Process Optimization for Mass Production of Marine Yeast *Candida* sp. S 27 and its Nutritional Characterization

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JULY 2009

.........................at the feet of Saint Antony of Padua

DECLARATION

I hereby do declare that the thesis entitled **"Process Optimization for Mass Production of Marine Yeast *Candida* sp. S 27 and its Nutritional Characterization"**, is a genuine record of research work done by me under the supervision of Dr. Rosamma Philip, Senior Lecturer, School of Marine Sciences, Cochin University of Science and Technology, Cochin-682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title of any University or Institution.

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CERTIFICATE

This is to certify that the thesis entitled **"Process Optimization for Mass Production of Marine Yeast *Candida* sp. S 27 and its Nutritional Characterization"** is an authentic record of research work carried out by Ms. Simi Joseph P. under my supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy and no part thereof has been presented before for the award of any degree, diploma, or associate ship in any University.

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Chapter 1

General Introduction

The alarming rate of population growth and rapidly dwindling natural resources has resulted in scarcity of food, specifically protein shortages in third-world countries since the latter half of 20th century. The main source of protein for human and animal consumption is from the agricultural sector, where the production is vulnerable to diseases, fluctuations in climatic conditions and deteriorating hydrological conditions due to water pollution. Thus to alleviate the food shortages due to growing imbalance between food production and increasing population in developing countries, Single Cell Protein (SCP) production has evolved as an excellent alternative. The dried cells of microorganisms produced commercially as source of protein and used as human food or animal feed are collectively known as 'microbial protein' or 'single cell protein'. The criterion for coining this term is the single celled habit of microorganisms used as food and feed. Therefore, in the light of protein shortage; microorganisms offer possibilities for protein production. They can be used to replace totally or partially the valuable amount of conventional vegetable and animal protein feed.

The use of microbes as a food source may not be acceptable world wide, but the idea is certainly an innovative way to solve global food scarcity. The consumption of microorganisms by man and animals is not a revolutionary new suggestion. For thousands of years man has consumed either intentionally or unintentionally the microbial biomass along with products such as alcoholic beverages, cheese, yoghurt and soya sauce, which is responsible for their production.

Organisms used

Bacteria, actinomycetes, yeasts, algae, moulds and fungi are widely used in single cell protein production. However, among all sources of microbial protein, yeast has attained global acceptability and has been preferred over bacteria for SCP production especially for human consumption because they are more familiar to humans in foods like bread or beer.

Advantages of SCP

Roth (1982) has described a number of advantages of single cell protein or bioprotein, over plant and animal sources of protein which include: Short generation time (algae, 2-6 hr; yeast, 1-3 hr; bacteria, 0.5-2 hr i.e. high productivity), easily modifiable genetically (eg. for composition of amino acids); flexibility in the use of substrates, high proportion of cell mass as protein (about 43-85 % in dry mass), a good profile of desirable amino acids, good performance in feeding livestock and no toxic or carcinogenic compounds.

One of the features of SCP is that they can be produced from the by products from human and animal activities (uneaten food, non-food grade organic materials) which are currently discarded or pose an environmental hazard. SCP can be produced through fermentation technology so as to produce specific products or nutrients, including carotenoids, amino acids, immunostimulants and growth promoters.

Yeast as SCP

Yeasts occupy a unique place in science and technology: being a unicellular microorganism readily amenable to cultivation and to manipulation to reflect process needs. Thus in the wake of considerable advancement in biotechnology, yeast based single cell protein production stands as the best alternative to supplement the requirements of food and feed-grade protein, vitamins and amino acids.

The need to design feasible and financially viable processes and the utilization of low cost industrial wastes as raw materials for edible yeast biomass production is extremely important, as it gives a solution to the management of these wastes and the environmental pollution caused by their disposal. Moreover apart from providing alternative sources of food for humans or animals and reducing pollution, yeast based SCP production using waste materials is attractive to manufacturers as it leads to increased profits from the use of low cost raw materials, value addition and reduction of waste treatment costs. Marine yeasts are reported to be truly versatile agents of biodegradation (De souza and D’souza, 1979; Kobatake et al., 1992). Utilization of this potential in the biotransformation of cheap raw materials or waste matter into value added products will be a highly rewarding endeavour. Biotransformation and

the partial conversion of raw material into yeast biomass (single-cell protein) are highly significant due to the nutritional quality of yeast and its possible utilization as animal or aquaculture feed (Rhishipal and Philip, 1998). Among SCP, yeasts have been the most widely used among aquafeeds (Tacon, 1994).

Yeasts also have immunostimulatory properties by virtue of their complex carbohydrate and nucleic acid components (Anderson et al., 1995). Both cellular and humoral responses have been induced by dietary yeast, depending on the environmental conditions. Yeasts have been used to improve the growth rate of *Litopenaues vannamei* larvae (Intriago et al., 1998). Probiotic properties of yeasts like *S. cerevisiae* have been reported and displayed as the ability to survive in the gastrointestinal tract and interact antagonistically with pathogens such as *E. coli*, *Shigella* and *Salmonella*. *S. boulardii*, a thermophylic, non-pathogenic yeast has been used for more than 50 years as a livestock feed supplement as well as therapeutic agent for the treatment of a variety of gut disorders like diarrhoea (Bekatorou et al., 2006). Other yeasts commonly used in animal feeds as probiotic additives are *Candida pintolopesi* and *C.saitoana* (Bovill et al., 2001; Leuschner et al., 2004). In older fish, dietary yeast stimulates metabolism and growth (Gatesoupe, 2007).

Providing an acceptable feed with reliable availability, proper size and nutrient composition remains a major challenge in aquaculture. It is becoming increasingly evident that the development of low-cost, high quality protein food stuff is crucial for the future success of the aquaculture industry (Rumsey, 1978). The main protein sources used in aqua feeds are fish meal for which the major constraints are their increasing cost and instability in supply in the long term. Biotechnological advances in the past few decades have provided methods and products that can be used to assist in the successful replacement of conventional feed sources by alternate abundant protein sources (microbial or single cell protein). The use of microbial protein to replace part of the protein required in fish feed could be considered a promising and innovative solution to this problem (Martin et al., 1993). Successful experimental trials indicated that yeast SCP served as inexpensive protein supplement in diets for pigs (Tegbe and Zimmerman, 1977) and in feeds for rabbits (Faria et al., 2000) and egg laying pullets (Ayanwale et al., 2006).

