (Scholz et al. 1999, Sritunyalucksana et al. 1999) could be explained with the structure of the glucan they used. The immunostimulatory property of glucan depends on the structure, solubility, ratio of 1,3 and 1,6 bonds and the molecular weight which depends on its mode of extraction (Kim et al. 2000, Lowman & Williams 2001). Engstad & Robertson (1994) studied the immunostimulatory effect of different yeast β glucans in Atlantic salmon, and noticed that β-1,6-glucan is not having any immunostimulatory properties. They concluded that number of β-1,3- linked side chains is decisive for the immunostimulatory effect of yeast β glucan in Atlantic salmon. In the present work the effect of different glucan extraction protocol and its process modifications on the immunostimulatory property, structure and its environmental impacts were evaluated.

### 1.2. History of bacterins

History of bacterins for aquaculture dates back to 1954 when Schaperclaus attempted to vaccinate fishes with killed and live *Aeromonas hydrophila* cells. Since then Vibrionacea associated with fish has been exclusively studied with much progress in furunculosis vaccine in the 1980's. Attempts have also been made to control vibriosis using vaccine in cultured *Anguilla anguilla* (Kusuda et al. 1978, Itami & Kusuda 1980) salmonids (Antipa et al. 1980, Hastein et al. 1980), Carp and sea bream (McKay & Jenkin 1969, Joosten et al. 1995).

McKay and Jenkin achieved the first vaccination in crustacean (McKay & Jenkin 1969), who could induce increased resistance to *Pseudomonas* on fresh water Cray fish *Parachaeraps bicarinatus*. Lewis & Lawrence (1983) demonstrated higher levels of agglutinins in immunized *Penaeus setiferus* and showed that bacterins of *Vibrio alginolyticus* induced some protection against subsequent challenge by increasing the levels of non-specific agglutinins towards Gram-negative bacteria. Itami et al. (1989) vaccinated *Penaeus japonicus* with formalin-killed cells of *Vibrio* species by immersion, injection and spray vaccination.

Teunissen and coworkers (Teunissen et al. 1998) while studying the influence of vaccination of *Penaeus monodon* against vibriosis, resistance to the pathogens was found enhanced even with bacterins alone, and was higher than those treated with glucan. They explained it by the partial specificity of the 'vaccine induced' bactericidins. Adams (1991) found bactericidins in the haemolymph of *P. monodon* after exposure to heat killed *Vibrio alginolyticus*. However, this would only hold true if there existed some memory for different pathogens in the shrimp immune system. According to Adams (1991), these bactericidins persisted in the shrimp haemolymph until five days after exposure and the haemagglutinin titre, part of the shrimps non specific response, peaked up for 7 days after exposure to the heat killed bacteria. Huang and colleagues (Huang et al. 1981) stated the possibility that the agglutinins observed in *Macrobrachium rosenbergii* behaved similarly to antibodies by facilitating recognition of foreign particles by phagocytes or by transporting them to areas of bactericidal activity. The possibility of the presence of such molecules in *Penaeus indicus* was proposed by Singh and coworkers during their attempts to vaccinate shrimps against white spot syndrome virus (Singh et al. In Press).

The first attempt to vaccinate *Macrobrachium rosenbergii* was done by Huang and coworkers (Huang et al. 1981) with *Vibrio anguillarum*. It was basically an immunological study and on injecting formalin killed cells of *V. anguillarum*, the animal did not respond to the vaccination indicated by absence of an increase in the level of circulating agglutinins or the $LD_{50}$ value after 6 days of post vaccination.

Vici and coworkers (Vici et al. 2000) prepared two types of bacterins against vibriosis in larvae of *Macrobrachium rosenbergii*. On applying it along with yeast *Acremonium diospyri* to the hatchery system, they obtained 15% increase in the post larval production in the experimental group compared to the control group. Later Anas & Singh (2003) continued the study by using glucan extracted from *Acremonium diospyri* as adjuvant and observed further increase in seed production.

One of the major objectives of the present study was to evaluate the impact of interval of administration of glucan cum bacterin preparation in increasing the larval production of *Macrobrachium rosenbergii*. It is an established facts that immunostimulation in crustacean aquaculture is for a short time (Sung et al. 1994), which proposes the need of booster doses at regular interval. Sung & Song (1996) studied the residence time of heat killed *vibrio* antigen in tiger shrimp *Penaeus monodon* using indirect fluorescent antibody technique. They could trace the antigen up to 7 days after immersion in gill, 1 day after in plasma and haemocytes, and 3 days in hepatopancrease and hematopoetic tissue, and by day 14, the antigen was completely undetectable. While studying the effect on different routes of administration of *Vibrio anguillarum* bacterins in post larvae of *Penaeus monodon*, Azad and coworkers (Azad et al. 2005) observed enhanced growth rate and disease protection with booster doses of bacterin through feed. The concept of booster dose was further established by Singh et al. (In Press), who observed maximum protection against white spot syndrome virus in *Penaeus indicus*, when repeatedly administered with inactivated virus preparation (IVP) once in seven days.

Bacterins are the inactivated bacterial cells, which can elicit an immune response in the recipient. Mean while vaccine can be defined as the suspension of an attenuated or killed disease causing microorganism as of virus when inoculate stimulate the production of antibodies and therefore conferring immunity against the virulent microorganism. The process of bacterin preparation consist of 5 steps

1. Selection of the pathogenic strains
2. Mass culturing of the selected pathogen
3. Harvesting
4. Inactivation
5. Confirmation of the inactivation

### 1.2.1 Selection of pathogenic strains

Most immunostimulants used in aquaculture are live bacteria or killed bacterial or cell wall components like lipopolysaccharide, peptidoglycan or glucan. The degree of protection offered increases with the virulence of the pathogen from which the vaccine was prepared (Alabi et al. 1999b). But the isolation of such a strain is very difficult because most of the bacterial diseases are of secondary nature (Lightner & Lewis 1977), other predisposing factors like nutritional deficiency and extreme stress play the key role.

Baticadose (1986) suggested good environmental management for control of vibriosis, as the pathogen isolated from diseased shrimp couldn't induce disease on injection in to healthy animals under ideal environmental conditions. The same observations was made by Pillai & Jayabalan (1993) who injected advanced post larvae of *Penaeus indicus* with *Vibrio harveyii* and it couldn't induce clinical signs of vibriosis. However, the successful isolation of the organism from the haemolymph after four days made him conclude that they were opportunistic pathogens.

Lightner in his review (Lightner 1988) reported that a relatively massive inoculum has to be administered to overcome the natural defense and to cause disease and death in experimental animal. Subsequently, the isolation and confirmation of pathogenicity in aquaculture systems become extremely difficult. However, satisfying Koch's postulates is the only option left even though it happens to be cumbersome.

### 1.2.2 Mass culturing

After selecting the pathogenic strain for the bacterin, it has to be cultured in a medium under experimental conditions. An appropriate bioprocess technology has to be evolved to get maximum yield of cells. Preference of solid or liquid medium varies with the organisms cultured.

### 1.2.3 Harvesting

Antipa and coworkers (Antipa et al. 1980) cultured *Vibrio anguillarum* in Trypticase Soya broth for 24 h, the bacterial cells were separated by centrifugation at 2500g and

washed twice with sterile 0.85% saline. The cells were centrifuged again and finally re-suspended in physiological saline at a concentration of 6.7mg dry weight bacterial cells/ml

Vici and coworkers (Vici et al. 2000) cultured the pathogenic strains in Nutrient Agar (Peptone 0.5%, Beef extract 0.5%), prepared in aged sea water (15ppt) for 48h and harvested in Phosphate buffered saline (PBS) composed of Na $H_2PO_4$ 6.42g., $Na_2HPO_4$ 34.32g ., NaCl 10g , distilled water 1000ml. The cultures were diluted in PBS to obtain 0.5 OD ($Abs_{600}$).

**1.2.4 Inactivation**

Out of the five steps most important in bacterin preparation is the inactivation of the organism. There are many physical and chemical methods applicable for the inactivation as follows.

**1.2.4.1 Chemicals**

**1.2.4.1.a Aziridines**

The aziridines owe much to the development of mustard gas in world war II, and I and in particular, the β Chloro ethylamines or Nitrogen mustard. Indeed, ethyleneimine (EI) is commonly prepared by cyclization of bromoethylamine hydrobromide under alkaline condition (Doel 1985)

Ethyleneimine is known to react with α and ε amino, imidazole, carboxyl, sulphhydryl and phenolic groups of proteins, inorganic phosphates, glycero and hexose phosphates and amino groups of adinine and thiamine

Clearly the aziridines are highly reactive group of substances and they have been used to mutagenize a wide range of organisms. Although undoubtedly toxic, there is no published evidence of tumor induction in man. Further more Fellowes (1965) failed to produce tumors in rats given 0.5mg of acetyl ethyleneimine and kept for 515 days.

According to the knowledge of the authors, no works has been published in the area of inactivation of bacteria using ethyleneimine. This method is widely used for the inactivation of viruses.

**1.2.4.1.b Formaldehyde**

Formaldehyde has been used more widely for the inactivation of bacteria than any other chemicals (Song & Sung 1990, Sung et al. 1991, Hoel et al. 1998, Alabi et al. 2000, Mikkelsen et al. 2004). The chemistry of commercial solutions of formaldehyde remains somewhat obscure. Most aldehydes including formaldehyde and glutaraldehyde readily polymerize to a whole series of derivatives and it becomes difficult to propose specific mechanism of inactivation against this background of aldehyde related substances. It is clear, however, that formaldehyde react with both nucleic acid and protein primarily through exposed amino groups. Frankel (1981) suggested that besides the reversible addition of formaldehyde to amino groups, there are slower and more stable cross-linking reactions of the resultant amino methlols through condensation with other amino acid side chains yielding methyl bridges. Similar reactions occur with the amino groups of nucleic acids and probably give rise to cross links with the nucleic acid and between the nucleic acid and any adjacent protein

**1.2.4.1.c β Propio lactone (BPL)**

The alkyl and acyl bonds at each end of the lactose structure made β Propiolactone (BPL) highly reactive. This molecule is relatively stable in pure and concentrated forms, but it degrades quickly in the presence of cellular debris and cell culture medium.

**1.2.4.1.d Chloroform**

Chloroform has long been as widely accepted as a preservative. Although chloroform can inhibit the activity of microorganism, it has no effect on their enzyme. Chloroform kills many non spore-bearing bacteria with no important modification of their biochemical characteristics. The use of chloroform thus conserves their potency. But the preparation of bacterin from spore bearing organism and non-spore bearing resistant type bacteria are not practical by this method. Bunyea (1927) first reported the use of

chloroform for bacterin preparation. Using this method Kawakami et al. (1997) prepared bacterins of *Pasteurella piscicida*, a potential pathogen of Yellow tail (*Seriola quinqueradiata*) by exposing the bacterial cells to 0.5% chloroform-PS for 24h at 4°C. The residual chloroform layer was removed by evaporation for 30 minutes at 50°C and the inactivated cells were separated by centrifugation at 5000g for 30 minutes.

### 1.2.4.2 Physical Methods

#### 1.2.4.2.a UV rays

UV rays are absorbed by many cellular materials but most significantly by the nucleic acids. The absorption and sequence of reaction are predominantly in the pyrimidines of the nucleic acids. One important alteration is the formation of pyrimidine dimers in which two adjacent pyrimidines get bound.

There is however, more significant problem associated with UV radiation. The most relevant problem is that of the critical nature of the dose of UV required to inactivate. This is compounded by the technical difficulties involved in dosing large volume UV opaque solution. Considering that the opacity of the bacterial preparation may vary significantly from batch to batch, the technical problem of UV inactivation is formidable.

#### 1.2.4.2.b X-rays

X-rays are lethal to microorganisms as they have considerable energy and penetration ability. The practicability of this method is less because it is very expensive to produce in quality and very difficult to utilize.

#### 1.2.4.2.c γ radiation

γ Radiation is emitted from radioisotope. They have high penetration power. Thus high-energy particles make a direct hit on the DNA particle and cause ionization. This inactivation is attained by the formation of either cross links or breaks in the structure of nucleic acid (Pollard 1960). Stewart & Zwicker (1974) inactivated the *A. viridans* (Var) *homarii* cells by exposing the suspension to γ rays from a $Co^{60}$ source for 30 minutes.

#### 1.2.4.2.d Heat

Heat is the second widely used method for the inactivation of bacterial cells. Antipa et al. (1980) prepared *Vibrio anguillarum* bacterins by heating the cultures in a boiling water bath for one hour. Azad et al. (2005) prepared the bacterin in the same way by heating the *Vibrio anguillarum* suspension at 70°C for 30 minutes. The heat is destructive to the products of bacteria. The use of heat in the preparation of bacterins therefore reduced their potency

### 1.2.5 Confirmation of Inactivity

In Europe during the period of 1970's and 1980's, large proportion of Foot and Mouth disease outbreaks have been attributed either due to improperly inactivated vaccine or the escape of virus from vaccine production plant, rather than the introduction of new strains from other parts to the world.

Therefore strict quality control measures have to be adopted for assuring the quality of bacterin. Vici et al. (2000) confirmed the inactivation of bacterin prepared by inoculating an aliquot of bacterin to a fresh medium, and incubating for 6 days to confirm that there was no bacterium alive.

## 1.3 Microencapsulation – Theory and Practice in Aquaculture

Microencapsulation is a process in which very thin coatings of inert natural or synthetic polymeric materials are deposited around micro sized particles of solids or droplets of liquids. Products thus formed are known as microcapsules. Recently, there has been an extensive and increasing interest in this field and has played a significant role in a variety of industries like pharmaceutical, cosmetic, food, agricultural, plastic, paper, photographic printing, paint, adhesive, and textiles, for many years (Remunan-Lopez & Badmeier 1996, Gonzalez Siso et al. 1997, Remunan - Lopez et al. 1998, Park & Chang 2000, Nelson 2002, Sinha & Trehan 2003). These encapsulated preparations range in size from one micron to seven millimeters and release their contents at a later time by means appropriate to the application.

Microencapsulation science evolved from polymer chemistry and was developed in response to the need for controlled release of materials (active ingredient) from encapsulated state. The objective of microencapsulation is to contain the active ingredient inside the capsule wall for a specified time or in a specified environment. Modification to the composition of the capsule wall and to the microencapsulation process technique can determine how, when or whether the active ingredient inside the capsule is released.

The first reference describing a microencapsulation process (preparation of gelatin microspheres by coacervation) dates from 1930 (Dziezak 1988). However, it was only two decades later when this approach was applied to the microencapsulation of drugs. These microcapsules were initially designed to mask the taste and protect drugs to the gastric fluids and, some years later, to sustain the release of drugs. Many of these formulations are currently in the market. These microencapsules, intended for oral administration, are composed of non-biodegradable polymers from which the drug is frequently released by diffusion. These microspheres are not, however, adequate for the controlled release of peptides and proteins, since these macromolecules cannot diffuse through polymers. It was only in the eighties when several investigators realized that in order to control the release of a macromolecule a biodegradable polymer is required. The polymer erodes over the time upon exposure to biological fluids thus releasing the encapsulated molecules. Even vaccines could be microencapsulated to release only at a particular site. There are varieties of coating materials used to produce microcapsules. Some of them are gum arabic, carageenan, starch, caboxymethyl cellulose, paraffin, silicates, albumin, gelatin etc.

The feeds/ drugs used in aquaculture have to be microencapsulated because of the following reasons. 1. Since the feeds/drugs in aquaculture is applying to the water, there is every possibility of loosing the ingredients without giving the desired effect. 2. The leaching of high protein ingredients leads to water pollution making the conditions unfavorable for the animal growth. 3. Many of the drugs like vitamins have to be guarded against light induced reactions or oxidation.

In aquaculture there is a growing need for microparticulate artificial diet for fish/prawn larvae, as well as other marine-suspension feeders such as crustacean and bivalve mollusks (Villamer & Langdon 1993, Lopez - Alvardo et al. 1994, Bustos et al. 2003). An unstable diet that leaches out rapidly affects larval growth and survival, since it not only diminishes diet availability, but also increases water fouling and bacterial production (Amjad & Jones 1989). This demands the need of a microencapsulated drug delivery system, which can protect the drug from the external environment of the animal and releases the drug inside the intestine at right time and right site. Crustacean larvae, having an underdeveloped digestive system, require a finite amount of time for consuming a diet. It is estimated that digestion, from intake to evacuation, occurs from 12 to 20 min (Pedroza-Islas et al. 2000). This necessitates the microcapsules to release the core material immediately after reaching the intestine rather than a slow controlled release.

Majority of trials of experimental oral vaccines produces variable protection or no protection at all (Smith 2002). The main reason for this fact is thought to be that protective antigens are inactivated by the high acidity of the stomach which prevent them for being absorbed in the lower gut of the fish and, therefore, for stimulating a response (Smith 2002). To improve the effectiveness of oral vaccination, development of an efficient delivery method, which protects the antigen against digestive degradation in the anterior part of the digestive tract, is of major importance (Johnson & Amend 1983, Dunn et al. 1990).

The art of microencapsulation includes three steps 1. Design of wall material, 2. Selection of encapsulation method, and 3. Design of release mechanism.

### 1.3.1 Design of wall material

There are varieties of wall materials used to produce microcapsules. Some of them are alginate, gum arabic, chitosan, gellan, pectin, carageenan, starch, caboxymethyl cellulose, paraffin, silicates, albumin, gelatin etc. In most microcapsules, the shell materials are usually organic polymer; however, waxes and fats have also been used, particularly in

food and drug applications where the shell must meet food and drug administration specifications.

### 1.3.1.1 Alginate

Alginic acid is a linear, 1,4-linked copolymer of β-D-mannuronic acid (M unit) and its C5 epimer, α-L-glucuronic acid (G unit). The G and M units are joined together in homopolymeric and heteropolymeric sequentially alternating blocks. The proportion of the various blocks depends on the seaweed origin, the season of harvest, and the part of the algae from which the alginate is extracted. When the monovalent ion of sodium is replaced by divalent ions or trivalent ion, ionic cross-linking among the carboxylic acid groups occur and the polysaccharide molecule form a polymeric network.

### 1.3.1.2 Carrageenan

Carrageenans are isolated from cell walls of red seaweeds and one of these, k-carrageenan, is the more suitable for microencapsulation. It is a condensation product of 1,3-linked α-galactose-4-sulphate and 1,4-linked 3,6-β-anhydrogalactose. It is well known that alkali metal ions ($K^+$, $Rb^+$, $Cs^+$), alkaline-earth metal ions ($Ca^+$) or trivalent ions ($Al^+$), and also $NH_4^+$and amines promote the gelation.

### 1.3.1.3 Chitosan

Chitosan α (1,4)-2- amino 2- deoxy β-D glucan is a deacetylated form of chitin, an abundant polysaccharide present in crustacean shell. Even though the discovery of chitosan dates from the 19$^{th}$ century, it has only been over the last two decades that this polymer has received attention as a material for biomedical and drug delivery applications (Janes et al. 2001). The accumulated information about the physicochemical and biological properties of chitosan led to the recognition of this polymer as promising material for drug delivery and, more specifically, for the delivery of delicate macromolecules (Hirano et al. 1988, Hirano & Nagao 1989, Hirano et al. 1990, Sudarshan et al. 1992, Aspeden et al. 1996, Chandy & Sharma 1996, Carreno-Gomez & Duncan 1997, Gonzalez Siso et al. 1997, Lim et al. 1997, Shepherd et al. 1997, He et al.

1999, Blanco et al. 2000, Ravi Kumar 2000, Janes et al. 2001, Ko et al. 2002, Zheng & Zhu 2003).

### 1.3.1.4 Gellan

Gellan gum is an anionic polysaccharide produced by extracellularly by diverse genera of bacteria including *Pseudomonas*, *Xanthomonas*, and *Sphingomonas*. It is a linear homopolymer with tetrasaccharide repeating unit consisting of two β-D-glucose, one β-D-glucuronic acid, and one α-L-rhamnose residue. The gellation occurs around 40°C and large variety of cations such as $Ca^{+}$, $Mg^{+}$, $K^{+}$ or $Na^{-}$ has been also reported to gellify the beads and citrate has been used to solubilize gellan gel (Doner & Becard 1991)

### 1.3.1.5 Pectin

Pectin is an important constituent of the cell walls and soft tissues of higher plants. It is composed of long, regular sequence of 1,4-linked α-D-galcturonate residues, which, in nature, may be partially esterified. The level of esterification, which varies with the source and the method of extraction, profoundly affects the gelling and thickening properties of commercial pectins.

### 1.3.1.6 Gum Acacia

Gum acacia (arabic) is the traditional carrier used in spray drying. It is a natural exudate from the trunk and the branches of leguminous plants of the family Acacia (Thevenet 1988). There are several hundred species of Acacia; however, only a few species are gum producers and these are located in the sub desert regions of Africa.

## 1.3.2 Selection of encapsulation method

Numerous encapsulation processes have been developed during the past several years. These include coacervation, interfacial polymerization, spray drying, air suspension, centrifugal extrusion and rotational suspension separation. The broad ranges of capabilities available through these processes are important because, according to Baken & Anderson (1970), no single encapsulation process is adaptable to all core material conditions.

### 1.3.2.1 Coacervation

This method is based on the ability of cationic and anionic water-soluble polymers to interact in water to form a liquid, polymer rich phase called a coacervate. Gelatin is normally the cationic polymer used. A variety of natural and synthetic anionic water-soluble polymers interact with gelatin to form complex coacervate suitable for encapsulation (Thies 1996). When the coacervate forms, it is in equilibrium with a dilute solution called the supernatant. In this two phase system, the supernatant act as the continuous phase, where as the coacervate acts as the dispersed phase. If a water insoluble core material is dispersed in the system and the complex coacervate wets this core material, each droplet or particle of dispersed core material is spontaneously coated with a thin film of coacervate. When this liquid film is solidified, capsules are formed.

This is the most widely used method of encapsulation in aquaculture. Yufera et al. (2002) encapsulated the free aminoacids by this method. They dispersed the dietary material in a basic pH buffered tris HCl aqueous solution. Two parts of this solution were emulsified in five parts of soy lecithin and cyclo hexane solutions. The cross-linking agent trimesoyl chloride, dissolved in diethyl ether, was then added to the emulsion. The microcapsules formed were allowed to settle, and the cyclohexane lecithin solution was decanted. After washing with cyclohexane, the microcapsules were dispersed in a gelatin solution while stirring. Distilled water with a temperature approximately 38°C was added while stirring. The capsules were then repeatedly washed with fresh water, then in a pH 8-buffered saline solution in order to remove the debris. Considering the high cost and potential toxicity of cyclohexane and trimesoyl chloride used in the above microcapsule preparation (Yufera et al. 1999), they avoided the scale up of the particles and developed (Yufera et al. 2005) another type of food micro particles avoiding these chemicals, for the larvae of *Sparus aurata* and *Solea senegalensis*.

Langdon & Waldock (1981) encapsulated dietary lipids by complex coacervation using gelatin acacia as wall material. This encapsulated lipid in combination with algal foods were fed to the juvenile *Crassostrea gigas*

### 1.3.2.2 Interfacial polymerization

The unique feature of this technology is that the capsule shell is formed at or on the surface of a droplet or particle by polymerization of reactive monomers. This approach to encapsulation has evolved in to a versatile technology able to encapsulate a wide range of core material, including aqueous solutions, water immiscible liquids, and solid. The wall of the nylon protein capsule is prepared by interfacial polymerization and is made up of protein cross linked with 6,10 nylon (Chang et al. 1996b). The use of nylon – protein walled microcapsules for delivering nutrition to aquatic filter feeders was first described by Jones et al. (1974) who cultured *Artemia* on a non defined encapsulated diet. Jones and coworkers (Jones et al. 1974, Jones et al. 1979a, Jones et al. 1979b) showed that it was possible to feed other crustacean species on nylon – protein encapsulated diets and to study some of their nutritional requirements. They succeeded in growing larvae of the prawn *Penaeus japonicus* from zoea to the post larval stage on nylon – protein encapsulated diets of chicken egg and powdered short-necked clam, *Tappes philippinarum*. Several scientists modified the nylon-protein-encapsulation method so as to eliminate the need for toxic chemicals in the preparation of the capsules (Hayworth 1983, Levine 1983, Jones et al. 1984). Maugle et al. (1983) reported that nylon-protein encapsulated supplements of bovine trypsin improved the growth of *P. japonicus* fed on artificial diet. Furthermore, they found that encapsulated trypsin supplements increased the total endogenous protease activity in the hepatopancrease of the prawn, possibly by activating protease zymogens. Similar improvements in prawn growth were observed with supplements of amylase (Maugle et al. 1983).

### 1.3.2.3 Spray drying

It is the most commonly used method in the food industry. The process is economical and flexible, uses equipment that is readily available and produces particles of good quality. The process is conducted in a spray dryer, and involves three major steps, such as preparation of dispersion, or emulsion to be processed, homogenization of the dispersion and atomization of the mass into the drying chamber (Judie 1988).

The material to be atomized is prepared by dispersing on active material in to a solution of the coating with which it is immiscible. The coating is generally food grade hydrocolloids such as gelatin, vegetable gum, modified starch, dextrin or non-gelling protein (Balassa & Fanger 1971). Following the addition of an emulsifier, the dispersion is homogenized to give oil – in water type of emulsion, and then atomized in to a heated air stream supplied to the drying chamber (Sparks 1981). As the atomized particle falls through the gaseous medium, they assume a spherical shape with the oil encased in the aqueous phase. This explains why most spray-dried particles are water-soluble. The rapid evaporation of water from the coating during its solidification keeps the core temperature below 100°C, in spite of high temperature used in the process (Brenner 1983). The particle exposure to heat is in the range of few seconds at most (Balassa & Fanger 1971). The chief advantage of this method is its ability to handle many heat labile materials.

Villamer & Langdon (1993) prepared capsules for the delivery of dietary components to larval shrimp (*Penaeus vannamei*). They developed a novel food particle type that retained low molecular weight, water soluble nutrients (Glucose and vitamins) within lipid wall microcapsules embedded with dietary ingredients in particles of gelled alginate gelatin. Same way Onal and Langdon (Onal & Langdon 2004) prepared a lipid spray beads (LSB) for the delivery of riboflavin to first feeding larvae of Zebra fish, *Brachdanio rerio* and glowlight tetra, *Hemigrammus erythrozonus*. They observed 96.7% mean inclusion efficiency (IE) of riboflavin within the LSB, which was further increased by the addition of emulsifiers to lipid matrix.

Romalde and coworkers (Romalde et al. 2004) enhanced protection of rainbow trout against fish lactococcosis when administered booster doses of bacterial vaccines incorporated in spray dried alginate acetone micro particles.

**1.3.2.4 Air suspension coating**

It is also known as fluidized bed or spray coating is accomplished by suspending solid particles of core material in an up ward moving stream of air, which may be heated or cooled (Baken & Anderson 1970). The coating is atomized through nozzles in to the

chamber and deposits as a thin layer of the surface of suspended particles. The turbulence of the column of air is sufficient to maintain suspension of coated particle allowing them to tumble and thereby becomes uniformly coated.

### 1.3.2.5 Extrusion

As a low temperature encapsulation method, extrusion involves forcing a core material dispersed in a molten carbohydrate mass through a series of disks in to a bath of dehydrating liquid. Upon contacting the liquid, the coating material, which forms the encapsulating matrix, hardens to entrap the core material. The extruded filaments are separated from the fluid bath, dried to mitigate hydroscopicity, and sized.

The extrusion process is particularly useful for heat labile substances and has been used to encapsulate flavours, vitamin C and colour. Using this technique (Murano et al. 1997) encapsulated formalin killed *Vibrio anguillarum* and administered to rainbow trout. They concluded that the oral vaccination of rainbow trout with alginate encapsulated *V. anguillarum* could be used as booster vaccination in combination with other vaccination methods as immersion or intra-peritoneal injection.

### 1.3.2.6 Centrifugal extrusion

This is another encapsulation technique that has been investigated and currently used by some vitamin manufacturers for the encapsulation of vitamins. The device consists of a concentric feed tube through which coating material and core material are pumped separately to the many nozzles mounted on the outer surface of the device (Sparks 1981). Core materials flows through the central tube and the coating material flows through the outer tube. The entire device is attached to a rotating shaft such that the head rotates around its vertical axis. As the head rotates, the core material and coating material are co extruded through the concentric orifices of the nozzles as a fluid "rod" of core sheathed in coating material. Centrifugal force impels the rod outward, causing it to break in to tiny particles. By the action of surface tension, the coating material envelops the core material, thus accomplishing encapsulation. The capsules are collected on a moving bed

of fine-grained starch, which cushions their impact and absorbs unwanted coating moisture.

#### 1.3.2.7 Rotational suspension separation

The process involves suspending core particles in a pure, liquefied coating material, then pouring the suspension through a rotating disc apparatus under such condition that excess liquid between the core particle spread in to a film thinner than the core particle diameter. The excess liquid is then atomized in to a very small particle, which are separated from the product and recycled. The core particle leaves the disc with residual liquid still around them, which forms the coating. Chilling or drying hardens the particles.

Rotational suspension separation is a continuous high capacity process that takes seconds to minutes to coat core particles. The process can handle a wide variety of core materials and coating materials. This process handles each particle only once and, under most conditions, produces no uncoated particles. The process has been used successfully to coat particle ranging from 30μ to 2mm. Coating have been produced with thickness ranging from 1- 200μ

### 1.3.3 Design of release mechanism

The release from the microcapsule/microsphere is dependent both on diffusion through the polymer matrix and on polymer degradation (Sinha & Trehan 2003). If during, the desired release time, polymer degradation is considerable, then the release rate may be unpredictable and erratic due to break down of microspheres. This may be most useful in crustacean larviculture, since the injection to evacuation time of food particles in crustacean larvae is very short (Pedroza-Islas et al. 1999). However, the release of core material from such system is depended diffusivity through the polymer barrier, solubility of core in bulk phase, size of drug molecule and distribution of core through out the matrix, etc. Nature of polymer plays a major role in release process. The possible mechanisms of drug release are (Sinha & Trehan 2003):

1. A comprehensive force breaks open the capsule by mechanical measures
2. Initial release from microsphere surface
3. Release through the pores dependent on microsphere structure
4. Diffusion through the intact polymer barrier which is dependent on intrinsic polymer properties and core solubility
5. Diffusion through a water swollen barrier dependent on polymer hydrophilicity, which in turn depends on polymer molecular weight
6. Polymer erosion and bulk degradation, release affected by the rate of erosion and hydrolysis of polymer chains, leading to pore formation in matrix.

All these mechanisms together play a part in release process. Nature of core also influences release kinetics either by increasing polymer degradation or by physical binding with polymer chain. Drug polymer interaction leads to decreased release.

All these factors were considered while selecting a wall material for the design of a microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*. The major decisive factors we considered for the selection of wall material are:

1. Biological degradability of the polymer
2. Capacity of the polymer to protect the core material from leaching to the external environment
3. Consequence of polymer on pathogenic bacterial growth
4. Mechanism of release of core material inside the intestine
5. Acceptability of the preparation to the larvae.

**Figure 1.1 Chemical structure of β-(1-3)-D- glucan**

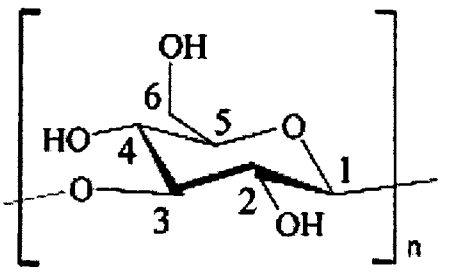



**Table. 1.1 Kinds of β-glucans extracted from fungus having application in medicine.**

| Glucan | Source | Reference |
|---|---|---|
| Pachyman | *Poria cocos* | Saito et al. 1968<br>Hoffmann et al. 1971 |
| Glucan component of zymosan | *Saccharomyces cerevisiae* | Manners et al. 1973a |
| Lentinan | *Lentinus edodes* | Sasaki & Takasaka 1976 |
| GU | *Grifola umbellate* | Miyasaki et al. 1978 |
| β-Glucan I | *Auricularia auricula-judae* | Misaki et al. 1981 |
| Schizophyllan | *Schizophyllum commune* | Tabata et al. 1981<br>Muller et al. 1995 |
| Scleroglucan | *Sclerotium glucanicum* | Rinaudo & Vincendon 1982<br>Pretus et al. 1991 |
| T-4-N | *Dictyophora indusiata* | Hara et al. 1983 |
| CO-1 | *Cordyceps ophioglossoides* | Yamada et al. 1984 |
| Pestalotan | *Pestalotia sp.* 815 | Misaki et al. 1984 |
| HA | *Pleurotus ostreatus* | Yoshioka et al. 1985 |
| Grifolan | *Grifola frondosa* | Ohno et al. 1985a |
| PVG | *Peziza vesiculosa* | Ohno et al. 1985c |
| VVG | *Volveriella volvacea* | Misaki et al. 1986 |
| SSG | *Sclerotina sclerotiorum* | Ohno et al. 1986 |

**Contd.**

| Glucan | Source | Reference |
|---|---|---|
| AS – 1 | *Cohliobolus miyabeanus* | Nanba & Kuroda 1987 |
| Tylopilan | *Tylopilus felleus* | Defaye et al. 1988 |
| Glomerellan | *Glomerella cingulata* | Gomaa et al. 1991 |
| PGG | *Saccharomyces cerevisiae* | Jamas et al. 1991 |
| AM-ASN | *Amanita muscaria* | Kiho et al. 1992 |
| H-3-B | *Cryptoporus valvatus* | Kitamura et al. 1994 |
| (1-3)-β-D-glucan | *Saccharomyces cerevisiae* | Williams et al. 1994 |
| Glucan Phosphate | *Saccharomyces cerevisiae* | Muller et al. 1995 |
| CSBG | *Candida* spp. | Ohno et al. 1999<br>Tokunaka et al. 2000 |
| Pullulan | *Cryphonectria parasitica* (Murr) | Molinaro et al. 2000 |
| PGL | *Ganoderma lucidum* | Bao et al. 2001 |
| OX-ZYM | *Saccharomyces cerevisiae* | Ohno et al. 2001 |
| *A*IBG | *Acremonium diospyri* | Anas & Singh 2003 |
| ASBG | *Aspergillus* spp | Ishibashi et al. 2004 |

## CHAPTER 2

# DEVELOPMENT OF A COMMERCIALLY VIABLE PROCESS OF EXTRACTION FOR GLUCAN AS IMMUNOSTIMULANT FROM *Acremonium diospyri*

### 2.1 INTRODUCTION

Complex polysaccharide molecules are known to stimulate the immune systems of vertebrates and invertebrates (Di Luzio et al. 1979, Di Luzio 1983, Ohno et al. 1985b, Kitamura et al. 1994, Williams et al. 1996, Williams 1997, Adachi et al. 1999, Chang et al. 1999, Chang et al. 2000, Dalmo 2000). These immunostimulatory agents are commonly known as Biological Response Modifiers (BRMs) (Henry et al. 1991). Among such molecules, β-(1-3)-D-glucan is the most extensively studied and widely used immunostimulant (Ohno et al. 1984, Ohno et al. 1986, Williams et al. 1987, Sandula et al. 1999).

The immunostimulatory activity of glucans relate to their ability to stimulate macrophage activity, hydrogen peroxide production and phagocytosis in fish (Brattgjerd et al. 1994, Jeney et al. 1994) as well as to increase production of proPhenoloxidase (Soderhall et al. 1990) and reactive oxygen intermediate (ROI) (Munoz et al. 2000) in crustaceans. Previous studies in our lab have also demonstrated that a water insoluble β-glucan extracted by alkali - acid hydrolysis from *Acremonium diospyri* possessed significant immunostimulatory effect in prawn larvae (Anas & Singh 2003). The type culture *Acremonium diospyri* (MTCC 1316) was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture produced mycelia mats in Sabouraud's dextrose broth and its exo-cellular glucan production was reported earlier (Seviour & Hensgen 1983).

Engstad and Robertson (Engstad & Robertson 1994) studied the immunostimulatory effect of different yeast β-glucans in Atlantic salmon, and noticed that the immunostimulatory property is directly linked to its structure. It has been indicated that the structure, molecular weight, degree of branching and solution conformation of β –

glucan, which depend on the method of extraction, are major determinants of immunostimulatory activity in animals (Falch et al. 2000, Tokunaka et al. 2000).

Majority of the methods developed for glucan extraction are based on the alkali - acid hydrolysis (Northcote & Horne 1952, Peat & Whelan 1958, Misaki et al. 1968, Williams et al. 1991, Muller et al. 1997) as initially promulgated by Hassid and coworkers (Hassid et al. 1941). The microparticulate glucan extracted by this method is chemically pure with no other carbohydrates, proteins or residual lipids (Muller et al. 1997) . However, there was a pharmacological need for a water-soluble β-glucan, and thus Ohno and coworkers developed a solubilization method using sodium hypochlorite oxidation and dimethyl sulphoxide extraction (Ohno et al. 1999).

The alkali acid extraction of glucan involves treatment of cell wall with alkali, acid and organic solvents to remove proteins, lipids and other polysaccharides. Different modifications could be made to this basic method. Freimund and colleagues (Freimund et al. 2003) reported that the hot water extraction instead of solvent extraction could remove a major portion of proteins, mannoproteins and lipids, preserving nearly all glucan. Meanwhile Sandula and colleagues (Sandula et al. 1999) proposed a supplementary ultrasonication step to the basic alkali acid hydrolysis method to further increase its purity, and solubility.

Song and coworkers (Song et al. 1997) reported that glucan treated shrimps gained enhanced resistance to white spot syndrome virus (WSSV) infection and increased tolerance to stress including catching, transportation and ammonia toxicity, when they were immersed in sonicated glucan prior to commencement of culturing and its subsequent application as dietary supplement. Same time Dehasque and coworkers (Dehasque et al. 1995a) reported that the partially purified yeast glucan enhanced the survival rate in European sea bass.

Having these as the methods of glucan extraction and their modifications the major concern is to evaluate the relationship between the immunostimulatory properties versus

structure and purity of the extracted glucans in the cultured species. In our investigation the efficacy of the preparations in activating a non-specific immune system response in crustaceans was quantified based on the changes in total haemocyte count, proPhenoloxidase (proPO) activity and reactive oxygen intermediates (ROI) using *Fenneropenaeus indicus* as the animal model.

One of the requirements in the development of a commercially viable process for the extraction of glucan was to make available a process of treatment of the waste generated. In general, regulatory bodies dictate that the effluent should have a pH of 5.5 – 7.5 and the COD should not exceed 75 mg/l before discharge to environment. However, glucan extraction wastewater (GEW) typically has a very high pH 13.07 – 13.08, with a COD of 58000 – 62000 mg/l and colour of 6000hazen (Anas 2000).

Several criteria should be considered when deciding on a treatment system for glucan extraction waste (GEW). These include an eco-friendly process that is flexible enough to handle variable concentration loads and characteristics. Various biological treatment process (Nicolella et al. 2000) are based on the use of three types of microbial aggregates: static biofilm (eg. Trickling filter), Particulate biofilms (eg. UASB) and flocs (eg. Activated sludge process). Biofilms are extensively used in environmental biotechnology where large volumes of dilute aqueous solutions have to be treated, and natural mixed populations of biofilms are used. The advantage of biofilm is that the process can be operated at a high biomass concentration.

In the present study experiments were conducted to compare strength of the waste generated during the extraction of water extracted non-sonicated glucan (AIBG-WENS) and ethanol extracted non-sonicated glucan (AIBG-WENS) and for the development of a microbial consortium for the treatment of AIBG-WENS wastewater. One of the major objectives of this study was to develop a biofilm forming microbial consortium, which can utilize the wastewater generated during glucan extraction (GEW) as sole source of carbon and nitrogen energy. A preliminary screening was conducted with a number of soil samples collected from different environments, based on its ability to grow in the

GEW and the selected consortia were further investigated for its potential to degrade the pollutants and its ability to form biofilms.

## 2.2. MATERIALS AND METHODS

### 2.2.1 Comparative efficacy of soluble and insoluble glucan from *Acremonium diospyri* as immunostimulants

#### 2.2.1.1 Production of *Acremonium diospyri* biomass

The *Acremonium diospyri* was inoculated into 500ml Sabauraud's-dextrose broth (Dextrose 20g; mycological peptone 10g; distilled water 1,000ml, pH 6.5 ± 0.2, autoclaved at 10lbs for 10min.) and incubated at room temperature (28 ± 2°C) for 30 days. On incubation, it developed into a mucilaginous mat on top of the medium and started settling down on attaining maximum growth and weight. The culture broth was filtered through muslin silk, the biomass was separated, washed twice with phosphate buffered saline (PBS) ($NaH_2PO_4$ 6.42g; $Na_2HPO_4$ 34.316g; NaCl 10g; distilled water 1,000 ml), dried at 80°C for 48hrs in a hot air oven, and the weight stabilized in a desiccator over silica gel at room temperature.