Nutritional aspects

The commercial value of SCP is linked to its protein content which in yeasts can account for up to 50 % of the dry weight, the remaining being constituted by lipids, polysaccharides etc. They can also supply the feed with vitamins, minerals and other components, which could stimulate the disease resistance of fish (Raa, 1990). Moreover yeasts serve as potential producers of enzymes, polysaccharides, organic acids, peptides, pigments, sterols etc. Yeasts as single cell protein sources have as advantage:1. their larger size, so easier to harvest 2. lower nucleic acid content 3. long history of use as food and so better public acceptance 4. high vitamin content and 5. ability to grow on substrates of low pH, which reduces bacterial contamination of the medium (Mitchel, 1974). The main nutritional contribution of SCP in animal feed is its high protein content and it varies depending upon the kind of microorganisms and substrates used for production. The mean crude protein in dry matter of yeasts, on conventional substrates lies between 50 and 60 percent. Because of high protein and fat content, the contribution of carbohydrates to the nutritional value of SCP is not of prime importance. Although yeast SCP products are deficient in the aminoacid methionine, they are a good source of vitamins; especially B vitamins (thiamine, riboflavin, biotin, niacin, pantothenic acid, pyridoxine, folic acid and p-amino benzoic acid) besides choline, streptogenin and glutathione (Frazier and Westhoff, 1990). However, the yeasts grown on molasses have high concentration of methionine (Bhalla et al., 1999).

Even though yeasts are the most commonly used source of microbial protein, work on marine yeasts is very much limited. The terrestrial yeasts have been receiving great attention in science and industry for over hundreds of years because their potential has been very well exploited. However, only in recent years, it has been found that marine yeasts have wide applications in aquaculture. It has been proved that marine yeasts serve as a high quality, inexpensive nutrient source for cultured shrimps (Sajeevan et al., 2006; 2009).The halotolerant property of marine yeast is an added advantage when selected as a candidate species as feed supplement in aquaculture because for baker's or brewer's yeast the seawater or the rearing water

for shrimp culture is hyperosmotic which cause cell rupture leading to water quality deterioration.

Bioprocess Technology

A good knowledge of growth conditions and medium formulation are necessary to obtain enough biomass in an application point of view. In bioprocess development, optimization of media represents a significant cost and time factor (Kennedy et al., 1994).

Biomass production is affected by factors like yeast strain, concentration and type of substrate, nitrogen source, temperature, inoculum size etc; and all these need to be standardized for the successful generation of biomass.

The development of an economic culture medium is necessary for large scale production of biomass. The constituents of the medium must satisfy the basic requirements for cell biomass and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance. The carbon substrate has a dual role in biosynthesis and energy generation, with carbohydrates being the usual carbon source for microbial fermentation processes.

Media prepared using pure compounds in defined proportions are called synthetic or defined. Alternatively media can be formulated using ingredients of natural origin which are not completely defined chemically such as blood, meat extract, malt extract, molasses, peptone, cottonseed flour etc., referred to as complex media. Defined media are usually preferred for research since they permit one to determine the specific requirements for growth and product formation by systematically adding or eliminating chemical species from the formulation. Other advantages of a defined medium include its reproducibility, low foaming tendency, translucency, and the relative ease of product recovery and purification.

The expense associated with the recovery of microbial cells from a spent medium is directly related to the physical and chemical characteristics of the medium. The complexity of the medium makes difficult the recovery of the product. Each step in recovery and purification usually involves a small to large loss of the

fermentation product. A complicated recovery and purification procedure caused by the chemical and physical nature of the fermentation medium thus can add up to considerable loss of fermentation product. By simplifying or changing the fermentation medium, the recovery becomes easier with less loss of product.

Fermentation yields are closely tied to the medium being used, and a change in the medium may well cause decreased fermentation yields. Thus, we must balance the total yields that can be obtained from various media against the losses that will occur during product recovery because of the characteristics of the media. In other words, a relatively lower yielding medium may well be economically more acceptable, if the final recovered yields of product are greater than for a higher yielding medium with poor product recovery characteristics, thus making complex media the preferred choice in industrial fermentations (Casida, 1968).

Optimization

To be economically feasible, it is necessary to engineer optimum culture conditions for maximum biomass productivity. The conventional method of medium optimization, involve the 'one factor at a time' technique, i.e. varying one parameter while keeping all the other factors at constant level. The disadvantages of this single variable optimization are that the approach is time-consuming, depends on many analyses and often does not bring about the effect of interaction of various parameters (Cochran and Cox, 1992).

In order to overcome this major problem, optimization studies are done using Response Surface Methodology (RSM) which is a combination of statistical and mathematical techniques widely used to evaluate the effects of several factors that influence the responses by varying them simultaneously in limited number of experiments. RSM and factorial design are important tools to study the effect of both the primary factors and their mutual interactions on the concerned response and thus determine the optimal process conditions (Box et al., 1958). Experimental designs for optimization have been commonly used for the optimization of multiple variables with minimum number of experiments (Leiro et al., 1995; Kalil et al., 2000; Montgomery, 2001).

The application of statistical experimental design techniques in bioprocess development can result in improved product yield, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall cost (Elibol, 2004).

In this context the present study was undertaken with the following objectives:

1. Screening of marine yeasts based on morphological and biochemical properties for the selection of a suitable strain for biomass production.
2. Evaluation of nutritional quality of the selected yeast.
3. Optimization of salinity, temperature and pH for biomass production by the selected marine yeast.
4. Optimization of media composition for yeast biomass production using defined and complex media.
5. Scale up and testing the efficacy of defined and complex media in yeast biomass production

Chapter 2

Molecular, Biochemical and Nutritional Characterization of Marine Yeast *Candida* sp. S 27

2.1 Introduction

Production of SCP by mass culture of microorganisms is yet to take momentum at industrial scale and deserve much attention to solve the problem of starvation in the coming decades. The criteria for microorganisms to be used as food or feed include 1.the organism must be genetically stable, non toxic and grow rapidly on a simple non specific medium.2. it should have high nutritional or vitamin content and should be edible to human and other animals. The organisms should also utilize the energy source without producing any side effects and any undesirable effects. 3. it should be easy to separate the cells from the medium and the product must have good quality and composition. Among the various groups of microorganisms used to produce SCP, yeasts are perhaps the most important groups because yeasts produce many bioactive substances such as proteins, amino acids, vitamins, polysaccharides, fatty acids, phospholipids, polyamines, astaxanthins, β-carotenoids, trehalose, glutathione, superoxide dismutase, chitinase, amylase, phytase, protease, killer toxin etc. which have been receiving much attention for many decades. The main nutritional contribution in either human food or animal feed is its high protein content. Because of high protein and fat content, the contribution of carbohydrates to the nutritional value of SCP is not of prime importance. But a major constraint for yeast as SCP is the thick cell wall which is difficult to digest leading to poor protein bioavailability.