#### 2.2.1.2 Extraction of glucan from *Acremonium diospyri*

*Acremonium* insoluble β-Glucan (AIBG) and *Acremonium* soluble glucan (ASG) were prepared from *Acremonium diospyri* following the methods of Williams and coworkers (Williams et al. 1991) and Ohno and coworkers (Ohno et al. 1999), respectively, with slight modifications. *Acremonium* insoluble β-Glucan (AIBG) was prepared as follows: One gram dried *Acremonium diospyri*, suspended in 20 ml 3% (w/v) aqueous sodium hydroxide (Himedia Laboratories, India) was maintained at 100°C for 6 hours in a serological water bath. Filtering through muslin silk and re-extracting with aqueous sodium hydroxide resulted in separation of alkali insoluble material. The insoluble material was again separated and extracted with 20ml 0.5N acetic acid (SRL, India) at 75°C for 6h. The resultant insoluble residue was separated by filtration through muslin silk and refluxed repeatedly with ethanol until the filtrate became colourless. The remaining precipitate (AIBG) was washed with distilled water and vacuum dried over silica gel at room temperature (28 ± 1°C) and designated as *Acremonium* insoluble β-glucan (AIBG).

*Acremonium* soluble glucan (ASG) was isolated by the sodium hypochlorite oxidation and dimethyl sulphoxide extraction method (Ohno et al. 1999). Briefly, One gram dried *A. diospyri* biomass was suspended in 100ml 0.1M NaOH and 12.5ml of the sodium hypochlorite (Merck, India) oxidizing agent was added immediately, incubated for one day at 4°C in a glass stopped conical flask, and filtered through muslin silk to separate the insoluble fraction. The insoluble fraction was washed with distilled water, dried first in ethanol and then in acetone (Hi media Laboratories, India), and recovered by decanting the fluid. The recovered insoluble fraction was macerated with dimethyl sulphoxide (Merck, India) solution and extracted for 60 minutes at 90°C with intermittent sonication (500W, 20KHz,VCX500, Sonics, USA). From this suspension the insoluble fraction was specifically removed by centrifugation at 1,000g for 15min. By treating the supernatant with 4 volumes of ethyl alcohol β-glucan was precipitated from the soluble fraction and designated as *Acremonium* soluble glucan (ASG).

### 2.2.1.3 NMR Spectroscopy

The proton NMR spectra of ASG and AIBG were collected on a JEOL Model Eclipse + 600 NMR spectrometer in 5-mm OD NMR tubes at 80°C with perdeuterated dimethylsulfoxide (DMSO-$d_6$) as the solvent. A few drops of trifluoroacetic acid-d were added to the solution to shift the resonance from the exchangeable protons downfield (Ross & Lowe 2000). Spectra were collected for either 4 or 24 hours depending on the amount of isolate available.

### 2.2.1.4 FT-IR spectra of ASG

Fourier transform infrared (FT-IR) spectrum of ASG was collected using a Digilab FTS-6000 spectrometer with a single–bounce Dursampler Attenuated Total Reflectance (ATR) accessory and a MCT detector with 256 scans collected for both the sample spectrum and the background spectrum. Care was taken to ensure good contact in order to obtain reproducible and the most photometrically accurate spectra possible using this technique.

### 2.2.1.5 Immunostimulatory potential of AIBG and ASG

The immunostimulatory potential of *Acremonium* insoluble β-glucan and *Acremonium* soluble glucan was assessed using *Fenneropenaeus indicus* as the animal model. The experimental diets were prepared by surface coating the commercially available pelleted feed (Higashimaru Pvt. Ltd., Cochin, India) with 0.2% (w/w) AIBG and ASG separately. Sixty animals were maintained in six tanks with 10 animals each. Animals maintained in two tanks were fed AIBG-coated pellets while animals in a second set of two tanks were fed ASG-coated feed for seven days. The remaining animals in two tanks were fed control diet (without glucan) during the same period. The experimental animals were returned to normal diet (without glucan) after completing the seven-day experimental feeding. Haemolymph of the animals from one tank in each feeding regimen was withdrawn on the 1st day following cessation of the experimental feeding for hematological assays. Haemolymph from the remaining animals of each feeding regimen was withdrawn on 7th day following cessation of the experimental feeding for hematological assays.

### 2.2.1.6 Haemolymph collection

Haemolymph was collected from the rostral sinus situated beneath the rostral spine and between the eyestalks by inserting a capillary tube, after blotting dries this region with sterile cotton swab. The withdrawn haemolymph was transferred into 2.2ml capacity microcentrifuge tubes each pre-filled with 200µl ice cold anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri sodium citrate 0.1M prepared in double distilled water, autoclaved and adjusted to pH 7.6) (Song & Hsieh 1994). To avoid clotting during collection of haemolymph the capillary tube was rinsed with the anticoagulant.

### 2.2.1.7 Haematological assays

The collected haemolymph was subjected to haematological assays such as haemocyte count and activities of proPhenol oxidase and reactive oxygen intermediate. The total haemocyte count was determined using Neubaeur's haemocytometer (Perazzolo & Barracco 1997). proPhenoloxidase activity was estimated spectrophotometrically by measuring the formation of dopachrome from L–DOPA at 490nm (Smith & Soderhall

1983). Reactive oxygen intermediate activity was measured based on the ability of haemocytes to adhere to plastic centrifuge tube and to reduce nitroblue tetrazolium (NBT) to formazan (Cheng et al. 2000). The proPO activity and ROI were expressed as absorbance/mg haemolymph protein/min. and absorbance/mg haemocyte protein respectively. Haemolymph and haemocyte proteins were estimated by Bradford method (Bradford 1976) using bovine serum albumin standard.

### 2.2.2 Effect of sonication and solvent extraction of *Acremonium* insoluble β-glucan (AIBG) on its immunostimulatory property

#### 2.2.2.1. Preparation of glucan

*Acremonium* insoluble β-glucan (AIBG) prepared following the method described in the section 2.2.1.2 was the base material used for preparing four categories of glucans. Briefly 1g dried *Acremonium diospyri* cells were suspended in 20ml 3% (w/v) aqueous sodium hydroxide and maintained at 100°C for 6hours in a serological water bath. Filtering through muslin silk separated the alkali insoluble materials, which was re-extracted with aqueous sodium hydroxide. The extraction was continued with 20ml 0.5N acetic acid at 75°C for 6 hours. The alkali acid insoluble residue was divided into two fractions. One fraction was refluxed with ethanol repeatedly and the residue designated as ethanol extracted non-sonicated glucan (AIBG-EENS). The other fraction was extracted with hot water for one hour and the residue designated as water extracted non-sonicated glucan (AIBG-WENS). AIBG-EENS and AIBG-WENS were sonicated in distilled water (1% w/v) for 15 minutes at 4°C (500W, 20KHz,VCX500, Sonics, USA). The resulting solution was centrifuged at 500g to separate ethanol extracted sonicated (AIBG-EES) and water extracted sonicated (AIBG-WES) glucans.

#### 2.2.2.2 Experimental design

The immunostimulatory potential of AIBG-EENS, AIBG-EES, AIBG-WENS and AIBG-WES was assessed in *Fenneropenaeus indicus* as the animal model. The experimental diets were prepared by surface coating in South India commercially available pelleted feed (Higashimaru Pvt. Ltd., Cochin, India) with 0.2% (w/w) each of AIBG-EENS, AIBG-EES, AIBG-WENS and AIBG-WES. The above preparations were administered to *F.*

*indicus* juveniles of 3 – 5g size maintained in fiberglass tanks of 25l capacity, filled with 20ppt seawater. Each of the feed preparations was administered to 12 shrimp maintained in 2 tanks. Two tanks were segregated for administering the control feed. The experimental feeding was continued for 7 days after which the control feed was used for another five days for all shrimp. Water exchange at the rate of 50 – 70% was provided to maintain congenial water quality.

**2.2.2.3 Haematological assays**

Haemolymph was collected from the rostral sinus of the animal using capillary tube pre rinsed with anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri sodium citrate 0.1M prepared in double distilled water, autoclaved and adjusted to pH 7.6) (Song & Hsieh 1994) and transferred to micro centrifuge tube containing 200µl ice cold anticoagulant. Haemolymph of two animals were pooled together and subjected to haematological assays such as proPhenoloxidase (proPO) activity and reactive oxygen intermediates (ROI) as mentioned in 2.2.1.7.

**2.2.2.4. NMR spectroscopy**

The proton NMR spectrum of both AIBG-EENS and AIBG-WENS was collected on a JEOL Model Eclipse+ 600 NMR spectrometer following the method described in the section 2.2.1.3

### 2.2.3 Characterization and treatment of the effluent generated during glucan extraction

**2.2.3.1 Chemical characterization of waste**

The wastewater generated after the extraction of AIBG-EENS and AIBG-WENS was compared based on pH, chemical oxygen demand, total alkalinity, Aromatic and Organic content ($A_{256}$) and colour. The pH was measured using digital pH meter (Systronics, 335), and colour by using Colour comparator (Merck India, Ltd.). Chemical oxygen demand (COD) was measured by open reflux method. Alkalinity was measured by simple titration following Standard Methods (APHA 1995). Aromatic and organic contents were expressed as absorbance at 254nm (Rivas et al. 2000). After selecting

AIBG-WENS as the immunostimulant for aquaculture, a total characterization of the waste water generated from this method was accomplished by monitoring the additional physical parameters such as Turbidity, Conductivity, T.S.S, T.D.S and Total solids (APHA 1995).

**2.2.3.2 Primary development of consortia for the treatment of GEW**

The prime objective of the process was to develop an appropriate consortium for the wastewater treatment. Soil samples collected from different environments were used for the development of consortia, which can utilize the waste as sole source of carbon and energy. For this 100ml aliquots of effluent was taken in four 250ml conical flasks, pH adjusted to 7 – 7.5 using acetic acid. The preparation was autoclaved and used as the medium for enriching organisms. The media were inoculated with one gram of the collected soil samples and incubated on a rotary shaker at 100rpm and re-inoculated to fresh media once in 7days. The pH of the preparation was monitored and adjusted as and when required. Two consortia FKB and POAB were selected based on its visual performances and subjected for further studies after nine consecutive passages.

**2.2.3.3 Comparison of the consortia**

Comparison of two selected consortia FKB and POAB was made separately in 250ml conical flasks. The flasks were inoculated with the consortia to a final strength of 1% (v/v) and incubated at room temperature (28 ± 2°C) in rotary shaker at 100rpm. A conical flasks of each treatment was withdrawn at 1,2,3,4, 5 and 6 day and then vacuum filtered through a 0.22μ filter paper. The filtrate was subjected for the analysis of COD, Colour and $A^{254}$. Differences with the initial levels of the waste led to the assessment of the rate of waste degradation

**2.2.3.4 Biofilm formation by the consortia**

Holders with microscope slides were immersed in waste treatment flasks. One slide each was withdrawn every day and stained with acridine orange (Wimmer 2001). The stain was prepared by adding 100mg of acridine orange to 10ml of ethanol. One milliliter of

the stain was added to 10ml of 20mM HEPES buffer, pH 6.96 and used for staining. The stained slides were viewed under epi-fluorescent microscope.

### 2.2.4 Statistical analysis

To determine significant differences existed between different treatment values, all results were analyzed using one – way analysis of variance (ANOVA). The significant differences were considered at $p < 0.05$ (Bailey 1995).

## 2.3. RESULTS

### 2.3.1 Comparative efficacy of soluble and insoluble glucan from *Acremonium diospyri* as immunostimulant

The ability of the yeast *Acremonium diospyri* to form mats on the surface of the medium and their subsequent sinking to the bottom on maturation, facilitated easy harvesting. It was observed that optimization of growth conditions and development of a bioprocess technology might increase the yield of yeast biomass to assure economic viability of the production process.

The structures of the AIBG and ASG extracted from *Acremonium diospyri* greatly affected their physical properties. The AIBG was only partially soluble in DMSO even at higher temperature of 150°C, whereas the ASG was completely soluble in DMSO at normal temperature. This facilitated only a partial characterization of AIBG by NMR while ASG could be analyzed using NMR and IR spectroscopy. Proton NMR spectra at 600 MHz of AIBG and ASG are shown in Figure 2.1. Resonances of AIBG at 4.54 ppm, 3.73 ppm and between 3.25 and 3.46 ppm (Figure 2.1 top) correspond to resonances of high purity β-(1-3)-D-glucan previously reported by Lowman & Williams (2001). This observation supports the presence of β-(1-3)-D-glucan in the AIBG preparation. Resonance from ASG at 5.09 ppm and between 3.42 to 3.86 ppm correspond to resonances in α-(1,3)-D-glucan (Figure 2.1 bottom) extracted from *Ganoderma incidium* and characterized by Chen and coworkers (Chen et al. 1998).

The IR spectrum of ASG shows two absorptions at 849 and 927 $cm^{-1}$, which are characteristic of the α conformation (Figure 2.2, Top) and are in agreement with the work of Chen and coworkers (Chen et al. 1998). For comparison, the IR spectrum of the highly purified β-(1-3)-D-glucan is shown in Figure 2.2 (Bottom). The characteristic IR absorption of the β-conformer is indicated by the absorption at $890cm^{-1}$ in the bottom spectrum.

Among the three immunological parameters measured in response to feeding with AIBG and ASG, significant variations were observed in the proPhenoloxidase system and NBT reduction but not in the haemocyte count (Tables 2.1 and 2.2). The group of animals fed with AIBG exhibited approximately 5-fold increased enzyme activity over the control group and nearly 2-fold increase over the animals fed with ASG ($p < 0.05$) on $6^{th}$ day of post experimental feeding. No significant difference could be seen between the group fed with AIBG and ASG on the first day of post experimental feeding ($p < 0.05$). In a similar pattern on the first day of post administration of AIBG and ASG, ROI did not show any significant variation between the groups. However, on $6^{th}$ day, the group of animals fed with AIBG exhibited significantly higher NBT reduction compared with the control groups and the one fed with ASG. Strikingly, no significant variation could be recorded between the control and the ASG-fed group.

### 2.3.2 Effect of sonication and solvent extraction of *Acremonium* insoluble β-glucan (AIBG) on its immunostimulatory property

The proPhenoloxidase activity and reactive oxygen intermediate production in animals fed with the various glucan preparations are presented in Figure 2.3 and 2.4 respectively. Significant increase in proPhenoloxidase activity ($p<0.05$) was observed in animals fed with AIBG-WENS registering 3.1 fold increase compared to the control group. Meanwhile, the animals fed on all the preparations more or less uniformly showed significantly higher ROI activity ($p<0.05$) compared to the control. However, ROI production in those fed on non-sonicated glucan preparations was higher (6.0 fold) than in the ones fed on sonicated preparations (4.5 fold) and to the control group.

NMR analysis was employed to assess the purity of AIBG-WENS and AIBG-EENS by comparing them with a highly purified standard alkali insoluble glucan extracted from *Saccharomyces cerevisiae* (Lowman & Williams 2001). Neither glucan sample was soluble in DMSO – $d_6$; therefore a complete structural characterization was not possible. The NMR spectrum of the DMSO soluble portion of AIBG-WENS (Figure 2.5, Top) indicated the high concentration of lipids and mannans in the sample. However, the AIBG-EENS (Figure 2.5 Middle) was predominantly a linear glucan, as indicated by the resonances at 4.54ppm, 3.73ppm, 3.5ppm and between 3.25 and 3.36ppm, which correspond to the resonances in the high purity glucan spectrum (Figure 2.5 bottom) (Lowman & Williams 2001), supporting the presence of more pure (1-3)-β-D-glucan in the isolate. Moreover the lipid contamination was also avoided in this sample by repeated ethanol extraction. Also present in the spectrum of AIBG-EENS is glucose as indicated by resonances at 4.94ppm, 4.31ppm, and 2.94ppm. The branching type and their level are difficult to determine from these NMR spectra.

### 2.3.3 Characterization and treatment of the effluent generated during glucan extraction

The strength of the wastewater generated during the extraction process varied greatly from treatment to treatment. The wastewater generated during hot water extraction of glucan showed a comparatively low concentration of COD and colour (Table 2.3), which enabled the treatment more easy. The chemical oxygen demand of the wastewater generated from WENS was within the range of 5184 – 7800 mg/l compared to 58000 – 62000 mg/ l of EENS.

The complete physico chemical properties of wastewater generated from AIBG-WENS are given in the Table. 2.4. The extraction of glucan from *Acremonium diospyri* generated large volume of wastewater with a high COD and pH and comparatively less dissolved and suspended solids.

Soil samples for the development of glucan extraction wastewater degrading consortia were collected from different locations, which were highly polluted with organic wastes.

In all enrichment flasks uniform (visual observation) turbidity was seen within 3 days of incubation along with shift in pH to 8.5. The consortia FKB and POAB were selected based on their comparatively high growth rate and floc formation compared to the other groups. These two were further passaged nine times and used for the study.

Even though the FKB showed better growth in the preliminary screening, its degrading capacity was trivial compared to POAB (Figure 2.6). POAB on the other hand showed a sharp decline in the chemical oxygen demand a day of inoculation after and the reduction was linear (Figure 2.6). With respect to the consortia FKB, no difference in the chemical oxygen demand was observed for 4 days and a slight difference was noticed only on the fifth day.

Absorbance at 254 nm is considered as an indication of the aromatic and organic content of wastewater (Rivas et al. 2000). The POAB had very less but measurable impact on the aromatic and organic content of the wastewater, where as FKB did not show any influence on the same (Figure 2.7). The consortia POAB decreased the $A^{256}$ from 4.0 to 3.3 during the 6 days of treatment.

Filtration of the wastewater reduced the colour significantly, from 3000 to 1000 hazen. Efficacy of the consortia was accounted after neglecting the reduction by filtration. Accordingly both the consortia were able to reduce the colour by 50% within a day.

The fluorescent microscopic pictures showed a population shift and biofilm formation by the consortia. In FKB consortium the total bacterial count was increasing steadily from the first day to the sixth day of treatment (Figure 2.8). The bacterial number increased and a biofilm was formed on fourth day, which on microscopic observation exhibited more yeasts and filamentous organisms. In POAB also the initial bacterial number and diversity was very less but it developed in to a biofilm (Figure 2.9) within a short period. The biofilm was found to have diverse population of bacteria, yeast and filamentous organisms.

## 2.4. DISCUSSION

### 2.4.1 Comparative efficacy of soluble and insoluble glucan from *Acremonium diospyri* as immunostimulants

β-(1-3)-D-glucans are BRMs that when administered in suitable quantities protect animals from stress and pathogenic invasion. However, over administration of glucan can lead to toxicity, therefore glucan concentration is an important parameter in developing a treatment regimen. In this investigation 0.2% (w/w) glucan was administered following Chang et al. (1996a) and Liao et al. (1996)

An important observation made from this study was the significantly higher immunostimulatory property of AIBG compared to ASG in shrimp. Eventhough several workers had investigated the effect of glucan extracted from different sources, the method of extraction of the glucans had not been discussed. This was primarily due to the use commercial grade glucan preparation for which the information about their extraction was not always available. Nevertheless, the outcome of some of those studies were enhanced phagocytic activity, cell adhesion and super oxide production in brooders of *Penaeus monodon* following the administration of glucan extracted from *Schizophyllum commune* (Cheng et al. 2000) and a 2.5-fold enhancement of reactive intracellular oxygen production in *P. monodon* haemocyte following administration of glucan extracted from *Saccharomyces cerevisiae* (Song & Hsieh 1994). Meanwhile, Sritunyalucksana and coworkers (Sritunyalucksana et al. 1999) could not observe any significant immunostimulatory property with the glucan preparation they administered to shrimp. Scholz and coworkers (Scholz et al. 1999) administered glucan prepared by rupturing cells of *Saccharomyces cerevisiae* with enzymes, separating the soluble fraction from insoluble, washing with ethanol, subjecting to acidification and drying. However they could not observe any significant difference in the proPhenoloxidase activity in *Penaeus vannamei*. These different observations point to the fact that significant variations do exist in the immunostimulatory property of glucan preparations.

It is an accepted fact that the immunostimulatory property of glucan is directly linked to its structural and physical properties such as degree of branching, conformation,

molecular weight and solubility in water (Ishibashi et al. 2004), which are partially related to the method of extraction. In the preliminary attempt to develop an alternative method for the extraction of soluble glucan, Ohno and coworkers (Ohno et al. 1999) proposed a two-step procedure using NaClO oxidation and dimethyl sulphoxide extraction which they applied to different strains of *Candida* and *Saccharomyces*. The structures of all the glucan isolates were identified as β-(1,3)-D-glucan with various amounts of β-(1,6)-D-glucan side chains. Later Ishibashi and coworkers (Ishibashi et al. 2004) extracted glucan from *Aspergillus* spp. using the same method and observed that the resulting glucan primarily contained α–(1,3)-linkages. In the present study the glucan extracted using the NaClO-DMSO method (ASG) primarily contained α–(1,3) linkages and exhibited negligible immunostimulatory activity.