Instability of the biochemical criteria and physiological characters and obscurity in the interrelationship of biochemical tests to the metabolism of the organisms has led to development of new techniques such as fatty acid profiling and rDNA sequencing for taxonomic identification of yeasts, although classical methods based on morphology, physiological and biochemical characteristics are still followed.

2.1.1 Molecular identification

The isolation and purification of DNA is a key step for most protocols in molecular biology studies (Sambrook et al., 1989). Several DNA extraction methods are widely used to isolate DNA from yeast including phenol extraction but they often

involve multiple, time consuming steps including the handling of toxic chemicals (Ausbel et al., 1995). An efficient, inexpensive method for obtaining yeast genomic DNA from liquid cultures or directly from colonies was developed by Harju et al. (2004). This protocol circumvents the use of enzymes or glass beads, and therefore is cheaper and easier to perform when processing large number of samples.

PCR based detection of fungal DNA sequences can be rapid, sensitive and specific (Makimura et al., 1994).The nucleotide sequence of the D1/D2 domain of the 26 S rRNA is sufficiently variable to allow reliable identification of yeast species (Kurtzman and Robnett, 1997). Fell and Kurtzman (1990) reported the nucleotide sequence analysis of a variable region of the large sub unit rRNA for identification of marine occurring yeasts. More recently, the differences in the rRNA internal transcribed spacer have been used to identify yeast species. Coding regions of the 18 S, 5.8 S and 28 S rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships. Between coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2 respectively) which evolve more rapidly and therefore vary among different species within a genus. From the conserved sequences of 18 S and 28 S rRNA genes at the ends of the ITS region two universal primers ITS1 (F) and ITS4 (R) were designed by White et al. (1990). This amplify a fragment of approximately 580bp containing the ITS 1, 5.8S and ITS 2 regions and are widely used for identification purpose. Differentiation of closely related species requires analysis of both D1/D2 and ITS regions (Fell, 2001).

2.1.2 Nutritional / Biochemical composition

The commercial value of single cell protein depends on their nutritional performance. The main nutritional contribution of SCP is its high protein content which varies depending upon the kind of microorganisms and substrates used for production. The mean crude protein content in dry matter of yeasts account for about 50 %. The amino acid composition of a protein primarily determines its potential nutritional value. The protein efficiency ratio and biological value of yeast protein are known to be relatively high (Munro, 1964). Composition of growth medium governs the protein and lipid contents of microorganisms. Yeasts, moulds and higher

fungi have higher cellular lipid content and lower nitrogen and protein contents, when grown in media having high amount of available carbon as energy source and low nitrogen (Litchfield, 1979). The lipid composition of microorganisms is very responsive to changes in the chemical and physical properties of the environment. Among the environmental factors that have been reported to affect the lipid composition of microorganisms are growth rate, composition of the medium, growth temperature, and dissolved O_2 tension in the culture. Since, with most organisms, the bulk of the cell lipids are in membranes, it is likely that these environmentally induced changes in lipid composition are of major physiological significance. Microorganisms contain a diverse range of fatty acid composition which can be useful as a chemotaxonomic tool for classifying species and strains. Fatty acids typically comprise 70-90 % of the lipids in yeast with oleic acid (18: 1 n-9) being the commonest found.

Fatty acids, the simplest of lipids are required for membrane structure, function, transport of cholesterol, formation of lipoproteins etc. Composition of growth medium governs the lipid content of microorganisms. Microbial fatty acid profiles are unique from one species to another. The fatty acids occur as esters in triacylglycerol, phospholipids, glycolipids or sterols in membranes and other cytoplasmic organelles, such as the mitochondria, plasmalemma, endoplasmic reticulum, nuclei, vacuoles, spores and lipid particles. The 14: 0 fatty acids are only seen as trace fatty acyl residues. The microbial identification system based on fatty acid methyl ester (FAME) analysis has been used in laboratories for the identification of clinical yeast strains (Peltroche-Liacsahuanga et al., 2000). The system analyses long-chain fatty acids containing 9–20 C atoms, identifying and quantifying the FAMEs of microorganisms. The database library searches for fatty acid composition, compares the FAME profile of the isolate with those of well-characterized strains and defines the most likely species of the isolate. Fatty acid composition of cold-adapted carotenogenic basidiomycetous yeasts was studied by Libkind et al. (2008). Total fatty acids of six yeast species isolated from the temperate aquatic environments in Patagonia ranged from 2 to 15 % of dry biomass. Linoleic, oleic, palmitic and α-linolenic acids were the major fatty acid constituents,

which accounted for as much as 40%, 34%, 13% and 9% of total fatty acids, respectively. The proportion of each varied markedly depending on the taxonomic affiliation of the yeast species and on the culture media used. The high percentage of polyunsaturated fatty acids (PUFAs) found in Patagonian yeasts, in comparison to other yeasts, is indicative of their cold-adapted metabolism.

Yeast proteins are easily digestible compared to those from bacteria. Chemical analysis of microorganisms tested for SCP reveal that they are comparable in amino acid content to the plant and animal sources with the exception of sulphur amino acid methionine which is low in some SCP sources, especially yeasts. However, this can be alleviated by culturing yeasts on molasses (Bhalla et al., 1999). The only species of yeast fully acceptable as food for humans is *S. cerevisiae* (baker's and brewer's yeast) (Bekatorou et al., 2006).

The majority of the SCP are either deficient in one or more amino acids or they suffer from an amino acid imbalance (Tacon and Jackson, 1985; Kiessling and Askbrandt, 1993). The supplementation of yeast-based diets with the deficient amino acids was shown to have beneficial effects on fish growth (Nose, 1974; Spinelli et al., 1979; Murray and Marchant, 1986).

Another concern with SCP is their high concentration in nucleic acids, ranging from 5 % to 12 % in yeast and 8 % to 16 % in bacteria (Schulz and Oslage, 1976). In rapidly proliferating microbial cells, RNA forms the bulk of nucleic acids. The RNA content of yeast cells is known to be dependent on the culture conditions and C/N ratios. The marine yeasts with high levels of nucleic acids could be used as a feed to marine animals because some of them can produce uricase which convert uric acid, the toxic intermediate of nucleic acid catabolism into the non-toxic allantoin.

The nutritional value of the yeast depends also on the concentrations of other micronutrients such as sterols, vitamins and minerals. Yeasts have usually high concentrations of sterols (typically 1-10 % of total lipids) which are required for growth and survival of molluscs (Brown et al., 1996).

Proteins of *Kluyveromyces marxianus* obtained by culturing on whole whey along with the unconsumed whey products yielded a SCP with high crude protein and low ash content. The product was also rich in sulphur-containing amino acids (Galvez et al., 1990; Singh et al., 1991). Sanderson and Jolly (1994) reported that the genus *Phaffia* contained 23 % fat and 22 % protein. Yeasts being a rich source of proteins and B-complex vitamins have been used as a supplement in animal feed to compensate for the aminoacid and vitamin deficiencies of cereals and are recommended as a substitute for soyabean oil in diets for fowl (Gohl, 1991). The profile of HPLC analysis from the extract of marine yeast strain YF12b grown in YPD medium showed that it synthesized vitamin C from glucose (Chi et al., 2006).

Single cell proteins, including yeast and bacteria, have been viewed as promising substitutes for fish meal in fish diets (Li and Gatlin, 2003). Brewers yeast could replace up to 25-50 % of fish meal protein without adversely affecting the growth of lake trout (Rumsey et al., 1990), rainbow trout (Rumsey et al., 1991) and sea bass (Oliva-Teles and Goncalves, 2001).

In brewers yeast, nucleic acid nitrogen is present mostly in the form of RNA and represents about 20 % to 25 % of the nitrogen (Rumsey et al., 1991). Sakai et al. (2001) reported that the nucleotides from brewers yeast RNA were capable of enhancing the phagocytic and oxidative activities of kidney phagocytic cells, serum lysozyme in common carp as well as resistance to *Aeromonas hydrophila.* Burrells et al. (2001) also reported that dietary nucleotides, extracted from brewers yeast, could enhance resistance to various pathogenic infections in Atlantic salmon. Choudhury et al. (2005) reported that dietary yeast RNA supplementation at 0.4 % reduced mortality by *Aeromonas hydrophila* in *Labeo rohita* juveniles and increased their immunological responses. .

Rishipal and Philip (1998) isolated 33 strains of marine yeasts from the coastal and offshore waters off Cochin. They were inoculated into prawn- shell waste and the protein content of the final product was 70.4 % after the transformation of the waste by *Candida* sp. M15. This finding promises wide application in aquaculture, particularly as a feed supplement and such transformation will facilitate the abatement of pollutant and recycling of waste.

Brown et al. (1996) compared the biochemical composition of seven strains of marine bacteria and six strains of marine yeast, isolated from an Australian waterway with two well studied yeast strains, *Candida utilis* and *Saccharomyces cerevisiae* to assess their nutritional quality for bivalve aquaculture. They found that the marine yeasts *Debaryomyces hansenii* ACM 4784, *Dipodascus capitatus* ACM 4779 and *Dipodascus* sp. ACM 4780 contained 23 %, 32 % and 36 % of crude protein respectively, while terrestrial *Candida utilis* ACM 4774 contained 42 % crude protein. They concluded that high protein content, high levels of carbohydrates and good amino acid composition characterized all the marine yeasts while high levels of saturated fats characterized only some marine yeast. However, all the marine yeast strains lacked the 20:5n-3 and 22:6n-3 polyunsaturated fatty acids (PUFA), which makes them unsuitable as a complete diet for larval culture. They could distinguish yeast and bacteria based on the position of double bond of 18:1 fatty acid; with the double bond at n-9 position for yeast and at n-7 position for bacteria. The amount of carbohydrate ranged from 21 % (*Debaryomyces hansenii*) to 39 % (*Saccharomyces cerevisiae*) of dry weight. Although the nutritional value of polysaccharides from microorganisms is likely to be more related to digestibility than sugar composition per se, the sugar composition could provide an indication of the polysaccharides present.

Moina macrocopa, a potential alternative live food for fish larvae was cultivated using different diets: marine yeasts (*Debaryomyces hansenii* Yeast-14 and *Candida austromarina* Yeast-16) and a commercial diet (*Erythrobacter* sp. Sπ –I) (Kang et al., 2006). They found that the yeast-fed *M.macrocopa* had essential amino acid profiles similar to the documented values for rotifers and Artemia enriched in microalgae and commercial diets. The commercial diet lacked n-3 polyunsaturated fatty acids, whereas PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) though in low amounts, were present in marine yeasts. The result was more pronounced in *Moina* fed with *C. austromarina* which had a high DHA: EPA ratio.

Chi et al. (2007) were successful in selecting *Yarrowia lipolytica* strains with high protein content from yeasts isolated from different marine environments. They

found that seven strains of marine yeasts grown in soy bean cake hydrolysate with 20g/L of glucose for 48 hr at 28 ^{0}C contained more than 41 g protein per 100g of cell dry weight. The highest crude protein content was reported with strain SWJ-1b (47.6 ± 0.7 %).

Glucan

It has been well known that yeast cells have a rigid thick cell wall of about 200 nm in thickness outside the plasma membrane (Ueda and Tanaka, 2000). The cell wall of yeast cells is mainly composed of mannoproteins and β-linked glucans and has β-1,3- and β-1,6-linked glucose and a fibrillar or brush-like outer layer composed predominantly of mannoproteins (Ueda and Tanaka, 2000). However, in general, the animals can not synthesize enzymes which hydrolyze mannoproteins and β-linked glucans. Therefore, it is difficult for yeast cell wall to be attacked in the guts of animals, especially in guts of marine animals.

The cell wall composes an appreciable part of fungal cell mass; where in yeasts it represents about 15 % to 25 % of the dry weight of cell. The composition of yeast cell wall usually comprises of glucan (50 % of which 20 % is alkali soluble), mannan (30 %), protein (10-15 %), lipid (8-9%) and chitin (1-2 %). Yeast cell wall has a complex structure with an outer amorphous layer of mannan, phosphorylated to different degrees in different yeast species, a middle layer of alkali- soluble β –glucan, and an inner rigid layer of alkali-insoluble β –glucan. The latter gives shape and rigidity to the cell i.e. it forms the structural skeleton of yeast cell wall.