The basis of alkali - acid hydrolysis is removal of contaminating groups from β-glucan by using repeated alkali and acid treatments and ethanol extraction, which leaves the β-(1,3)-D-glucan as the insoluble fraction. Whereas, in the NaClO-DMSO method, the contaminating groups are removed by sodium hypochlorite oxidation and the glucan is extracted by dissolving in DMSO. However, this process is greatly dependent on the solubility of the glucan. From the data generated here, it appears that the β-glucan present in the *Acremonium diospyri* is insoluble in DMSO under normal conditions. Therefore, it may not be feasible to extract this glucan using the sodium hypochlorite oxidation and dimethyl sulphoxide extraction method as suggested by Ohno and coworkers (Ohno et al. 1999).

The administration of glucan in the present study was for 7 days and the hematology was studied on the 1st and 6th day after completing the experimental feeding schedule. This experimental design was adopted based on the reported time required for development of immune response of crustaceans to an immunostimulant (Cheng et al. 2000). During our investigation to elicit immunological response to inactivated white spot syndrome virus (WSSV), maximum immune response was obtained during 5th to 10th day after completion of the schedule of its 7-day administration (Singh et al. In press). Similarly, in the present study the immunological response was significantly higher on the 6th day than

on the 1st day after completion of the 7-day schedule of administration of AIBG. The cascade of events that might take place during this period of post administration shall be a subject of investigation of great importance.

It is clear from this work that an analysis of the structure of isolated glucans is critical to our understanding of structure/property relationships in imunostimmulatory responses of shrimp to β-glucans. β-(1-3)-D-Glucan in AIBG exhibited a greater immunostimulatory response than the glucan in ASG, most likely due to the difference in conformer structure in these two glucan isolates. In addition, this study demonstrated that it took five days for the animals to express the immunostimulatory response to the glucan isolates after completion of the schedule of administration.

### 2.4.2 Effect of sonication and solvent extraction of *Acremonium* insoluble β-glucan (AIBG) on its immunostimulatory property

The study indicates that AIBG-WENS elicits significantly higher proPhenoloxidase activity in *Fenneropenaeus indicus*. All of the preparations stimulated ROI activity, with the highest activity observed in those animals fed a non-sonicated glucan preparation. This suggests that non-sonicated water extracted glucan preparations (AIBG-WENS) are more potent immunostimulant in *Fenneropenaeus indicus*.

The glucan extracted in this study was sparingly soluble in NMR solvent (DMSO – $d_6$) and, thus, the NMR data could be used only for confirming the presence of glucan in the sample, but not for elucidating its structure. However, it was possible to interpret the purity of AIBG-WENS and AIBG-EENS. It has to be pointed out that glucan could not be detected in the hot water extract, AIBG-WENS, because of the high concentration of lipids remaining. However, the lipid contamination was lower in the ethanol-extracted glucan (AIBG-EENS) compared to the AIBG-WENS.

Freimund and coworkers (Freimund et al. 2003) suggested that the hot water extraction could remove 80% of the total proteins and 6% mannoproteins from the starting yeast biomass, preserving nearly all glucan (98%). They also reported the depletion of even

26% lipids by hot water extraction. The remaining lipids could be removed by treating with different solvents such as ethanol and methanol (Hassid et al. 1941, Northcote & Horne 1952, Manners et al. 1973b, Freimund et al. 2003). However, the present study indicated that removal of lipids by treating with ethanol is not necessary for eliciting a better stimulatory effect in *Fenneropenaeus indicus*, as the water-extracted glucan (AIBG-WENS) gave better immunostimulation than the purer ethanol extracted glucan (AIBG-EENS).

The drastic alkali-acid and ethanol treatments lead to some degradation of the glucan chains particularly when oxygen is involved (Aspinwall et al. 1975, Young & Liss 1978). This was observed in our NMR results of AIBG-EENS also, as glucose content was indicated by the presence of resonances at 4.94 ppm, 4.31 ppm, and 2.94 ppm. The presence of glucose could in turn affect the immunostimulatory properties of the glucan isolate as evident in the results. Meanwhile, the relatively mild treatment employed in the extraction of AIBG-WENS resulted in a glucan with more stimulatory activity.

The widely used alkali-acid extraction of glucan leaves 5 – 10% impurities, mainly mannans, proteins, water soluble glucans and amorphous β - 1,6 – glucan trapped in the β-glucan fibrils (Sandula et al. 1999). Sandula and coworkers (Sandula et al. 1999) showed that the glucan could be further purified to the maximum of 96 – 99% by ultrasonication for 10–15 minutes, which also reduced the molecular weight of the compound without altering the average molecular composition. In the present study it was observed that the immunostimulation was lower in animals when fed sonicated glucan preparations such as AIBG-EES and AIBG-WES. The low immunostimulatory property of these glucans may be attributed to the reduction in molecular weight of the glucan due to sonication (Bohn & BeMiller 1995).

While studying the immunostimulatory effect of whole yeast cell (*Saccharomyces cerevisiae*) and glucan extracted from the same yeast, Scholz and coworkers (Scholz et al. 1999) observed enhanced growth rate, *Vibrio* clearing efficacy and Phenoloxidase activity in animals fed with raw yeast compared to isolated β-glucan. Dehasque and

coworkers (Dehasque et al. 1995b) reported that Atlantic salmon fry, fed on a diet containing Baker's yeast at 2% level displayed better survival than control animals when challenged with *Vibrio anguillarum*, while juvenile European sea bass showed similar results when fed on a diet containing experimentally treated yeast at 1% level (Dehasque et al. 1995a). They (Dehasque et al. 1995a) further demonstrated that brewer's yeast, chemically treated to expose its glucans on the surface gave better survival than fresh yeast fed European sea bass.

These results further support the argument that high purity glucan is not necessary for aquaculture purpose as partially purified glucans gave similar or better performance with less cost of production. This argument is supported by results of the present study as well since partially purified glucan AIBG-WENS gave better immunostimulation in *Fenneropenaeus indicus* compared to the other preparations. Based on these results the water extracted non-sonicated *Acremonium* insoluble beta glucan (AIBG-WENS) can be recommended for application in *F. indicus* by oral route.

### 2.4.3 Characterization and treatment of the effluent generated during glucan extraction

The fungus *Acremonium diospyri* has 4.82 ± 1.71 % of glucan (Dry weight) as their cell component. The glucans impart rigidity to the cell wall of fungus and the extraction of which consist of harsh alkaline and acid treatments, which generates large volume of high strength wastewater. The waste generated has very high COD (5184 – 7800 mg/l) bringing it to the classes of biological wastes, which may not be released in to the environment without any treatment. The highly alkaline nature of the wastewater might lead to corrosion of the treatment systems as well. Invariably the effluent has to be neutralized and in the present study acetic acid was used for neutralization. It has to be pointed out that at commercial scale acetic acid can be replaced with any acidic biological wastewater such as winery wastewater (pH 3 – 4) (Malandra et al. 2003).

It has been observed from the experiments that the microbial consortia POAB can degrade around 70% of the organics in the wastewater in a 6-day period. This long

duration required in degradation may be due to the high loading rate, which do not normally occur in industrial treatment plants (Kargi et al. 2000). To enhance the rate of degradation amendments of the medium with growth factors was has not been attempted. It is hoped that with such modifications the duration required for the treatment shall be minimized. It has to be pointed out that even though the consortia FKB could grow well in the wastewater, its degradation potential was comparatively lesser.

Both the consortia were able to reduce the colour of the effluent by 50% of the initial concentration on 1st day of the treatment. Thereafter, it stable without any remained deviation. Similar results were obtained when Sayadi and Ellouz (Sayadi & Ellouz 1993) treated oil mill wastewater with the fungi: *Pycnoporus cinnabarinus*, *Phlebia radiata* and *Polypporus frondosus*.

Microscopic analysis of the slides suspended in the treatment flasks inoculated with POAB was found covered with a thick biofilms on the surface of the slides, which constituted of a large number of yeast and filamentous bacterial cells. It was reported that filamentous microorganisms played an important role in biofilm formation as they could maintain the yeasts within their intertwined mycelial-like structures which acted as the support for yeast and bacterial attachments (Madoni et al. 2000).

The present study demonstrates the potential of the consortium POAB in degrading the glucan extraction wastewater. The biofilm potential of the consortium can be utilized for developing biofilm reactors for the treatment

**Figure 2.1: 600 MHz proton NMR spectra of AIBG (Top) and ASG (Bottom).**

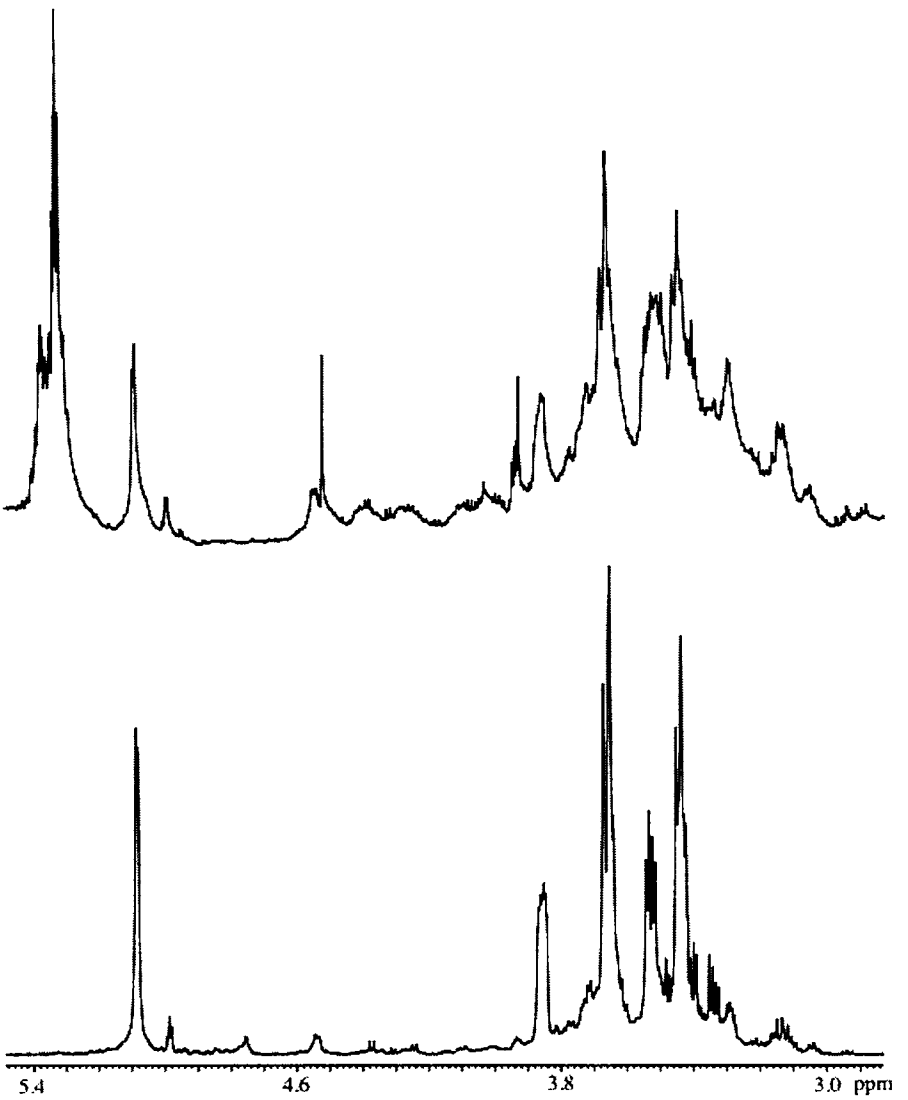


**Figure 2.2: Comparison of the IR spectra of *Acremonium* soluble glucan (Top) and β-(1,3)-D-glucan (Bottom). Absorptions supporting α – (849 and 927 $cm^{-1}$) and β-glucan (890 $cm^{-1}$) structures are indicated in each spectrum**

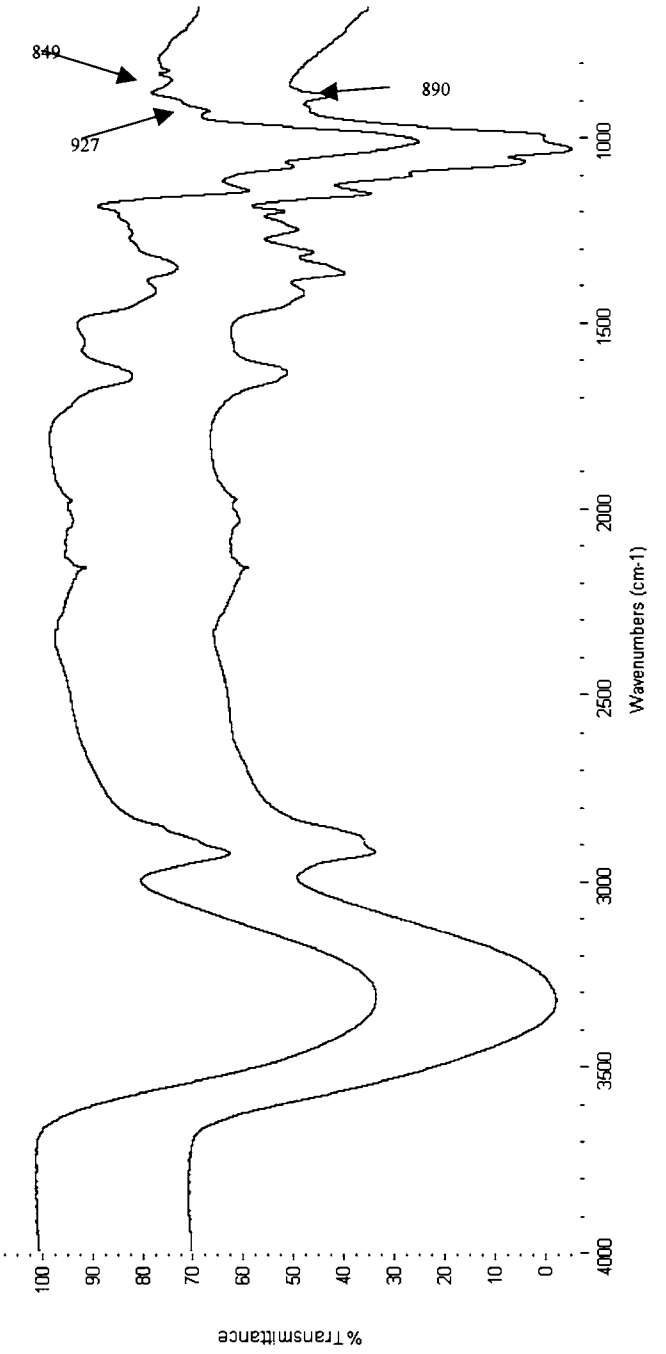


**Figure 2.3: Effect of feeding different glucan preparations on proPhenoloxidase activity in *Fenneropenaeus indicus* ($p<0.05$)**

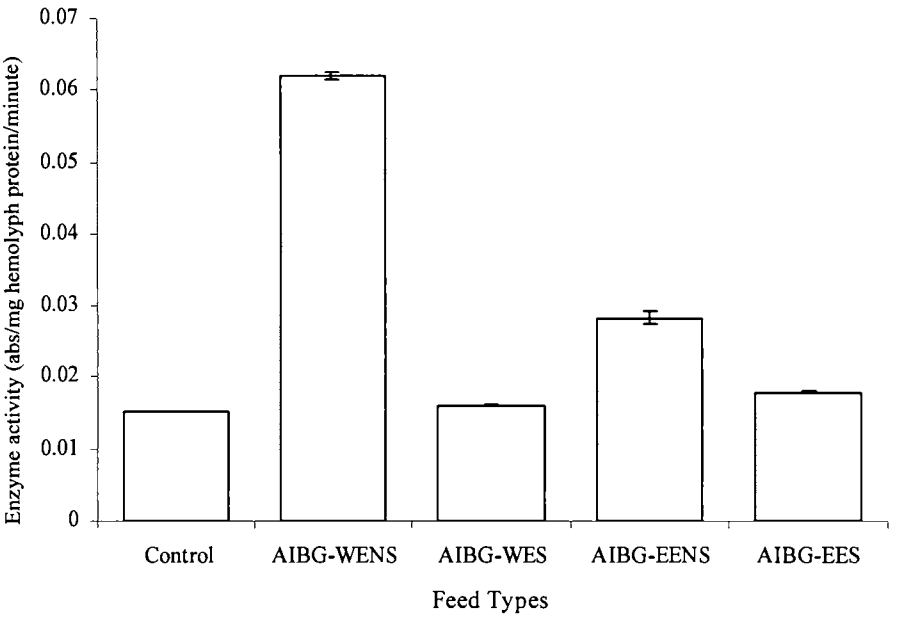



**Figure 2.4: Effect of feeding different glucan preparations on reactive oxygen intermediate production in *Fenneropenaeus indicus* ($p<0.05$)**

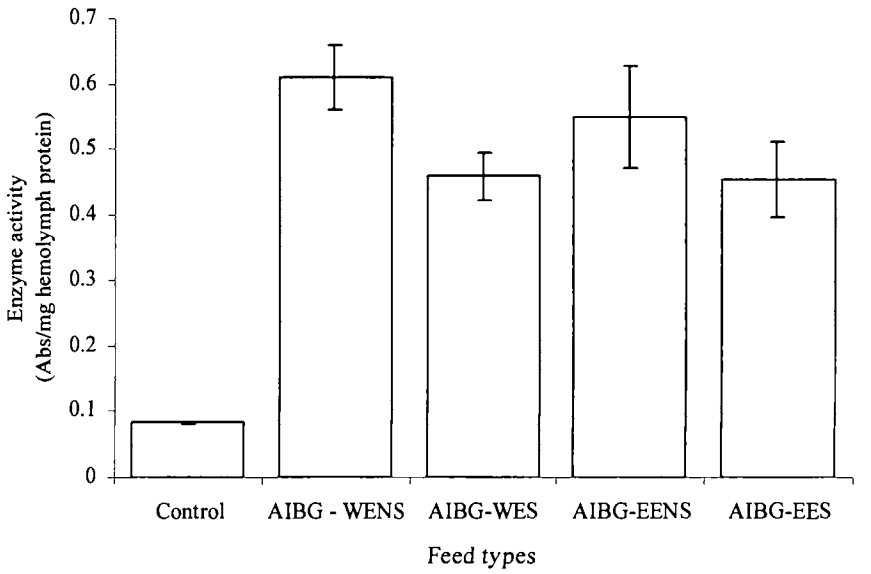


Figure 2.5: **Comparison of the carbohydrate proton NMR spectral regions of the water-extracted non-sonicated glucan isolate (AIBG-WENS) (Top) and ethanol extracted non-sonicated glucan (AIBG-EENS) (Middle). The spectrum of a high-purity glucan is also shown for comparison (Bottom). Glucan structure and assignment of major resonances in the high-purity glucan are also presented.**

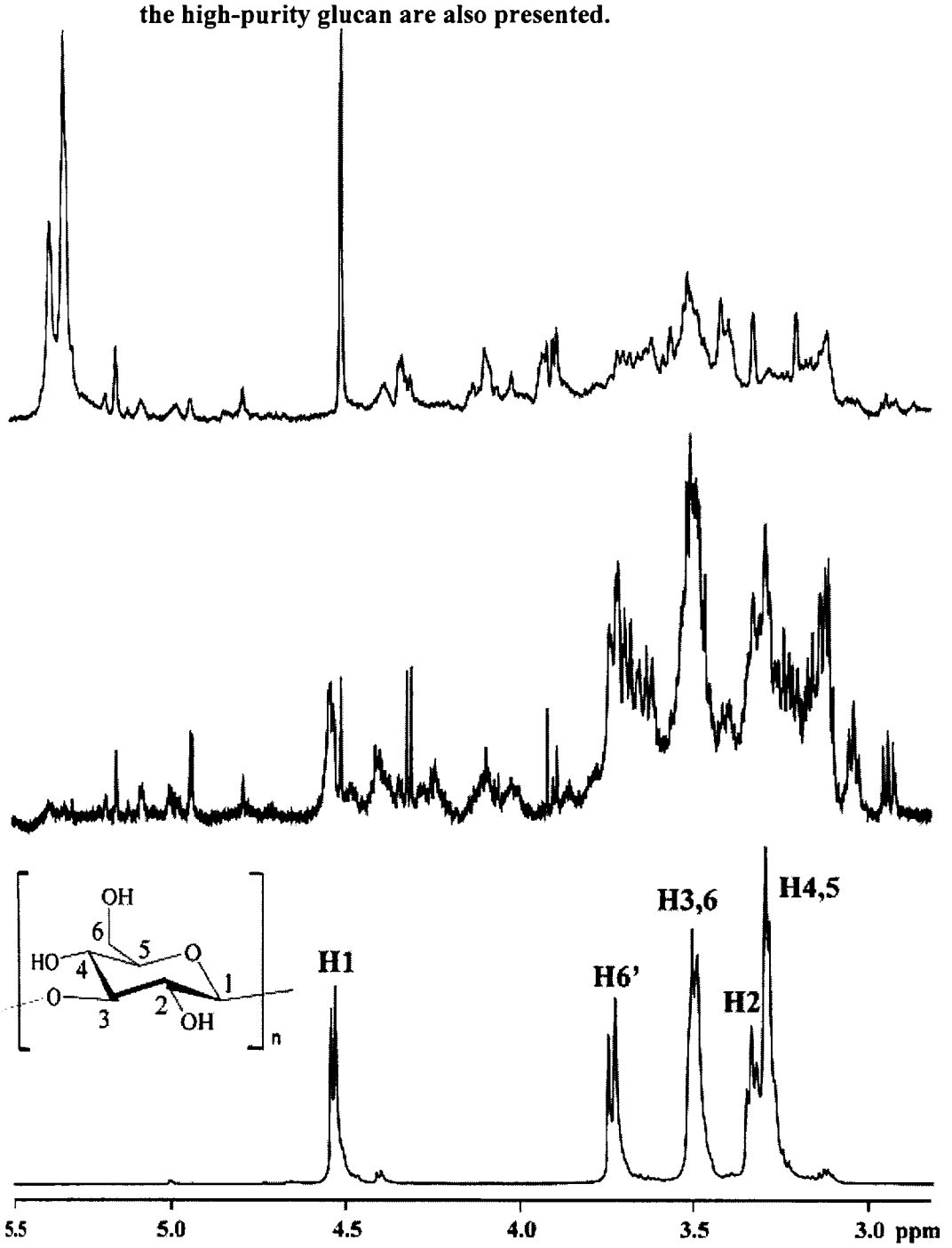


**Figure 2.6: Effect of microbial consortia on removal of COD from glucan extraction wastewater**

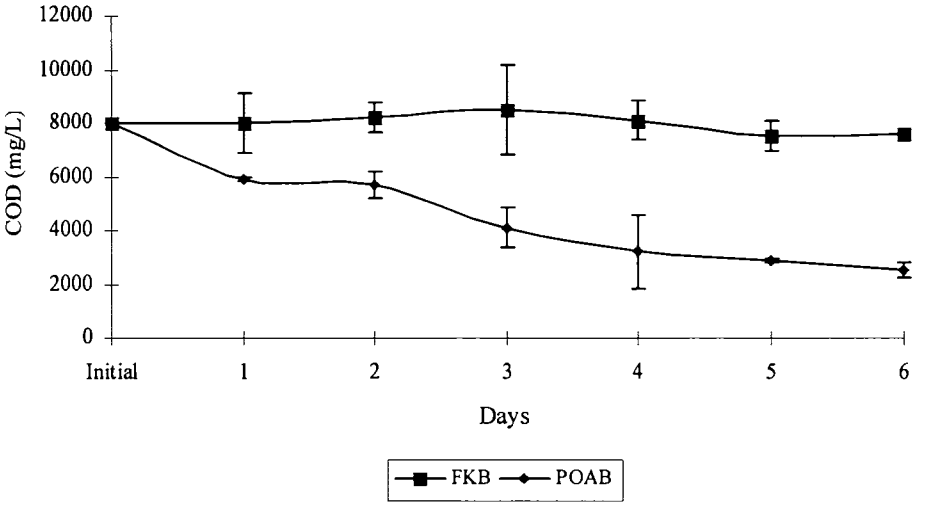



**Figure 2.7: Effect of microbial consortia on $A^{256}$**

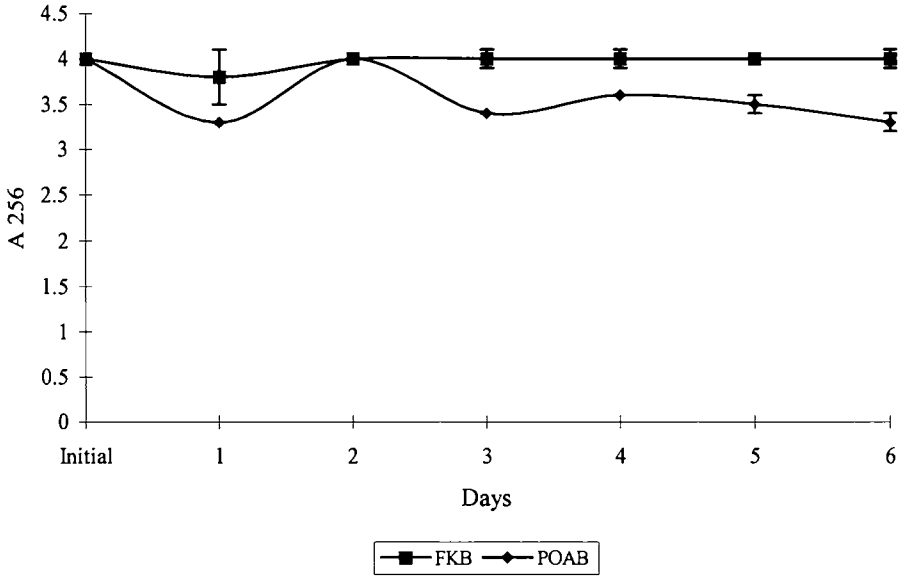
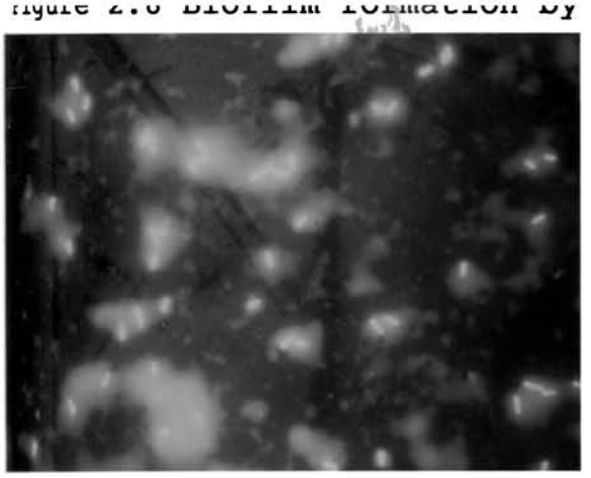
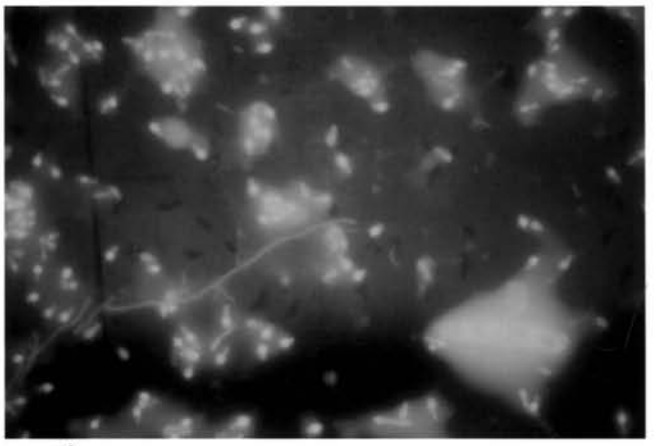


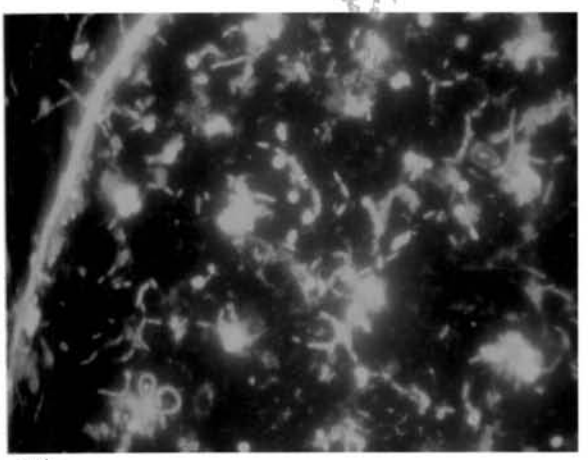
Figure 2.6 Biofilm formation by FRB consortium at different days



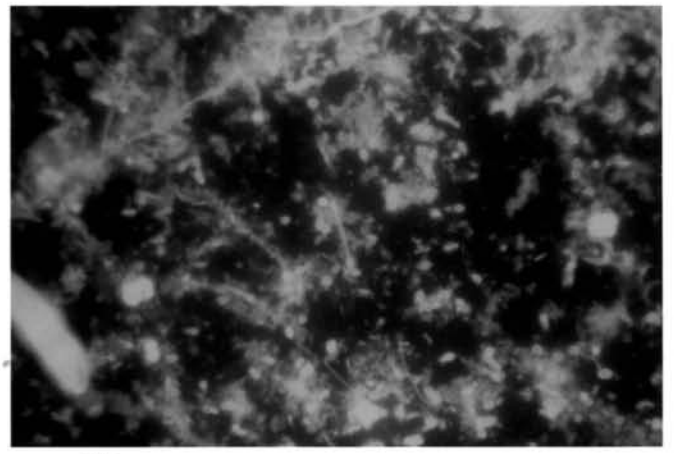
1st Day



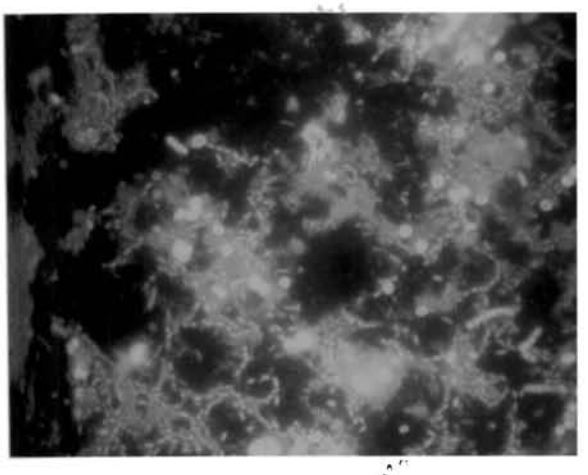
2nd Day



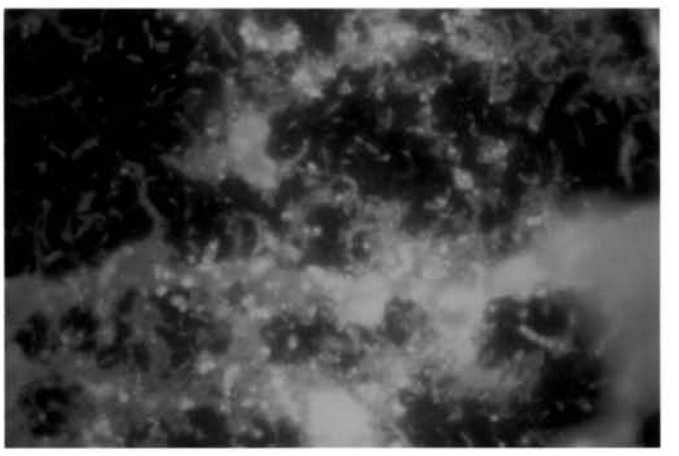
3rd Day



4th Day

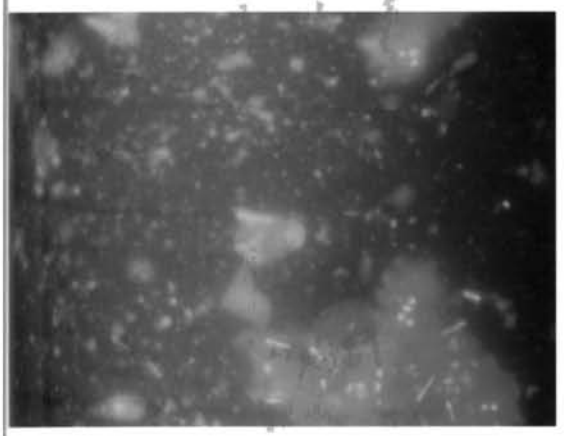


5th Day

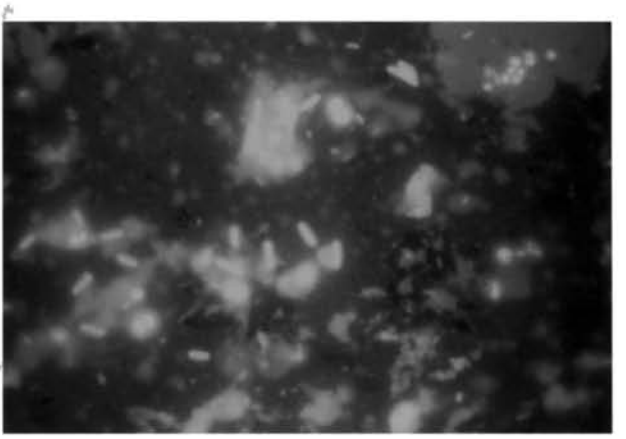


6th Day

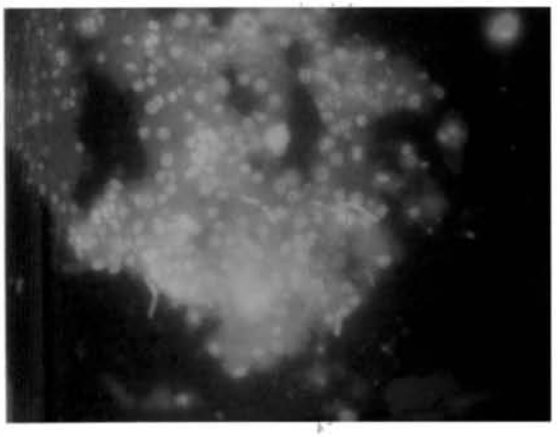
igure 2.9 Biofilm formation by POAB consortium at different days



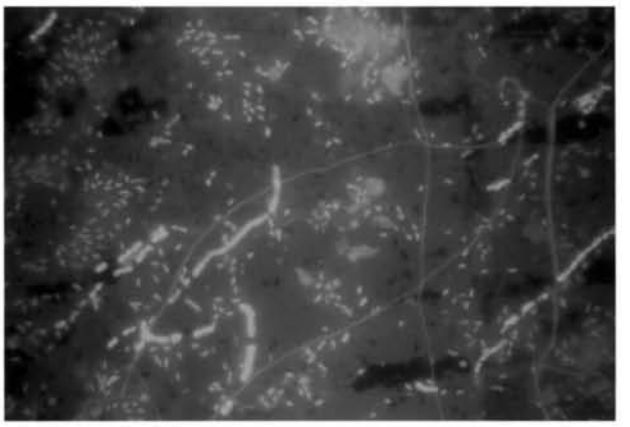
st Day



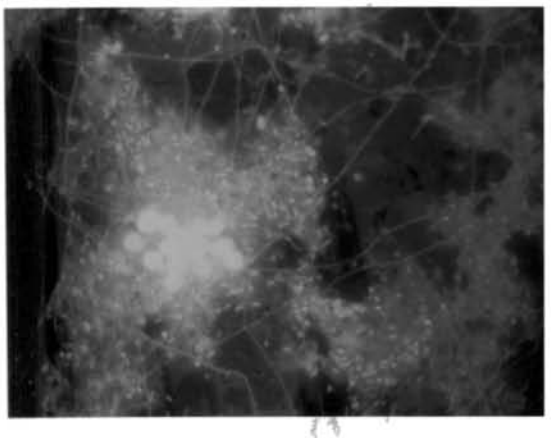
2nd Day



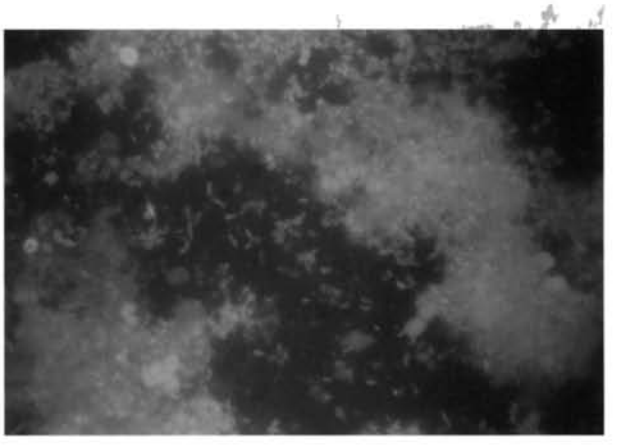
rd Day



4th Day



th Day



6th Day

**Table 2.1: Effect of Acremonium insoluble β-glucan (AIBG) and Acremonium soluble glucan (ASG) on haematological parameters on 1st day of completion of the feeding schedule (n=10) (Mean ± SD)**

| Treatment | Log of haemocyte count | ProPO (Abs/mg protein/ min.) | ROI (Abs/mg protein) |
|---|---|---|---|
| Control | 6.83 ± 0.11 | 0.051 ± 0.02 | 0.4 ± 0.26 |
| AIBG | 6.84 ± 0.19 | 0.041 ± 0.03 | 0.43 ± 0.31 |
| ASG | 6.91 ± 0.15 | 0.061 ± 0.04 | 0.35 ± 0.12 |

**Table 2.2: Effect of Acremonium insoluble β-glucan (AIBG) and Acremonium soluble glucan (ASG) on haematological parameters on 6th day of completion of the feeding schedule (n=10) (Mean ± SD)**

| Treatment | Log of haemocyte count | ProPO (Abs/mg protein/ min.) | ROI (Abs/mg protein) |
|---|---|---|---|
| Control | 6.72 ± 0.24 [a] | 0.014 ± 0.006 [a] | 0.423 ± 0.26 [a] |
| AIBG | 6.85 ± 0.23 [a] | 0.083 ± 0.05 [b] | 0.858 ± 0.39 [b] |
| ASG | 6.93 ± 0.21 [a] | 0.028 ± 0.013 [c] | 0.412 ± 0.236 [a] |

Data in the same column with different letters are significantly different between treatments ($p < 0.05$)

**Table 2.3: Comparison of wastewater generated during the extraction of ethanol extracted non sonicated (AIBG- EENS) and water extracted non sonicated glucans (AIBG- WENS)**

| Parameter | AIBG- EENS | AIBG- WENS |
|---|---|---|
| pH | 13.54 | 13.07 |
| Colour (Hazen) | 6000 | 3000 |
| $A_{256}$ | 4.0 | 4.0 |
| Alkalinity | 16000 | 14430.0 |
| COD | 58000 – 62000 | 5184 - 7800 |

**Table 2.4: Characterization of glucan extraction wastewater (GEW) generated during the extraction of water extracted non-sonicated glucan (AIBG-WENS)**

| Number of samples analyzed | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Yield of glucan (%) | 3.2 | 3.0 | 7.2 | 4.4 | 2.5 | 5.7 | 5.0 | 5.9 | 6.9 |
| Volume of waste produced per g of glucan (l) | 5.3 | 5.7 | 2.4 | 3.9 | 6.2 | 2.9 | 3.2 | 2.8 | 2.3 |
| pH | 13.08 | 13.07 | 13.07 | 13.07 | 13.08 | 13.08 | 13.07 | 13.08 | 13.07 |
| COD (mg/l) | 7800 | 5184 | 6120 | 7800 | 7800 | 7800 | 6400 | 7200 | 7800 |
| Colour (Hazen) | 3000 | 3000 | 3000 | 3000 | 3000 | 3000 | 3000 | 3000 | 3000 |
| $A^{356}$ | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Total Alkalinity (mg $CaCO_3$/l) | 14430.0 | 14430.0 | 14430.0 | 14430.0 | 14430.0 | 14430.0 | 14430.0 | 14430.0 | 14430.0 |
| Total suspended solid (mg/l) | 5.9 | 3.4 | 3.8 | 3.5 | 4.1 | 4.1 | | | |
| Total dissolved solids (mg/l) | 28.1 | 28.1 | 27.9 | 27.2 | 32.2 | 28.7 | | | |
| Total solids (mg/l) | 35.7 | 35.5 | 35.8 | 35.7 | 41.2 | 36.8 | | | |

# CHAPTER 3

# DEVELOPMENT OF A MICROENCAPSULATED DRUG DELIVERY SYSTEM FOR THE LARVAE OF *Macrobrachium rosenbergii*

## 3.1 INTRODUCTION

Development of appropriate vehicles for delivery of therapeutics, immunomodulants and nutrients to finfish and shellfish larvae has been a challenge to aquaculture scientists. Among the several strategies being explored for drug delivery, the most common has been administration with feed. However, instability of aquaculture feeds in water leads to leakage of the core material, diminishing availability to larvae (Pedroza-Islas et al. 2000). AS a result, microencapsulated particles have gained recognition as potential delivery vehicles, with the ability to transport specific substances to the digestive system. Major factors to be considered while designing such microcapsules for crustacean larvae are rate of dissolution of the substances in culture system, acceptance of the capsule by larvae, digestibility and, cost of production. However, the most crucial step in microcapsule design is the selection of appropriate wall material (Sankarikutty et al. 1988) because that the wall material should facilitate delivery of drugs within the short span of residence of the diet within the intestine (Pedroza-Islas et al. 2000).