There is increasing commercial interest in the polysaccharides of yeast cell wall i.e. their applications in food processing as thickening agents, fat substitutes, as sources of dietary fibre (Seeley, 1977; Dziezak, 1987). The ability of yeast wall glucans to stimulate the immune system (Williams et al., 1992; Jamas et al., 1996) and lower the serum cholesterol level (Robbins and Seeley, 1977), their antitumour activity (Bohn and Be Miller, 1995) and their potential use in cosmetics (Donzis, 1996) are also noteworthy. The mannoproteins exhibit biosurfactant properties (Cameron et al., 1988).

Yeasts have immunostimulatory property by virtue of their complex carbohydrate and nucleic acid components. Immunostimulant is an agent which stimulates the non-specific immune mechanism when given alone or the specific immune system when given with an antigen. Yeast glucan is the most extensively studied of the glucans (Sakai et al., 1999).The immunotherapeutic effects of biological response modifiers such as glucan largely depend on their structure. Most commonly employed method for glucan extraction is the alkali-acid hydrolysis method of Hassid (1941) later redefined by Williams *et al.* (1991) according to which more than 97 % pure form of glucan could be obtained.

In the 1950s and 1960s, β-glucan isolated from the cell wall of *S. cerevisiae* was subjected to investigation by the technique of sugar analysis, i.e., partial hydrolysis, methylation analysis, periodate oxidation, Smith degradation (periodate oxidation, reduction by $NaBH_4$, and subsequent partial hydrolysis) etc. However, the results obtained were rather discrepant (Bell and Northcote, 1950; Manners and Patterson, 1966). Only after finding that in the yeast cell wall several types of *β*-glucans exist (Bacon and Farmer, 1968), detailed fractionation of cell wall components and their characterization was made (Manners et al., 1973). It is believed now that the main component of β-glucan from the yeast cell wall is a slightly branched, high-molecular (1→3)-*β*- D-glucan (Degree of Polymerization (DP) about 1500, molecular weight ca. 240 kDa), with about 3% of β (1→6) branching. Of late, structural elucidation of the yeast glucan is being done with Nuclear Magnetic Resonance (NMR) Spectroscopy (Ensley et al., 1994; Kim et al., 2000; Lowman et al., 2003).

2.2 Materials and methods

2.2.1 Microrganism used

2.2.1.1 Selection of a suitable marine yeast strain for biomass production

Yeasts isolated from the offshore waters of the southwest coast of India and maintained in the Microbiology laboratory of School of Marine Sciences, CUSAT, Kochi were used for the study (Sarlin, 2005). These belonged to about 22 genera (*Candida, Williopsis, Lodderomyces, Debaryomyces, Leucosporidium, Kluyveromyces,*

Aciculoconidia,Oosporidium, Rhodotorula, Schizosaccharomyces, Cryptococcus, Filobasidium, Pichia, Geotrichum, Dekkera, Lipomyces, Hormoascus, Arxioxyma, Saccharomycopsis, Torulaspora, Mastigomyces and *Dipodascus*) with more than 100 strains. Representative strains from these 22 genera were subjected to various tests for the selection of a potent strain for biomass production.

2.2.1.1.1 Based on Growth

Medium used

Malt extract agar

Malt extract	-	17 gm
Peptone	-	3 gm
Agar	-	20 gm
Sea water	-	1000 ml
pH	-	5.5

Preparation of Inoculum

Malt extract agar slants were prepared and sterilized at 121.5 ^{0}C for 15 minutes in an autoclave. The selected yeast strains were streaked on to malt extract agar slants. Incubation was done at room temperature (28± 2^{0}C) for 24 hours. The cells were harvested using 30 $^0/_{00}$ sterile sea water. Optical density (OD) of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan). OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

Inoculation and Incubation

Malt extract broth

Malt extract	-	17 gm
Peptone	-	3 gm
Sea water	-	1000 ml
pH	-	5.5

Malt extract broth (10 ml) was prepared in triplicate and sterilized at 121.5 ^{0}C for 15 minutes in an autoclave. 10 µl from 1 OD (1.829 x 10^7 cells/ml) cell

suspension was inoculated into the tubes so that the initial OD of the culture medium was 0.001. The tubes were incubated at room temperature for 48 hours.

Estimation of growth

Growth was determined by measuring the optical density of the culture suspension at 540 nm in a UV-VIS spectrophotometer.

2.2.1.1.2 Based on Morphology/ Size

Wet mounts of the yeasts were made and examined the morphology and size under Microscope. Filamentous /non filamentous growth was observed by preparing slide cultures. Small blocks of medium (malt extract agar) 50 mm x 50 mm were cut from a poured plate and transferred them to the centres of sterile slides. Inoculated the edges of each block with yeast cultures and cover glasses were applied. Incubated in petri dishes supported by pieces of glass rod over filter papers soaked in 10 % glycerol in water to prevent drying up. When growth was adequate, the cover glass was removed from the slide culture. Fixed the growth on slide and cover glass with ethanol, placed a drop of lactophenol cotton blue on each and the slide was mounted with a clean cover glass and examined under the microscope.

2.2.1.1.3 Enzyme production

The yeast strains were checked for production of various enzymes viz, amylase, gelatinase lipase and catalase. Nutrient agar (Peptone- 5g, Beef Extract – 3g, pH -7.2, Agar -20 g, 50 % Filtered sea water- 1000 ml) supplemented with starch (1%), gelatin (2%) and tributyrin (1%) were prepared separately. Plating was done and spot inoculation was performed on the various media plated. After incubation at room temperature (28 ± 2 ^{0}C) for 3-5 days, observations were made. Starch agar plates were flooded with Gram's iodine solution and the appearance of a halo zone around the colonies was noted for a positive result. Gelatin agar plates were flooded with mercuric chloride solution (15 % $HgCl_2$ in 20 % Conc. HCl) and the appearance of a halo zone around the colonies was noted for a proteolysis. Lipid agar (tributyrin agar) plates were checked for a clear zone around the colonies, which indicated the production of the enzyme lipase. Effervescence or bubbling by a smear of 24 hr yeast culture on addition of a drop of H_2O_2 indicated the capability to produce catalase.

2.2.1.1.4 Antagonistic activity against bacteria

Antagonistic action of yeasts to certain bacterial prawn pathogens like *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus*, *Aeromonas salmonicida* and *Pseudomonas aeruginosa* was examined by Kirby Baur disc diffusion method. Presence of clearing zone around the disc indicated a positive result.