Microencapsulated drug delivery in aquaculture has so far been accomplished with biopolymers of different origin such as natural gums (Kanazawa 1981), proteins (Petitjean & Csengeri 1995, Yufera et al. 2003) and lipids (Lopez - Alvardo et al. 1994, Onal & Langdon 2004). However, chitosan has not been evaluated as a wall material, despite its favorable characteristics like gelation on contact with counter anions (Bodmeier et al. 1989), formation of films soluble in acidic pH (Remunan - Lopez & Bodmeier 1996) and, its susceptibility to digestive enzymes (Remunan - Lopez et al. 1998). Being a natural polymer, its degradation products are non-toxic to animals also (SuheylaKas 1997). These properties of chitosan prompted us to explore the possibilities of using it to develop an appropriate drug delivery system for larvae of *Macrobrachium rosenbergii*.

Chitosan is a cationic polysaccharide derived from chitin, a natural polymer of N – acetyl glucosamine found commonly in crustacean and insect exoskeletons, and in fungal cell walls (Shepherd et al. 1997). Among the biocompatible and biodegradable natural polymers, chitosan has interesting biological activities (Akbuga 1995). There are several reports on its anti-microbial activity against several species of bacteria, yeasts and fungi (Allan & Hadwiger 1979, Kendra & Hadwiger 1984, Sudarshan et al. 1992, Wang 1992, Roller & Covill 1999, Zheng & Zhu 2003). It has been suggested that its antibacterial effect is based on its ability to increase permeability of the outer membrane of Gram negative bacteria (Sudarshan et al. 1992, Chirkov 2002).

Since most of the protein based wall materials designed for the drug delivery harbor the growth of bacteria (Muir & Sutton 1994) and may cause unsafe effects to the larvae, chitosan with its reported antibacterial properties could be a better alternative. In this work, studying its antibacterial activity against 48 isolates of *Vibrio* spp isolated from *Macrobrachium rosenbergii* larval rearing systems assessed the impact of chitosan on the pathogens in larval rearing system.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Chitosan based microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*

#### 3.2.1.1 Preparation of microcapsules

One micro-bound and two microencapsulated diets were prepared using potato starch and dried shrimp powder as the core material. Nutritional value of the preparations was not considered, because of the exclusive focus on drug delivery. The Microbound diet (MBC) was prepared by mixing shrimp powder (70% w/w) and potato starch (30% w/w) for 10 minutes in a food processor with sufficient quantity of 2% potato starch solution as binder. The preparation was mixed well, dried in a vacuum oven (60°C, 48h), and sieved to particle sizes ranging from 300 to 700μm.

The microcapsules were prepared by coating the micro-bound particles with 1% chitosan prepared in 5% acetic acid. The micro-bound particles were spread over a plastic tray and coated with chitosan using an air gun (Super Mech Engineering Works, India). Force of the air was adjusted to have the particles suspended in air to attain overall coating. Intermittent manual shaking of the tray was also provided to achieve proper mixing and distribution of particles while coating. The preparation was dried in a vacuum oven at 40°C for 24 hours and one part cross-linked with acetone following (Kubota 1993) and labeled as MEC-A and the other part treated with 3% NaOH following (Chandy & Sharma 1996, Lim et al. 1997) and labeled as MEC- N. Both the preparations were dried in vacuum oven (Labline, India) at 40°C for 24 hours.

### 3.2.1.2 Morphology of capsules

The surface topography of both micro-bound and microencapsulated preparations was analyzed by scanning electron microscope (Leo 435 VP Sem, UK). The samples were prepared by placing the micro-spheres on a microscope sample holder and gold sputtering in an argon atmosphere. Adequate care was exercised to obtain homogenous micro-sphere gold coating.

### 3.2.1.3 Dissolution properties of the preparation

Leaching characterestics of micro-bound and microencapsulated preparations were estimated in seawater following Pedroza-Islas et al. (2000). Briefly, 0.3g each of the preparations was weighed and introduced into five test tubes, to which 25ml seawater (15ppt salinity, pH 8.0) was added. A control of 25ml seawater (15ppt salinity, pH 8.0) was kept for all the experiments. The tubes were incubated at room temperature (28 ± 2°C) and one tube was sequentially removed at 1, 2, 3, 4 and 5h intervals, and the contents were vacuum filtered through Whatman No 40 filter paper. The filtrate was dried at 60°C until constant weight. The amount of leached material was calculated based on differences in dry weight between the treatments and the control. All determinations were done in triplicate.

### 3.2.1.4 Leaching of total Free Amino Acids (FAA)

The initial FAA concentration of the feed preparation was quantified as follows: 15mg of each preparation was mixed with 30ml distilled water, homogenized for 15 minutes, and sonicated for 10min at 4°C (500W, 20KHz,VCX500, Sonics, USA) to disintegrate the particles, filtered through 0.2μm pore size cellulose-acetate membrane filter (Sartorius) and analyzed for FAA (Baer et al. 1996). The leaching experiments were carried out in a 500ml conical flask containing 250ml seawater (15ppt salinity, pH 8.0) stirred continuously at 60rpm (Yufera et al. 2002). At zero time point, 500mg of each of the feed preparations was added to the flasks and 10ml aliquots were removed from each at 1, 5, 15, 30 and 60min intervals using a syringe. The samples were filtered through a 0.2μm pore size cellulose-acetate membrane filter (Sartorius) and analyzed for FAA (Baer et al. 1996). The leaching rate was calculated as the percentage loss of FAA from the initial concentration.

### 3.2.1.5 Feed acceptance by *Macrobrachium rosenbergii* larvae

Acceptance of microencapsulated diet by the larvae of *Macrobrachium rosenbergii* was assessed by estimating frequency of the feed intake following Barros & Valenti (2003) over a period of time. Briefly, *M. rosenbergii* larvae at the 8$^{th}$ moult stage were transported to the laboratory and acclimatized. Batches of five larvae were placed into 100ml beakers containing 80ml seawater (15ppt salinity, pH 8.0) previously drawn from the same larval-culture tank, filtered through 125μm pore size nylon mesh screen. Each beaker was provided with aeration to maintain the feed particles in suspension. After 30 minutes, the digestive tract of each larva was examined under a light microscope for feed consumed. Number of larvae with any quantity of feed in the digestive tract was recorded. The experiments were conducted in duplicate and the frequency of feed intake was calculated as follows.

Frequency of Food Intake (FFI) % = $(N_1/N_2)$ 100

Where,

$N_1$= Number of larvae with ingested feed

$N_2$= Total number of larvae in the beaker

As an alternate approach to assess the acceptance of MEC-N by larvae in the larval rearing facility 10g feed was added to a 5 tonne tank containing 400,000 larvae. One hundred larvae from the tank were observed individually under a light microscope to evaluate the ingestion and disintegration of feed.

### 3.2.2 Antibacterial activity of chitosan against *Vibrio* spp. isolated from *Macrobrachium rosenbergii* larval rearing system

#### 3.2.2.1Chitosan

Chitosan, used in this study, was obtained from M/s South India Sea Foods, Kochi, Kerala, India. It was extracted from crustacean exoskeletons, had an average molecular weight of 180kDa and was 80% de-acetylated. Different concentrations of chitosan (0.25, 0.5, 0.75 and 1.0%) were prepared by dissolving in 50ml 5% glacial acetic acid (v/v) (Kubota 1993) making up to 100ml using distilled water. The pH was adjusted to 5.5 – 6.0 using 1 N NaOH.

#### 3.2.2.2 Vibrio

The forty-eight isolates of vibrios used in this study were taken from the culture collection of the Centre for Fish Disease Diagnosis and Management, Cochin University of Science and Technology. These isolates were accumulated over time from freshwater prawn larval rearing systems, and characterized phenotypically. All the isolates were grown in ZoBell's Marine Broth (2216E) prepared in 15ppt salinity seawater for 12 – 15h on rotary shaker at 100rpm. They were harvested at the exponential phase of growth, diluted to $10^{-6}$, and used for assaying the antibacterial properties of chitosan.

#### 3.2.2.3 Antibacterial Assay

Antibacterial activity was measured following the method of Zheng and Zhu (Zheng & Zhu 2003) with slight modification. Briefly, ZoBell's Marine Agar (2216E) plates were prepared using 15ppt seawater. Then 100µl *Vibrio* suspension was spread on the plates followed by 100µl of chitosan preparation in 5% glacial acetic acid (pH 5.5 – 6.0). Controls were identical except that 100µl of acetic acid solution (pH 5.5 – 6.0) replaced

the chitosan solution. All plates were incubated at 28 ± 1°C for 24h before total number of colonies was enumerated. Inhibition rate (η) was calculated using the equation

$$\eta = \frac{N_1 - N_2}{N_1} \times 100\%$$

Where $N_1$ and $N_2$ were the number of colonies developed on the control and experimental plates respectively.

### 3.2.3. Statistical Study

Differences in dissolution rates and FAA leaching at different immersion times and between micro-diets were analyzed by one-way ANOVA. A significance level $p < 0.05$ was considered. Karl Pearsons coefficient of correlation was used to assess the relationship between the concentration of chitosan and antimicrobial activity. The student's t test was used to assess differences at $p < 0.005$ (Bailey 1995).

## 3.3 RESULTS

### 3.3.1 Chitosan based microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*

The particle size of the micro-bound and microencapsulated preparations ranged from 400 – 900μm as observed under a scanning electron microscope (Figure 3.1 – 3. 3). The lack of uniformity in the morphology of these particles is due to the mechanical milling operation involved in the feed preparation. A layer of chitosan is seen deposited on the microencapsulated preparations, MEC-A and MEC-N.

Significant differences could be observed in the dissolution pattern of the micro-bound and microencapsulated diets, MEC-A, MEC-N (Figure 3.4) ($p < 0.05$). Microcapsules cross-linked with NaOH (MEC-N) exhibited 14.43 ± 2.0% dissolution during the first hour immersion in seawater, subsequently increasing to 23.33 ± 3.4% at 5h. Dissolution of microcapsule cross-linked with acetone (MEC-A) was also similar having 18.9 ± 3.8% by the first hour immersion and 24.4 ± 2.0% at the 5$^{th}$ hour. However, a significantly

higher dissolution rate was observed from the micro-bound diet (MBC) under the same experimental conditions, with an initial dissolution of 60.32 ± 9.7% during the first hour, subsequently attaining a maximum of 69.72 ± 5.1% after 5h.

Substantial difference was observed in the FAA leaching pattern between micro-bound and each of the microencapsulated preparations ($p < 0.05$) (Figure 3.5). During the first minute of immersion 2.93 ± 0.5% FAA was found to have leached out from MEC-N, 36.0 ± 3.6% from MEC-A and 81.07 ± 2.9% from MBC. However, after sixty minutes of immersion, 65.2 ± 2.7% of FAA had leached out from MEC-N, 75.03 ± 1.6% from MEC-A, and there was complete release from MBC.

The feed acceptance studies indicated that the frequency of feed intake by the larvae was similar for MEC-N (75%) and MEC-A (73.5%) over a period of 30 minutes. Disintegration of the microcapsules in the larval intestine could be noticed through microscopic observations. In the hatchery system, the majority of larvae were found to capture the microcapsule (MEC-N), and hold it at least for some time. However, only 50–60% had accepted the diet.

### 3.3.2 Antibacterial activity of chitosan against *Vibrio* spp. isolated from *Macrobrachium rosenbergii* larval rearing system

Results showed that there was an increase in antimicrobial activity with increasing chitosan concentration ($p < 0.005$) (Table 3. 1). Even though, many cells survived at 1% chitosan (Figure 3.6), the highest concentration of chitosan used inhibited *Vibrio vulnificus* by 88.8 ± 14.6% whereas the same concentration inhibited *Vibrio alginolyticus* by only 50.8 ± 19.8%.

## 3. 4 DISCUSSION

### 3.4.1 Chitosan based microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*

The study was aimed at the development of an economically practical and easy to prepare microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*.

Microcapsules with a particle size ranging from 400 – 900μm, within the acceptable range of the larvae (Barros & Valenti 2003), were prepared for this study. The lab trials revealed that 75% of the larvae were able to ingest and digest the MEC-N capsules. In hatchery trial, even though all the larvae had captured the MEC-N capsules, only 50 – 60% were found to ingest and digest them. This may be because of the differences in stage of the larvae in the same tanks. *M. rosenbergii* larvae starts effective feeding from stage VI onwards (Agard 1999), when yolk reserves disappear and the digestive tract is developed. During the stages of VII to VIII, development of the digestive system of larvae gets completed and it changes from carnivorous to omnivorous (Kamarudin et al. 1994), and the larvae can then accept and digest artificial diets (Barros & Valenti 2003). Therefore, it may be suggested that the microencapsulated diets may be useful during the advanced larval stages.

Based on the total nutrient loss during immersion, chitosan microcapsule MEC-N was found to be a promising delivery vehicle as it showed only 23.33 ± 3.4% dissolution after 5 hours of immersion in seawater. Alabi et al. (1999a) measured the leakage rates of protein and total nutrients (by weight) from a commercial microbound diet and the one encapsulated in cross-linked protein wall material. They observed 50 – 70% protein loss within 1h of suspension in seawater for all particle types. The total nutrient loss from the encapsulated diets was 37-39% and increased to 58% in the microbound diet during a 6h period of suspension. ~~Pedroza-Islas and coworkers~~ (Pedroza-Islas et al. 1999), after studying the kinetics of leaching in a series of microcapsules with different biopolymer treatments demonstrated that a microcapsule with a minimum of 60 minutes in water would provide ample time for shrimp larvae to achieve an adequate intake of the diet. Under this condition, chitosan microcapsule (MEC-N) would be a promising drug delivery vehicle.

Looking at the amino acid leaching property, only 3% was lost from MEC-N capsule after 1min of immersion in seawater and increased to 65% after 60 minutes of immersion. The amino acid leaching rates are much less than those reported by Lopez - Alvardo et al. (1994). ~~They reported that~~ more than 80% of free amino acids were lost from alginate,

carrageenan and zein microbound particles after 2 minutes of suspension in aqueous medium and as much as 60% of dietary free aminoacids had been lost from carrageenan bound and zein coated and gelatin bound diets within 1 min of suspension. Similarly, Ozkizilcik & Chu (1996) reported free lysine loses of 80% in protein walled micro-capsules after 60 minutes of rehydration. Meanwhile, Yufera and coworkers (Yufera et al. 2002) proposed a complex protein walled microcapsule prepared by emulsification of dietary compounds dispersed in a basic pH buffered Tris-HCl with soy lecithin and cyclohexane, and cross-linked by using trimesoyl chloride dissolved in diethyl ether. The capsule, created throught this process of interfacial polymerization showed FAA leaching of 17% after one-hour immersion in distilled water. The high cost of these protein walled microcapsules, use of organic solvents and highly reactive chemical cross-linking agents diminish the prospects of long term commercial production and use (Langdon 2003). Moreover, a high bacterial load associated with the breakdown of commercial diets encapsulated within cross-linked, protein walled capsules also has been reported (Muir & Sutton 1994). Antimicrobial properties of chitosan are well documented (Allan & Hadwiger 1979, Kendra & Hadwiger 1984, Sudarshan et al. 1992, Wang 1992, Roller & Covill 1999, Zheng & Zhu 2003) and our studies also have demonstrated the same against vibrios associated with *Macrobrachium rosenbergii* larval rearing systems. Considering all its potential characteristics such as non-toxicity, film forming and antimicrobial properties, chitosan – a biocompatible biopolymer – may be considered as an appropriate wall material to be used for preparing microcapsules as vehicles for delivering drugs and nutrients to *M. rosenbergii* larvae.

### 3.4.2 Antibacterial activity of chitosan against *Vibrio* spp. isolated from *Macrobrachium rosenbergii* larval rearing system

Major factors believed to contribute to the antimicrobial properties of chitosan are concentration of the chitosan in solution, molecular weight, degree of deacetylation and the level of protonation of the free groups in the chitosan. Using chitosan at different viscosity average molecular weights ranging from less than 5 to 350kDa on *Escherichia coli* and *Staphylococcus aureus* (Zheng & Zhu 2003) concluded that its antibacterial properties were directly related to its concentration.

Chitosan in the larval rearing system may function as a *Vibrio* growth depressant. As the risk of infection is directly related to pathogen density, depressed cell counts may help to prevent larval vibriosis. Besides, chitosan is recognized as an immunostimulant in fish (Siwicki et al. 1994, Sahoo & Mukergee 1999). Thus, it may be worthwhile to test it as an immunostimulant in prawn larvae also.

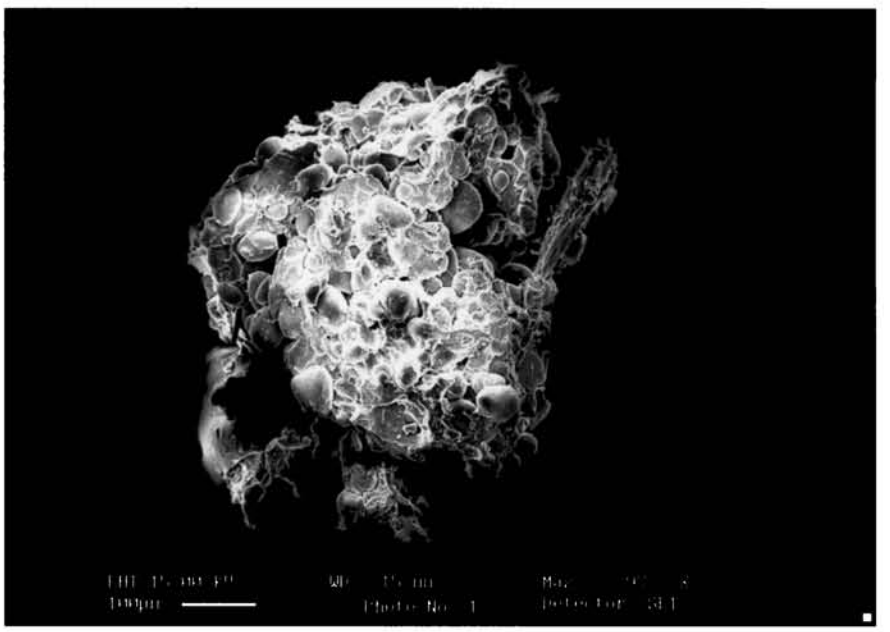


Figure 3.2 Scanning Electron Micrograph of microcapsule cross linked with NaOH (MEC N)

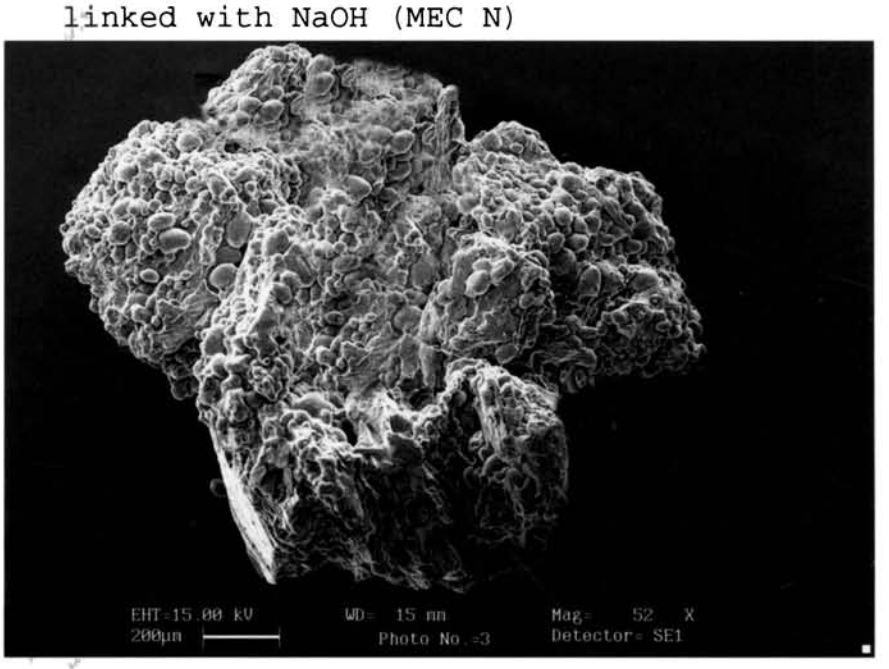



Figure 3.3 Scanning Electron Micrograph of microcapsule cross linked with acetone (MEC A)