2.2.1.1.5 Haemolytic assay

Haemolytic activity of the marine yeasts on prawn blood agar was tested to check their non- pathogenicity to cultured prawns (Chang et al., 2000). Approximately 1 ml of haemolymph was collected using a sterile capillary tube and transferred immediately to a sterilized tube containing 0.2ml of citrate-EDTA buffer (0.01M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA dissolved in 20 $^{0}/_{00}$ sea water; pH 4.6) and stained by addition of 133 µl of 3 % (w/v) Rose Bengal (dissolved in citrate-EDTA buffer). It was gently rotated to achieve complete mixing. The basal agar medium (Bactopeptone-10g, NaCl-5g, Bacto agar-15 g dissolved in 1000 ml water; pH 6.8) was prepared and autoclaved at 121 ^{0}C for 15 minutes. It was cooled to 45-50 ^{0}C in a water bath. Aseptically 1ml of the stained haemolymph preparation was added to 15 ml of this prepared basal medium followed by gentle mixing and pouring into a petri dish. The 5 marine yeast strains were spot inoculated on the plate along with a reference pathogenic strain (*Vibrio* sp. MBCS 6). It was incubated at 23 ^{0}C for 48 hours and observed for the result.

2.2.1.1.6 ***Selection of an yeast for biomass production***

Based on morphological and physiological characterization of the marine yeasts, *Candida* sp. S 27 was selected for biomass production.

2.2.2. Characterization of the selected marine yeast (*Candida* sp. S 27)

2.2.2.1 Identification

2.2.2.1.1Based on phenotypic characteristics

Biochemical characterization of the marine yeast strain *Candida* sp. S 27 (MTCC 8176) was done at IMTECH, Chandigarh (Table 2. 3)

2.2.2.1.2 DNA based identification

2.2.2.1.2.1 Yeast genomic DNA isolation

By employing a rapid isolation protocol, yeast genomic DNA was isolated (Harju et al., 2004). 1.5 ml of the overnight culture of yeast grown at 30^0C in YPD (1% yeast extract, 2% peptone and 2% dextrose) was centrifuged at 20000 x g for 5 minutes and the cell pellets were resuspended in 200µl of lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl, 1mM EDTA of pH 8). The tubes were placed in -80^0C freezer for 2 minutes, then immersed in a 95^0C water bath for 1 minute to thaw quickly. The process was repeated once; the tubes were vortexed for 30 seconds. Added 200 µl of chloroform, vortexed for 2 minutes and then centrifuged 3 minutes at room temperature at 20000 x g. the aqueous layer was transferred to a tube containing 400 µl of ice-cold 100 % ethanol. The tubes were allowed to precipitate 5 minutes at room temperature and centrifuged 5 minutes at room temperature at 20000 x g. Supernatant was removed and DNA pellets were washed with 0.5 ml of 70 % ethanol followed by air-drying at room temperature. DNA was resuspended in 20 µl of TE buffer (10 mM Tris, 1mM EDTA of pH 8) and stored at 4^0C for future use. The isolated DNA was quantified spectrophotometrically (A_{260}) and the purity of DNA was assessed by calculating the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}), the value of which determined the amount of protein impurities in the sample. Electrophoresis was done using 0.8 % agarose gel and the DNA marker used was that of 1 kb (NEB) (Fig 2.4).

Concentration of DNA was calculated from the following formula:

Conc. of DNA (µg/ml) = OD at 260 nm x 50 x dilution factor.

2.2.2.1.2.2 Amplification of the ITS Region

Internal Transcribed Spacer (ITS) sequences are considered to be the best tool for rapid and accurate identification of yeast isolates (Kutty and Philip, 2008). ITS primers (Forward ITS 1-5’ TCC GTA GGT GAA CCT GCG G 3’ and Reverse ITS 4- 5’ TCC TCC GCT TAT TGA TAT GC 3’) by White et al. (1990) which amplify a fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions were used for the purpose.

The amplification reaction was performed by using a DNA thermal cycler (Eppendorf). PCR was performed in a final volume of 25 µl containing 2.5 µl of dNTPs, 1 µl each of forward and reverse primers, 2.5 µl of buffer, 1 µl of the extracted DNA of concentration 600 ng/ µl, 1 µl of Taq DNA polymerase and 16 µl of Milli Q water. After an initial denaturation at 95 ^{0}C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 94 ^{0}C for 1 minute, annealing at 56 ^{0}C for 45 seconds, extension step at 72 ^{0}C for 1 minute and a final extension at 72 ^{0}C for 10 minutes.

The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TBE buffer and stained with ethidium bromide (Fig 2.5).

2.2.2.1.2.3 Cloning of the PCR product onto pGEM-T Easy vector system.

Ligation

Fresh PCR product of ITS region was used for cloning into the pGEM-T Easy vector

(Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2X), 0.5 µl of the vector (50 ng/ µl), 3.5 µl of PCR product (600 ng/ µl) and 1 µl of T4 DNA ligase (3U/ µl). It was incubated at 4^{0}C overnight.

Transformation

JM 109 High Efficiency competent cells of *E.coli* (transformation efficiency ≥ x10 ^{8} cells/ µg DNA) were used.

The ligation mix was added to 10 ml glass tube previously placed in ice to which 50 µl of competent cells were added and incubated on ice for 20 minutes, a heat shock at 42^{0}C was given for 90seconds to facilitate the entry of recombinant DNA to the host cells, then the tubes were immediately placed on ice for 2 minutes. Added 600 µl of SOC media and incubated for 2 hrs at 37^{0}C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM), and X-gal (80 µg/ml). The plates were incubated at 37^{0}C overnight. The clones/transformants were selected using the blue/white screening. Successful cloning of an insert into the pGEM-T easy vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening

on indicator plates. The white colonies were selected and streaked on LB-Amp + X-gal + IPTG plates and incubated overnight at 37 ^{0}C.

Colony PCR

Colony PCR was done to confirm the presence of the insert DNA (DNA fragment to be cloned). All the individually streaked colonies were tested with colony PCR using universal vector primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'- GATTTAGGTGACACTATAG-3'). , to confirm the presence of the gene of interest and electrophoresis was done on 1 % agarose gel prepared in 1X TBE buffer and stained with ethidium bromide (Fig 2.5).

White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µl) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1 µl of 10 pmol/ µl of T7 and SP6 primers, 0.5 U of taq polymerase and the remaining volume was made up with Milli Q. The thermal cycling conditions were as follows: After an initial denaturation at 95 ^{0}C for 5 minutes, amplification was made through 35 cycles, each consisting of a denaturation at 94 ^{0}C for 15 seconds, annealing at 57 ^{0}C for 20 seconds, extension step at 72 ^{0}C for 1 minute and a final extension at 72 ^{0}C for 10 minutes following which the temperature was brought down to 4 ^{0}C.

2.2.2.1.2.4 Plasmid extraction and Purification

Plasmid extraction and purification was done using 'GenElute HP' plasmid miniprep kit (Sigma). Cells were harvested by centrifuging 2 ml of overnight recombinant *E.coli* culture at 16000 x g. Resuspended the pellet in 200 µl resuspension solution with RNase. Lysed the resuspended cells by adding 200 µl of the lysis buffer. Immediately mixed the contents by gentle inversion until the mixture becomes clear and viscous. Precipitated the cell debris by adding 350 µl of the neutralization buffer. Gently inverted the tube and the cell debris was pelleted by centrifuging at 16000 x g for 10 minutes. Column was prepared by inserting a Gen Elute HP Miniprep Binding column into a provided microcentrifuge tube. Added 500 µl of the column preparation solution to miniprep column and centrifuged at 16000 x g for 1 minute. Discarded the flow through liquid. Transferred the cleared lysate to the column and centrifuged at 16000 x g for 1 minute. Discarded the flow through

liquid. Added 500 µl of the wash solution 1 to the column and centrifuged at 16000 x g for 1 minute. Discarded the flow through liquid. Added 750 µl of the wash solution 2 to the column and centrifuged at 16000 x g for 1 minute. Discarded the flow through liquid. Centrifuged at 16000 x g for 1 minute to remove excess ethanol. Transferred the column to a fresh collection tube. Added 100 µl of elution solution (10 mM Tris-HCl) to the column. Centrifuged at 16000 x g for 1 minute. The DNA present in the eluate (plasmid DNA) was stored at -20^{0}C.

2.2.2.1.2.5 Sequencing and Phylogenetic analysis

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland. The primers used were T7 and SP6. Sequenced DNA data was complied and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information' (NCBI). The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov/). The sequences were multiple aligned using the programme Clustal W (Thompson et al., 1994). Then the aligned ITS-rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987), using the MEGA4 package (Tamura et al., 2007). Bootstrap analysis was based on 1000 replicates.

2.2.3 Nutritional characterization of the selected marine yeast strain *Candida* sp. S 27

2.2.3.1 Estimation of proximate composition

The biochemical composition of the selected strain was determined to assess the nutritional quality.

2.2.3.1.1 Preparation of yeast biomass

The selected yeast strain *Candida* sp. S 27 was swab inoculated into malt extract agar plates, incubated at room temperature (28± 2^{0}C) for 72 hrs and harvested with sterile saline. The cell suspension was centrifuged at 7000 rpm for 15 minutes in a refrigerated centrifuge (Remi C-30, Mumbai). It was washed repeatedly with sterile

saline to remove the media components. The yeast biomass was stored at 4^0C in a refrigerator.

2.2.3.1.2 Estimation of Protein

The protein content of the biomass of marine yeast strain *Candida* sp. S 27 was estimated by the method of Lowry et al. (1951). The sample was subjected to alkali while being warmed in a water bath. To the resulting solution, first an alkaline solution of copper sulphate and then Folin's reagent were added. The resulting solution was blue in colour, and the intensity measured at 750 nm was proportional to the protein concentration of the sample. The crude protein content was verified by the equation Total Nitrogen x 6.25 after determining the former by Microkjeldahl method (Lang, 1958).

2.2.3.1.3 Estimation of Lipids

The lipid content of the yeast biomass was analysed by Phosphovanillin method following chloroform-methanol extraction of the sample (Barnes and Blackstock, 1973). 500 mg of sample was mixed well with 10 ml of chloroform: methanol solution (2: 1) in a homogeniser. Filtered the homogenate through Whatman No. 1 filter paper. Added 2 ml of 0.9 % NaCl and shaken well. Transferred the mixture to a separating funnel, allowed to stand overnight at 4^0C. Removed the lower phase that contained all the lipids and adjusted the volume to 10 ml by the addition of chloroform. 0.5 ml of the extract was taken in a clean tube, allowed to dry in vaccum dessicator over silica gel. Dissolved in 0.5 ml of Conc. H_2SO_4. Mixed well, plugged and placed in a boiling water bath for 10 minutes and cooled to room temperature. To 0.2 ml of the acid digest, added 5 ml of vanillin reagent, incubated for 30 minutes and measured the colour at 520 nm. 0.2 ml of chloroform served as blank and cholesterol (10 mg in 10 ml of (2: 1) chloroform: methanol solution) as standard.

2.2.3.1.4 Estimation of Total Carbohydrates

Total carbohydrates in the yeast biomass was determined spectrophotometrically by Anthrone method (Hodge and Hofreiter, 1962). 100 mg of the sample was hydrolysed in a boiling water bath for 3 hours with 2.5 N HCl and

cooled and neutralised with solid Na_2CO_3 until the effervescence ceased. The volume was made up to 100 ml, centrifuged and the supernatant was collected. Added 4 ml of anthrone reagent (200 mg anthrone in 100 ml ice-cold Conc. H_2SO_4), heated for 8 minutes in a boiling water bath, cooled rapidly and read the green colour at 630 nm. Glucose was used as the standard and distilled water as blank. Amount of carbohydrate was expressed as g/100gm dry weight.

2.2.3.1.5 Estimation of Nucleic acids

DNA estimation was done by Diphenylamine method and RNA by Orcinol method (Kochert, 1978). Pentose of RNA or deoxyribose of DNA reacts with orcinol reagent forming a bluish green colour at 660 nm. But only DNA gives colour with diphenylamine reagent at 600 nm. Diphenylamine produces a blue colur by reaction with deoxyribose moiety in DNA. From the difference in colour density by 2 methods, composition of the mixture can be calculated.

2.2.3.1.6 Determination of ash content

Ash content was determined by incineration of yeast biomass in a silica crucible at 550^0C in a muffle furnace for 5 hours. The weight of the residue in the crucible was the ash content.

2.2.3.1.7 Estimation of fibre content

The detergent method (Goering and Van Soest, 1970) with a modification by Van Soest and Mc Queen, 1973) was used to estimate the fibre content. A weighed quantity of yeast biomass was dried at 60 ^{0}C for 8 hours and weighed again. It was ground to pass through a 1mm sieve. Added a definite volume (100 ml for 1g dried sample) of the detergent solution which is a mixture of Sodium lauryl sulphate (3% at final concentration), Disodium ethylene-diamine tetraacetate dehydrate (1.86% at final concentration), anhydrous Disodium hydrogen phosphate (0.456% to final volume), sodium borate decahydrate (0.681% to final volume) and 2-Ethoxy ethanol (1ml per 100 ml solution). Added Decalin (2ml per 100 ml of detergent solution) and Sodium sulphite (0.5% to final concentration) to the mixture and placed in a Berzelius beaker for refluxing. The mixture was heated to boiling and refluxed for one hour. Cooled and washed with hot water (80 ^{0}C) and centrifuged. The mixture

was washed with water and acetone. The residue was collected, dried at 105 ^{0}C for 8 hours and weighed. The content of total dietary fibre was expressed as g/100g of the dry weight of the sample.