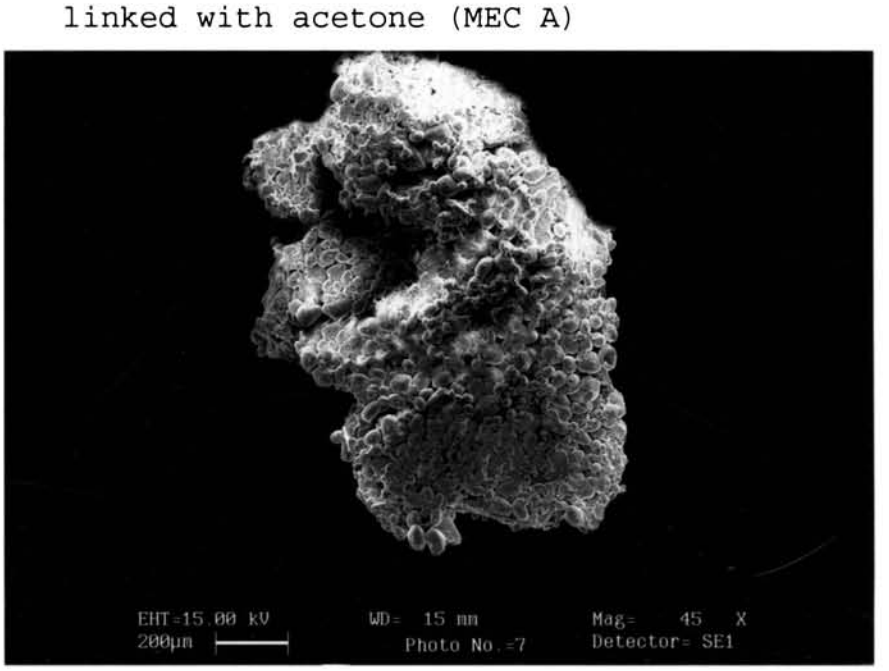


**Figure 3.4 Total dissolution of nutrients from microbound (MBC) and microencapsulated preparations (MEC-N & MEC-A)**

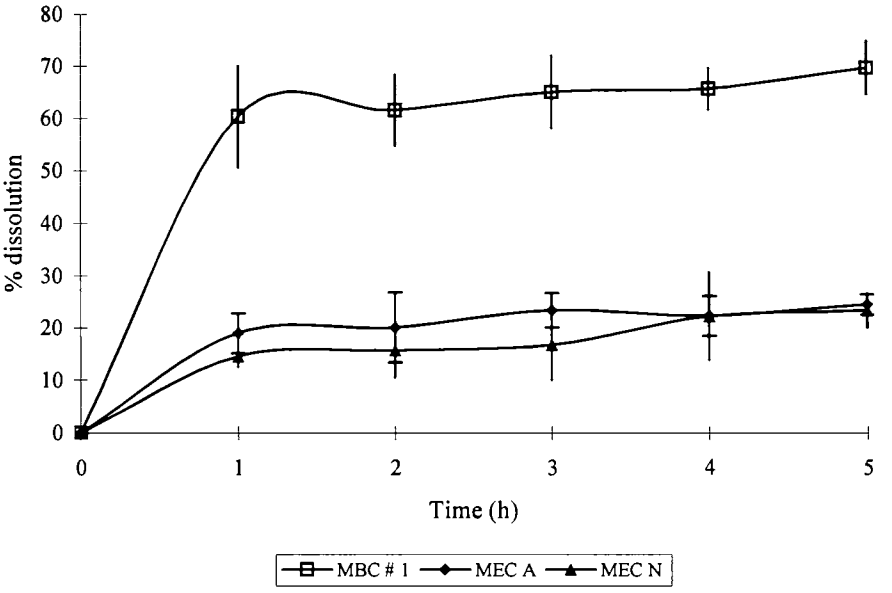



**Figure 3.5 Leaching of Total free amino acids from microbound (MBC) and microencapsulated preparations (MEC-N & MEC-A)**

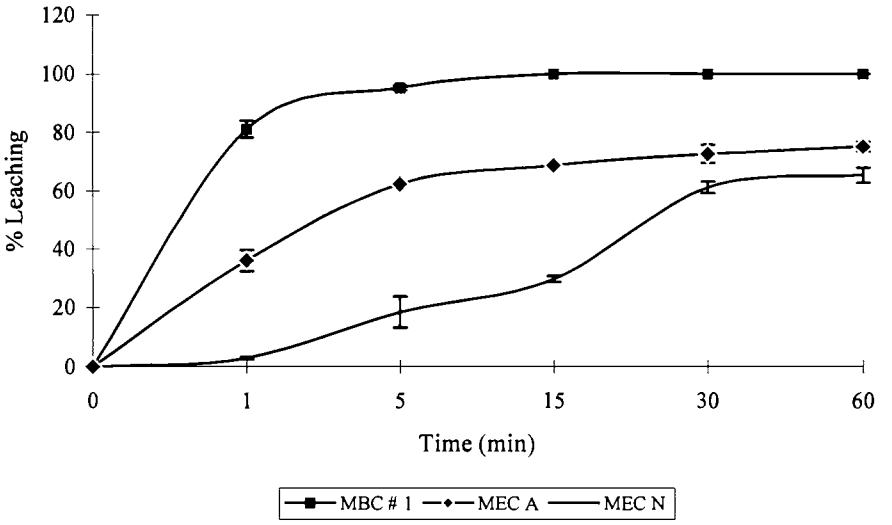


Figure 3.6 Effect of different concentrations of chitosan solution on its antimicrobial activity to an isolate of *Vibrio*

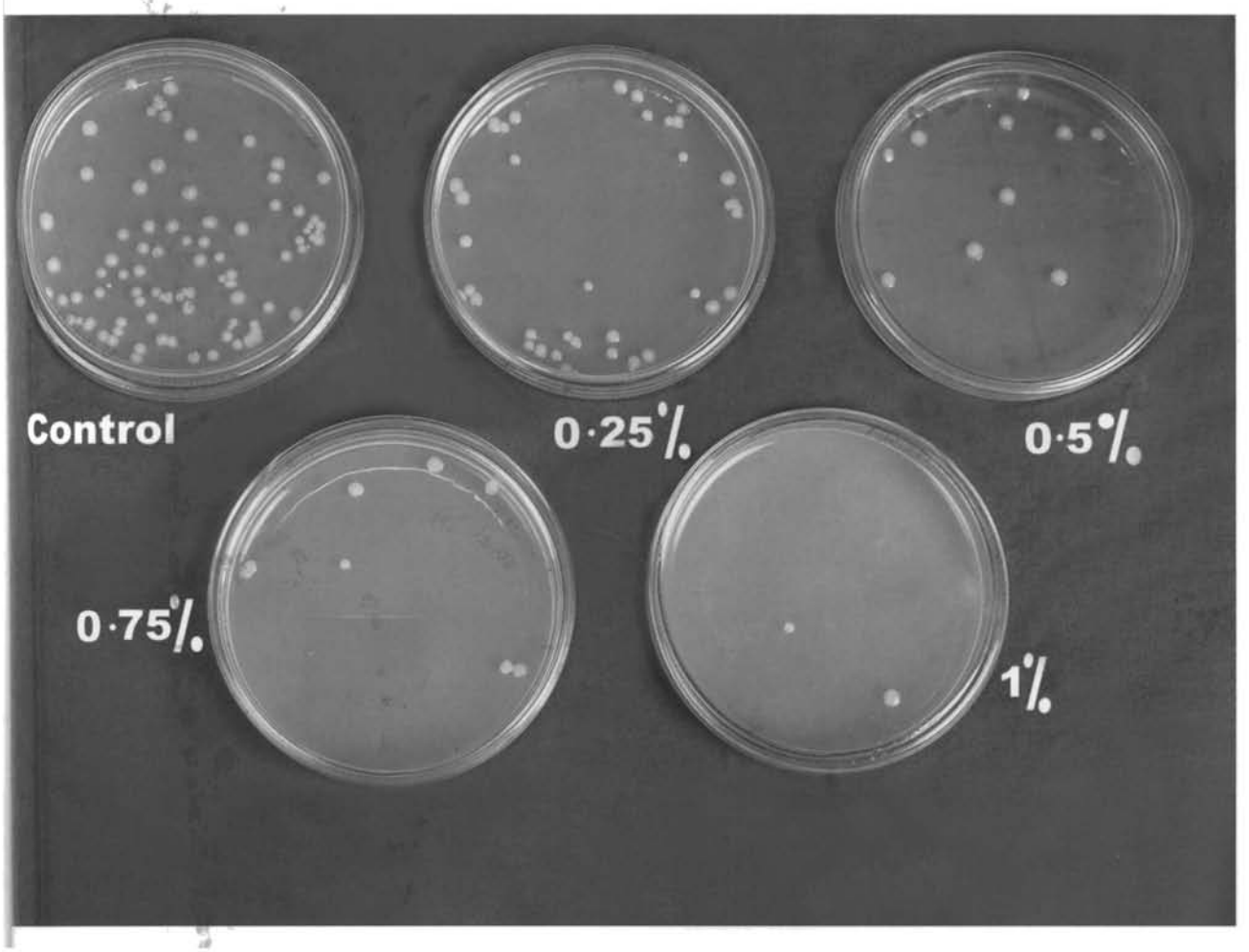


**Table 3.1 Response of *Vibrio* isolates to different concentrations of chitosan**

| Vibrio isolates | No of strains | Inhibition rate (η) (Mean ± SD) at different concentrations of chitosan (W/V) | | | | Correlation Coefficient* |
|---|---|---|---|---|---|---|
| | | 0.25 % | 0.5 % | 0.75 % | 1.0 % | |
| *V. cholerae* | 11 | 55.7 ± 30.8 | 75.5 ± 23 | 81.5 ± 19.6 | 85.4 ± 21.2 | 0.931 |
| *V. parahaemolyticus* | 5 | 39.2 ± 26.3 | 50.2 ± 25.8 | 57.4 ± 30.8 | 69.1 ± 22.3 | 0.996 |
| *V. mediterranei* | 6 | 51.1 ± 27.2 | 63.7 ± 26.7 | 73.9 ± 20.5 | 80.4 ± 19.7 | 0.990 |
| *V. nereis* | 11 | 63.6 ± 20.7 | 73.1 ± 13.9 | 81.8 ± 9.6 | 84.2 ± 10.3 | 0.973 |
| *V. proteolyticus* | 2 | 59 ± 36.3 | 65.2 ± 27.2 | 72.7 ± 22 | 88.1 ± 12.4 | 0.975 |
| *V. splendidus* | 2 | 26.2 ± 9.8 | 29.3 ± 2.8 | 49.7 ± 2.7 | 68.0 ± 13.7 | 0.966 |
| *V. vulnificus* | 3 | 46 ± 41.2 | 63 ± 29.3 | 80.1 ± 26.1 | 88.8 ± 14.6 | 0.990 |
| *V. alginolyticus* | 8 | 21.9 ± 19.8 | 29.2 ± 16.1 | 41.1 ± 19.9 | 50.8 ± 19.8 | 0.996 |

* Coefficient of correlation between concentration of chitosan and cell count of *Vibrio*

## CHAPTER 4

# MICROENCAPSULATED GLUCAN WITH BACTERIN PREPARATION FOR INCREASED SEED PRODUCTION IN *Macrobrachium rosenbergii* LARVAL REARING SYSTEM

### 4.1 INTRODUCTION

*Macrobrachium rosenbergii*, the giant fresh water prawn, has proven potential as an aquaculture species (Willis et al. 1976, Sandifer & Smith 1977). Kurup in 1994 estimated the seed requirement as 200 million per year in India, and it had been increasing every year due to the greater emphasis given to the species after the outbreak of white spot disease (WSD) in Penaeid culture. To satiate this requirement 71 fresh water prawn hatcheries with a built-in production capacity of 1.83 billion post larvae per year are under operation (Bojan 2003). However, the larval production technology especially with regard to disease management has not yet been perfected. One of the most important disease problems is the recurrence of vibriosis ( Sindermann 1977, Singh 1990) which reduces larval survival rate to a level of economic non-viability. Currently this problem is addressed by prophylactic use of antibiotics and other chemotherapeutics (Karunasagar et al. 1994), which leads to the emergence of antibiotic resistant bacteria (Tendencia & delapena 2001) and also to the transference of resistance to human pathogens. Therefore, the industry is on the look out of alternate strategies to combat vibriosis with minimal impairment of the environment.

One of the widely accepted alternate methods is to enhance the non-specific immune system of animals by using immunostimulants (Soderhall 1981, Soderhall et al. 1985, Sung et al. 1994, Vici et al. 2000). Among these compounds, β-glucans have been shown to increase the survival rate and enhance the resistance to bacterial and viral pathogens in crustaceans (Itami et al. 1994, Su et al. 1995, Liao et al. 1996). Glucan can also be used as vaccine adjuvant, particularly with bacterin. Indeed Aakre and coworkers (Aakre et al. 1994) showed that the presence of adjuvant in an anti – *Aeromonas* vaccine gave a high relative per cent survival in Atlantic salmon after a viral challenge. Further more, these authors showed that the use of β - glucan as an adjuvant could lead to higher serum

antibody levels compared to vaccine given alone. Most of these studies were in fishes (Nikl et al. 1991, Chen & Ainsworth 1992, Rorstad et al. 1993).

McKay & Jenkin (1969) achieved the first vaccination in crustaceans, inducing increased resistance to *Pseudomonas* on fresh water Cray fish *Parachaeraps bicarinatus*. Vici and coworkers (Vici et al. 2000) hypothesized the potential of using bactrerin and immunomodulant in the larval rearing of *Macrobrachium rosenbergii*. In their study the dried cells of glucan producing fungus *Acremonium diospyri* was given along with inactivated bacterial cells to the larvae of *M. rosenbergii* and observed an enhanced rate of metamorphosis, with no significant difference in the survival rate. In continuation to this, glucan was further extracted from *A. diospyri* and administered along with bacterins to the larvae of *M. rosenbergii* and observed 17 % increase in seed production (Anas & Singh 2003). The major impediment in both our previous studies was the inability of egg custard to deliver the cells and compounds safely to the intestine of the animal as the preparations were unstable in water. The present study was envisaged to deliver glucan and bacterins safely to the intestine of the larvae by employing microencapsulation technique and to evaluate its impact in terms of total days required for the emergence of post larvae, total post larvae produced, length, weight and specific growth rate. Importance of frequency of administration of the preparation was also examined in this study.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Preparation of glucan

Water extracted non-sonicated isolate of *Acremonium* insoluble β glucan was extracted following the method described in the previous section 2.2.2.1. Briefly 1g dried *Acremonium diospyri* cells were suspended in 20 ml 3% (w/v) aqueous sodium hydroxide and maintained at 100°C for 6h in a serological water bath. Filtering through muslin silk separated alkali insoluble materials, and re-extracted with alkali. The extraction was continued with 20ml of 0.5N acetic acid at 75°C for 6h. The precipitate was extracted with hot distilled water and dried under vacuum.

### 4.2.2 Preparation of bacterins

Two bacterial isolates from moribund larvae of *Macrobrachium rosenbergii* belonging to the family of Vibrionaceae were used for the development of bacterins. These strains (MRNL – 1 and MRNL – 3) were made available from the culture collection of Centre for Fish Disesae Diagnosis and Management, Cochin University of Science and Technology. Bacterins were prepared according to Vici and coworkers (Vici et al 2000). Briefly, 48h old bacterial culture was harvested in phosphate buffered saline and inactivated by adding formalin to a final concentration of 0.2% v/v. The inactivation was confirmed by inoculating an aliquot of one-milliliter bacterin to ZoBell's Marine Broth (2216 E) prepared in 12 ppt salinity seawater and observed 7 days for growth. Subsequently the bacterin was preserved at 4°C till used.

### 4.2.3 Preparation of microencapsulated glucan with bacterin

The ingredients used for the preparation of microencapsulated glucan with bacterin are shown in the Table 4.1. The nutrient value of the preparation was not considered because it was desired only for delivery of the glucan with bacterin preparation. The volume of bacterin given in the Table 4.1 is the volume needed to give $10^{10}$ cells $g^{-1}$ feed. The ingredients A and B were mixed in a food processor for 10 minutes, supplemented the glucan with bacterin preparation and continued the mixing for 10min. Sufficient quantity of starch solution (2% w/v) was added to the above preparation and ensured proper binding of the drug to the core material by continued mixing. The particle size of the preparation was adjusted to 250 – 700μ after drying in vacuum oven at 50°C for 24h. The particles were suspended on a plastic tray and coated with 1% chitosan solution prepared in 5% (w/v) acetic acid (Kubota 1993) using an air gun (Super Mech Engineering Works, India). The air pressure was adjusted in such a way that the particles were suspended in air to attain complete coating. Intermittent shaking was given to the tray to achieve proper distribution of the particles. The preparation was dried in a vacuum oven at 50°C for 24 hours and cross-linked with 3% NaOH (Chandy & Sharma 1996). The chitosan-coated microcapsules thus prepared were dried in a vacuum oven and sieved to separate the particles of 250 - 700μ size.

**4.2.4 Experimental conditions**

The experimental evaluation of the microencapsulated glucan with bacterin preparation was conducted at M/S Rosen Fisheries prawn hatchery, Thrissur, Kerala, India following the commercial larval production technique. Three tanks of 5 tonne capacity each, filled with 12ppt seawater were allotted for the experiment. Tank # 1, stocked with 410,000 larvae (stage VIII) was designated as the control and fed *Artemia nauplii* and egg custard. Larvae stocked in tank # 2 (410,000 larvae) were fed the experimental diet daily morning using 10g as a single dose. Meanwhile larvae stocked in tank # 3 (370,000 larvae) were fed experimental diet using 10g during morning hours once in seven days. The larvae were fed normal diets during the other times.

Growth and survival of larvae were evaluated at the termination of the feeding trials. Growth was determined by measuring average length and weight of 25 larvae from each tank. Measurement of length was made from rostral tip to the end of telson excluding the spines. The specific growth rate (SGR) was calculated in terms of dry weight following (Tacon 1990). SGR = 100 (ln $W_f$ – ln $W_i$) day $^{-1}$, where $W_f$ and $W_I$ were the final and initial weight.

pH, temperature, Salinity, Ammonia, Nitrite, Nitrate, Alkalinity and hardness of the rearing water were monitored at 3 days interval. Temperature was measured by using a maximum minimum thermometer, pH by digital pH meter (Systronics, 335), ammonium by phenol hypochlorite method of Solarzano (1969), nitrite by the method of Benschnerder & Robinson (1952), nitrate following hydrazine sulphate reduction (Strickland & Parson 1968), salinity using a refracto salinometer and total alkalinity by simple titration following Standard Methods (APHA 1995).

The water and animal samples were collected at 3-day interval for assessing the microbial quality of the system. The water samples were serially diluted to $10^{-5}$ in sterile seawater (15ppt salinity). The larval samples were washed gently in sterile seawater (15ppt) to remove loosely adhering particles. The samples were then separated using sterile filter paper and excess water was blotted out aseptically. Weight of the larvae were measured

aseptically, homogenized in sterile seawater (15ppt) and diluted to $10^{-6}$. Aliquots of 100 µl from each dilution were spread plated on ZoBell's Marine Agar (2216E) in duplicate.

ZoBell's Marine Agar (2216E) in 12ppt seawater employed for the enumeration of total heterotrophic bacteria and thiosulphate citrate bile salts sucrose agar (TCBS) prepared in distilled water was used for the selective enumeration of *Vibrio* spp. After incubation for 72h at 28 ± 2°C, plates with 30 – 300 colony forming units were used for counting.

### 4.2.5 Statistical study

The results obtained were compared by one way analysis of variance (ANOVA) and a significance level at $p < 0.05$ was considered (Bailey 1995).

## 4.3 RESULTS

The responses of larvae of *Macrobrachium rosenbergii* towards the application of microencapsulated glucan with bacterin preparation in a continuous mode and once in 7 days in comparison to a control are given in Table 4.2. Significantly higher post larval production could be attained in experimental tanks, 47.3% production in tank # 3 fed with the experimental feed once in 7 days and 30.1% production in tank # 2 fed with experimental feed continuously. Whereas the post larval production attained in the control tank (tank # 1) was only 24.1%. Rate of metamorphosis has been enhanced in animals reared in the experimental tanks. Even though the stage of the larvae was VII in tank # 3 at the time of the commencement of the experiment, the first post larvae appeared on 20$^{th}$ day of culture and facilitated the harvest on 30$^{th}$ day. Whereas in the tank # 2 (continuous administration of experimental feed) and tank # 1(control) the stage of larvae was VIII at the time of starting the experimental feeding and the first post larvae appeared on 22$^{nd}$ and 23$^{rd}$ day and facilitated harvest on 32$^{nd}$ and 33$^{rd}$ day of culture respectively. The specific growth rate also was significantly higher in animals fed with the experimental diets. The larvae reared in tank # 2 and 3 exhibited an enhanced specific growth rate of 9.3 and 10.7% dry weight per day respectively, compared to 3.1% dry weight per day of the animals reared in tank # 1. Similarly the average length and weight

of the post larvae reared in the experimental tanks (tank # 2 and 3) were also higher compared to those reared in control tank (tank # 1).

The enhancement in post larval production, specific growth rate, length and weight could be accomplished in a condition where no significant differences existed between the tanks in terms of physicochemical quality of rearing water (Table 4.3) and microbial quality of both rearing water and animal samples (Table 4.4) ($p>0.05$). However many of these parameters were above the permissible limit for *Macrobrachium rosenbergii* larval rearing.

## 4.4 DISCUSSION

Purpose of this study was to evaluate the potential of microencapsulated glucan with bacterin preparation in enhancing the seed production of *Macrobrachium rosenbergii* in a commercial hatchery system. The administration protocol also was subjected for validation in this study. Application of the preparation once in seven-day period resulted in significantly higher level of seed production compared to that from continuous application and the control. In our previous study (Anas & Singh 2003), glucan with bacterin preparation was administered to the larval rearing system daily by incorporating in egg custard and attained 37% post larval production compared to 20% in the control. Interestingly, in the present study when glucan with bacterin preparation was administered microencapsulated, the per cent post larval production has increased to 47.3% with an over all improvement in the health of the animal. The reduced survival rate observed in the tanks with continuous application would probably be due to the over dosage of glucan. Sung and coworkers (Sung et al. 1994) studied the protective effect of glucan against *Vibrio* infection in *Penaeus monodon* and observed shrinkage of gill tissues when treated with higher concentrations.

Application of the preparation, once in seven days reduced the total culture period of the larvae from 33 days to 30 days, which could invariably reduce the total cost of production also. In addition to the growth rate, total length and wet weight of larvae were also higher in the animals fed with microencapsulated glucan with bacterin preparation once

in 7 days. It has to be acknowledged that comparatively larger size of larvae would enhance growth rate under farm condition.

In almost all hatcheries of India, partial or total loss of larvae is a regular occurrence. The mortality is generally associated with the recurrence of bacterial diseases caused by *Vibrio* (Sindermann 1977, Singh 1990). The mortality problem supports the need for improved larval rearing techniques to ensure consistent survival rates at least in the range of 30 – 40% so that hatchery operation can be more viable commercially. In the present study the most interesting observation to be highlighted was that the enhancement in post larval production and growth rate could be attained in a condition where many of the water quality parameters were beyond the permitted level. Eventhough there was no significant difference in the water quality parameters between the tanks, the ammonia and nitrite concentrations were well above the permitted level (Sebastian 1996). However, comparatively better survival and post larval production was observed in the tank administered with the preparation once in 7 days. Also *Vibrio* in the system was relatively higher in all tanks. The administration of the preparation once in 7 days appeared to have facilitated the immune system of the larvae to overcome the environmental stress and the possible *Vibrio* invasion to attain a better growth and survival rate.

**Table. 4.1. Ingredients used for the preparation 250 gm glucan with bacterin microcapsule**

| Sl. No | Ingredient | Quantity |
|---|---|---|
| A | Dried Prawn Powder | 175 gm |
| B | Potato Starch | 75 gm |
| C | Glucan | 0.5 gm |
| D | Bacterins | 25 ml each |

**Table 4.2. Response of larvae of *Macrobrachium rosenbergii* to the administration of microencapsulated glucan with bacterin preparation**

| Parameter | Control Tank | Experimental Tanks | |
|---|---|---|---|
| | Without administration Tank # 1 | Continuous administration Tank # 2 | Administration at 7 d interval Tank # 3 |
| Initial Stocking (No) | 4,10,000 | 4,10,000 | 3,70,000 |
| Day on 1st Post larvae appeared | 23 | 22 | 20 |
| Specific growth rate (% Dry weight $day^{-1}$) | 3.1 | 9.3 | 10.7 |
| Average length of PL (cm) ($p<0.05$) | 1.036 ± 0.095 | 1.096 ± 0.143 | 1.152 ± 0.112 |
| Average wet weight of PL (mg) ($p<0.05$) | 5.289 ± 1.556 | 7.338 ± 1.974 | 7.743 ± 2.440 |
| Total day of culture | 33 | 32 | 30 |
| No of Post larvae harvested | 99,000 | 1,25,000 | 1,75,000 |
| % Post larvae production | 24.1 | 30.1 | 47.3 |

**Table. 4.3. Physico chemical quality of rearing water of control and experimental tanks**

| **Parameter** | **Control Tank** | **Experimental Tanks** | |
|---|---|---|---|
| | Without administration Tank # 1 | Continuous administration Tank # 2 | Administration at 7 d interval Tank # 3 |
| pH ($p = 0.243$) | 7.32 – 7.52 | 7.28 – 7.52 | 7.19 – 7.40 |
| Salinity (ppt) | 12 - 14 | 12 - 14 | 12 – 14 |
| Ammonia (ppm) ($p<0.05$) | 0.32 – 0.95 | 0.53 – 1.39 | 0.36 – 1.48 |
| Nitrite (ppm) ($p<0.05$) | 0.01 – 0.11 | 0.02 – 0.1 | 0.02 – 0.09 |
| Nitrate (ppm) ($p<0.05$) | 0.68 – 0.81 | 0.62 – 0.74 | 0.61 – 0.72 |
| Alkalinity (mg $CaCO_3/l^{-1}$) ($p<0.05$) | 60 - 80 | 70 - 75 | 65 – 80 |
| Total Hardness (mg $CaCO_3/l^{-1}$) ($p<0.05$) | 1800 – 2800 | 1750 - 2725 | 1700 - 2750 |

**Table. 4.4. Microbial quality of rearing water and animal samples**

| Parameter | Control Tank | Experimental Tanks | |
|---|---|---|---|
| **A. Water** | Without administration Tank # 1 | Continuous administration Tank # 2 | Administration at 7 d interval Tank # 3 |
| Total plate count (cfu. $ml^{-1}$) ($p<0.05$) | $1.7x10^5 - 5.8x10^6$ | $1.3x10^5$ – $3.8x10^6$ | $4.6x10^5$ – $1.5x10^7$ |
| Total vibrio count (cfu. $ml^{-1}$) ($p<0.05$) | $1x10^3 - 2.3x10^4$ | $2x10^3$ – $4.3x10^4$ | $1.5x10^3$ – $4.3x10^4$ |
| **B. Animal** | | | |
| Total plate count (cfu $g^{-1}$) ($p<0.05$) | $1.8x10^6 - 3x10^7$ | $6x10^5$ – $2.2x10^7$ | $1x10^6$ – $8.3x10^7$ |
| Total vibrio count (cfu $g^{-1}$) ($p<0.05$) | $1.5x10^3 - 5.7x10^4$ | $4x10^3$– $7.7x10^4$ | $2x10^3 - 5.7x10^4$ |

# CHAPTER 5

# CONCLUSION

# &

# THE NEED FOR FUTURE RESEARCH

*Macrobrachium rosenbergii* hatcheries in India incur financial loss due to low ~~per cent~~ post-larval production. Principal reason for the lower rate of larval production in hatcheries is the inadequacy of the technology available at present for disease management. Currently disease ~~management is~~ mainly ~~carried out~~ through antibiotics administration. Their indiscriminate use is likely to cause serious environmental problems. As part of the strategy built up to tackle such issues, most of the developed countries have banned the use of antibiotics in aquaculture, and started looking forward to augment organic shrimp production. At this stage, as Bachere and coworkers (Bachere et al (1995) pointed out, there is an urgent requirement to maximize the immuno-competence of the cultured stock whilst minimizing the use of therapeutic chemicals. Selective breeding programmes and the use of genetically modified strains are still a long way to come into practical level for an ethically acceptable and commercially viable means of reducing the problem posed by epidemics. It is not surprising, therefore, that there has been growing interest in finding ways to protect the cultured stock prophylactically in a manner conceptually equivalent to the use of immunostimulants and vaccines now routine for humans and live stock.

Present work is aimed at development of an appropriate microbial technology for protection of larvae of *Macrobrachium rosenbergii* from diseases and to increase survival rate in hatcheries. Application of immunostimulants to activate the immune system of cultured animals against pathogens is the widely accepted alternative to antibiotics in aquaculture. An immunostimulant may be defined as an agent, which stimulates the nonspecific immune mechanism when given alone, or the specific mechanism when given with an antigen. Many different types or groups of immunostimulants have been reported in aquaculture among which the most important one is glucan. Therefore a

research programme entitled "Extraction of glucan from *Acremonium diospyri* and its application in *Macrobrachium rosenbergii* larval rearing system along with bacterins as microspheres" was under taken with the following objectives:

1. Development of aquaculture grade glucan from *Acremonium diospyri*
2. Development of microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*
3. Development of a microencapsulated glucan with bacterin preparation for the enhanced production of *Macrobrachium rosenbergii* in larval rearing system

Even thought there are many studies elaborating the immunostimulatory potential of glucan in aquaculture, its use is still in debate mainly because of the non-immunostimulatory property reported by hardly any publications. The immunostimulatory potential of glucan depends mainly on the structure, molecular weight, degree of branching and solution-conformation of β –glucan, which in turn depends on the method of extraction. Strikingly the above decisive factors were not discussed in literature relating to aquaculture and it is not possible to conclude the immunostimulatory potential of glucan without its structural characterization. Therefore in the present study the impact of extraction method on the structure and immunostimulatory potential of glucan yielded has been evaluated. Glucan was extracted by following two different methods such as alkali-acid hydrolysis (AIBG) and sodium hypochlorite oxidation and dimethyl sulphoxide extraction method (ASG). Further modifications were made to AIBG to develop an aquaculture grade glucan by employing water or ethanol extraction and further purification by sonication.

The structural characterization of *Acremonium* insoluble beta glucan (AIBG) and its water extracted (AIBG-WENS) and ethanol extracted (AIBG-EENS) counterparts were accomplished by NMR studies whereas the structural characterization of *Acremonium* soluble glucan (ASG) was achieved by NMR and FT-IR spectroscopy. The efficacy of the preparations in activating a non-specific immune system in crustaceans was quantified based on the changes in total haemocyte count, proPhenoloxidase (proPO)

activity and reactive oxygen intermediates (ROI) using *Fenneropenaeus indicus* as the animal model.

The major stumbling block in the commercialization of the aquaculture grade glucan manufacturing technology is the large quantity of wastewater generated during the process of extraction. This was addressed by studying the physico chemical character of wastewater and developing a consortium for its treatment.

Development of appropriate vehicles for delivery of therapeutics, immunomodulants and nutrients to finfish and shellfish larvae has been a challenge to aquaculture scientists. This was experienced in the present study also, which necessitated the development of a microencapsulated drug delivery system. The major offshoot of this work was the realizations that the chitosan, the wall material used for the microcapsule can effectively prevent the growth of pathogenic *Vibrio* spp. isolated from larval rearing system of *Macrobrachium rosenbergii*. Subsequently glucan and bacterins were delivered safely to the intestine of larvae by employing microencapsulation technique and evaluated its impact in terms of total days required for the emergence of post larvae, total post larvae produced, length, weight and specific growth rate. Importance of regulating frequency of administration of the preparation was also examined in this study.

Contributions of this study are as follows:

### 5.1 Comparative efficacy of soluble and insoluble glucan from *Acremonium diospyri* as immunostimulants

In this segment the structural and immunostimulatory potential of two preparations, *Acremonium* insoluble β glucan (AIBG) extracted by the method of alkali acid hydrolysis and *Acremonium* water soluble glucan (ASG) extracted by sodium hypochlorite oxidation and dimethyl sulphoxide extraction were compared. The structural evaluation indicated that the major fraction obtained in AIBG contained a β-(1,3)-D-glucan backbone while the major fraction of ASG was identified as α-(1,3)-D-glucan. The immunostimulatory potential of these preparations was investigated in *Fenneropenaeus indicus* by

administering through feed for seven days. Total haemocyte counts (THC) and activities of proPhenoloxidase (proPO) and reactive oxygen intermediates (ROI) in the blood were measured on 1st and 6th day after completion of 7-day schedule of administration of the glucans. On the 6th day of post experimental feeding proPO and ROI were found to be significantly higher in animals fed with *Acremonium* insoluble β-glucan (AIBG) compared to those fed with *Acremonium* soluble glucan (ASG) and the control group maintained on normal diet. The study reveals that the alkali acid hydrolysis is suitable for extracting β-(1,3)-D-glucan from *Acremonium diospyri*, which can then be used as a potential immunostimulant in shrimps. Glucan isolated from *Acremonium diospyri* as β-(1-3)-D-glucan using the alkali hydrolysis method exhibited immunostimulatory property in shrimp. In addition, detailed structural characterization of the isolated glucans from both sources clearly enabled better understanding of the importance of glucan structure relative to immunostimulatory properties in shrimp.

### 5.2 Effect of sonication and solvent extraction of *Acremonium* insoluble β-glucan (AIBG) on its immunostimulatory property

In this work, the impact of various modifications to alkali acid hydrolysis method of glucan extraction on immunostimulatory potential was examined using *Fenneropenaeus indicus* as animal model. Two major groups of *Acremonium* insoluble β-glucan (AIBG) that differed in the solvent used for removing lipids were further subdivided as sonicated and non-sonicated and their immunostimulatory properties were investigated. The results indicated that partially purified water extracted isolate (AIBG-WENS) resulted in better immunostimulation than the ethanol extracted counter part (AIBG-EENS) or the sonicated preparations including water extracted sonicated isolate (AIBG-WES) and ethanol extracted sonicated glucan (AIBG-EES). These results suggest that high purity glucans are not necessary for aquaculture purpose as partially purified ones give similar or better performance with lower production costs.

### 5.3 Characterization and treatment of effluent generated during glucan extraction

The anticipated stumbling block in the commercialization of the glucan extraction technology in aquaculture is the high quantity of wastewater generated during extraction process. To find an appropriate wastewater treatment process a preliminary screening, with soil samples collected from different locations was conducted to develop microbial consortia, which could use the organics in the wastewater generated during the extraction of *A*IBG-WENS as the sole source of carbon and energy. Among the microbial consortia developed POAB was able to degrade approximately 70% of the waste within 6 days. Fluorescent microscopy demonstrated that the consortium formed biofilms within a short period facilitating its use in biofilm based treatment systems. Further studies are needed to validate the performance of the consortium in a large-scale treatment plant and its kinetics.

### 5.4 Bacterins

Apart from the glucan, inactivated bacterial preparation (Bacterins) also has been identified as one of the microbial technologies for the protection of larvae of *M. rosenbergii* from Vibriosis. The bacterins were prepared from the pathogenic strains of *Vibrio alginolyticus* and *Vibrio harveyi* isolated from diseased *Macrobrachium rosenbergii* larval samples. The inactivation was achieved by using formalin at a concentration of 0.2% (v/v) and the inactivated bacterins were used along with the glucan.

### 5.5 Chitosan based microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*

Major impediment in the medication of the larvae of *Macrobrachium rosenbergii* is the lack of a commercially viable and scientifically established drug delivery system. In this context a comparatively inexpensive and easy to prepare microcapsule for oral delivery of drugs specifically to larvae of *Macrobrachium rosenbergii* was developed using chitosan as the wall material. The chitosan was selected as the wall material because of its favorable characteristics like gelation on contact with counter anions, formation of biofilm soluble in acidic pH, its susceptibility to digestive enzymes and most

significantly its antimicrobial properties. Two types of chitosan-coated microcapsules were prepared using NaOH (MEC-N) and acetone (MEC-A) as the cross-linking agents. They were compared with a micro bound diet in terms of their morphology, total dissolution of nutrients and free amino acid. Amongst them microcapsule MEC-N showed the lowest level of total dissolution of nutrients (23.33 ± 3.4%) during 5h immersion and retained 35% free amino acids at 60 min. During laboratory trials 75% larvae accepted MEC–N capsule while under hatchery conditions it was 50–60%. The study suggested that chitosan-based microcapsule MEC-N could be used as a vehicle for delivering drugs to larvae of *M. rosenbergii.*

### 5.6 Antibacterial activity of chitosan against *Vibrio* spp. isolated from *Macrobrachium rosenbergii* larval rearing system

Chitosan is a biocompatible and biodegradable natural polymer with established antimicrobial properties against specific microorganisms. The present study demonstrated its antibacterial activity against 48 isolates of *Vibrio* species from prawn larval rearing systems. The antibacterial activity had a positive correlation with the concentration of chitosan and showed a maximum activity at 1% concentration. Therefore this concentration was selected for the preparation of microcapsules, as it could prevent colonization of pathogenic vibrio on the surface of the capsule and subsequent degradation of the microcapsules. This work opens up avenues for using chitosan as a prophylactic biopolymer for protecting prawn larvae from vibriosis

### 5.7 Microencapsulated glucan with bacterin preparation for increased seed production in *Macrobrachium rosenbergii* larval rearing system

*Acremonium* insoluble beta glucan and bacterins were microencapsulated using chitosan as the wall material and administered to the larvae of *Macrobrachium rosenbergii* in two modes, daily and at once in 7-day interval. The batch of larvae fed once in seven days performed better than either the control or the batch fed daily. This was measured in terms of overall survival and metamorphosis, the number of days required for emergence of post larvae, length, weight and specific growth rate. Based on the results it is

concluded that application of microencapsulated glucan and bacterins once in seven days will enhance the production of quality seed of *M. rosenbergii*

Concisely the objectives accomplished suggest the importance of the extraction method of glucan on its immunostimulatory property. It is hoped that this information will be useful to conclude the debate, which has been going on in the immunostimulatory potential of glucan in aquaculture. The study clearly indicated that the glucan would provide the required immunostimulation in aquaculture only if the following conditions were retained.

1. An appropriate extraction method, especially the modified alkali-acid hydrolysis is followed (as standardized in this programme).
2. The interval of application of glucan may be fixed as once in 7 days.
3. The dosage of glucan may be at a concentration of 2g $Kg^{-1}$ feed at a time.

Another technology developed in this study was the microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*. The study indicated that the microcapsules could safely delivery molecules having molecular weight higher than free amino acids to the intestine of the *M. rosenbergii* larvae. The possibility of extending the capsule for the delivery of drugs to the larval stages of other crustaceans may be worked out. The chitosan, the wall material of microcapsule, can independently be used as prophylactic agent also for the control of vibriosis.

Based on the results of field trials of microencapsulated glucan with bacterin preparation, it is concluded that application of the microencapsulated preparation at a concentration of 25g per million larvae once in seven days will enhance the production and quality seed of *Macrobrachium rosenbergii*.

Higher percentage of larval production obtained by the application of microencapsulated glucan with bacterin preparation ensures economic viability in the *Macrobrachium rosenbergii* hatcheries and help Indian aquaculture to attain the target of 50,000 mt of *M.*

*rosenbergii* production by 2010 with minimum investment. Moreover the research outcome of this work would give additional velocity to the growing organic aquaculture concept of our country.

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*** As available in original citation**

# Appendix

## Publications and patents arising out of this thesis

**Publications**

1. **Anas A**, Paul S, Jayaprakash NS, Philip R, Singh ISB (**In press**) Antimicrobial activity of chitosan against vibrios from freshwater prawn (*Macrobrachium rosenbergii*) larval rearing systems. Diseases of Aquatic Organisms

2. **Anas A**, Philip R, Singh ISB (Communicated) Chitosan based microencapsulated drug delivery systems for larvae of *Macrobrachium rosenbergii*

3. **Anas A**, Lowman D, Williams DL, Sajeevan TP, Philip R, Millen S, Singh ISB (Communicated) Comparative Efficacy of Soluble and Insoluble glucans extracted from *Acremonium diospyri* as Immunostimulants in Indian White Prawn, *Fenneropenaeus indicus*

4. **Anas A**, Lowman D, Williams DL, Sajeevan TP, Philip R, Millen S, Singh ISB (Communicated) Effect of sonication and solvent extraction of *Acremonium* insoluble β-glucan (*A*IBG) on its immunostimulatory property in *Fenneropenaeus indicus*

5. **Anas A**, Philip R, Singh ISB (Communicated) Microencapsulated glucan with baterin preparation for increased seed production in *Macrobrachium rosenbergii* larval rearing system

**Patents**

1. Aquaculture grade glucan from *Acremonium diospyri*

2. Chitosan based microencapsulated drug delivery system for the larvae of *Macrobrachium rosenbergii*