2.2.3.1.8 Determination of moisture

The moisture content of wet yeast biomass was measured by drying 10 g of freshly harvested yeast biomass at 105 ^{0}C for 18 hours or till constant weight is attained and calculating the difference in weight.

2.2.3.1.9 Determination of fatty acids

Identification and quantification of fatty acids were carried out by Fatty Acid Methyl Ester (FAME) methods. The fatty acids were converted to their methyl esters and heptane and were analysed by gas chromatography with flame ionization detection (GC-FID) equipped with silica capillary column (25m length, 2mm I.D X 0.33µm film thickness) (Agilent Technologies, model 6890 USA employing the software Sherlock, MIDI Inc, USA).

Materials and method

Reagents

Saponification Reagent

NaOH (certified ACS) - 45 g

Methanol (HPLC grade) - 150 ml

Deionized distilled water - 150 ml

Methylation Reagent

6 N HCl - 325 ml

Methanol (HPLC grade) - 275 ml

Extraction Solvent

Hexane (HPLC grade) - 200 ml

Methyl tertiary-butyl ether (HPLC grade) - 200 ml

Base Wash

NaOH (certified ACS)	- 10.8 g
Deionized distilled water	- 900 ml

Saturated NaCl

NaCl (certified ACS)	- 40 g
Deionized distilled water	- 100 ml

Procedure

The marine yeast *Candida* sp. S 27 was streaked on to Sabouraud Dextrose agar plate employing the Quadrant streak method. It was incubated overnight at 28 ^{0}C.

Quadrant streak method was designed to dilute the inoculum so that quadrant 4 will contain well-isolated colonies to serve as a check for purity. Colonies were harvested from the most dilute quadrant (Quadrant 3) exhibiting late log phase along the streaking axis. This area of harvesting typically yields the most stable fatty acid compositions since the inoculum has been diluted enough to result in abundant growth of colonies without a limiting nutrient supply. Yeast colonies weighing up to 100 mg were carefully harvested using a plastic inoculating loop or a Pasteur pipette melted into a small loop or a small diameter wire loop.

Saponification

A strong methanolic base combined with heat kills and lyses the cells. Fatty acids are cleaved from cell lipids and are converted to their sodium salts. Pipetted out 1 ml of reagent 1, the methanolic base into the culture tube. It was tightly sealed with a clean Teflon-lined screw-cap. The tube was vortexed for 5-10 seconds. The tube was placed in a boiling or circulating water bath at 95-100 ^{0}C. After 5 minutes, the tube was removed from the water bath and cooled slightly without loosening the cap. The tube was vortexed again for 5-10 seconds. The tube was returned to the water bath. The tube was heated in the water bath for an additional 25 minutes. After a total of 30 minutes of saponification, the tube was removed and placed in a pan of cold tap water and cooled.

Methylation

Methylation converts the fatty acids (as sodium salts) to fatty acid methyl esters, which accelerates the volatility of the fatty acids for the GC analysis. The tube was uncapped, then added 2 ml of Reagent 2, the methylation reagent to the tube.

The tube was tightly capped and vortexed the solution for 5-10 seconds. The tube was heated in an 80 ^{0}C water bath for 10 minutes. It was removed and quickly cooled to room temperature by placing in a tray of cold tap water. The tube was shaken to speed the cooling process.

Extraction

Fatty acid methyl esters were removed from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction procedure. The tube was uncapped, added 1.25 ml of Reagent 3, the extraction solvent to the tube. It was sealed tightly and placed in a laboratory rotator and gently mixed end-over-end for 10 minutes. The tube was uncapped, and using a Pasteur pipette, removed and discarded the lower aqueous phase.

Base Wash

A dilute base solution was added to the sample preparation tubes to remove free fatty acids and residual reagents from the organic extract. Residual reagents will damage the chromatographic system, resulting in tailing and loss of the hydroxyl fatty acid methyl esters. Added 3 ml of Reagent 4, the base wash to the tube. It was tightly capped and gently rotated end-over-end for 5 minutes.

The upper solvent phase was removed and placed in a sample vial suitable for automatic sampler mounted on the gas chromatograph. Uncapped the tube and using a clean Pasteur pipette, transferred about 2/3 of the upper organic phase to a clean GC sample vial. A cap was crimped onto the sampler vial, loaded the automatic sampler and it was run. 2µl sample was injected. The run lasted for 20.7 minutes. The oven temperature was 170^{0}C which was increased to 310^{0} C at the rate of initially 5^{0}C/minute and then up to 40^{0}C/minute. Hydrogen was used as the carrier gas at a constant flow rate of 1.3L/min. Methyl Tertiary Butyl Ether (MTBE) and hexane constituted the stationary phase.

2.2.3.1.10 Amino acid analysis

Qualitative and quantitative estimation of amino acids was done using HPLC-LC Shimadzu 10 AS automatic amino acid analyzer as per the procedure of Ishida et al. (1981). This procedure can be applied for the estimation of all amino acids except tryptophan. The amino acid tryptophan is not stable to acid digestion in the presence of even trace amount of oxygen and is estimated separately following digestion under alkaline conditions with thioglycolic acid reagent (Sastry and Tummuru, 1985).

Reagents

a. 6 N HCl
b. 5 % NaOH
c. 0.05 M HCl]
d. Buffer A: Dissolved 58.8 g of tri sodium citrate in 2 L of double distilled water, added 210 ml of 99.5 % ethanol, adjusted the pH to 3.2 by adding 60 % perchloric acid and made up to 3 L using double distilled water.
e. Buffer B: Dissolved 58.8 g of tri sodium citrate and 12.4 g boric acid in double distilled water, adjusted the pH to 10 by adding 4N NaOH and made up to 1 L using double distilled water.
f. Phthaldehyde (OPA)Buffer: Dissolved 122.1 g of Na_2CO_3, 40.7g of H_3BO_3 and 56.4g of K_2SO_4 in double distilled water and made up the volume to 3 L.
g. Phthaldehyde solution (OPA): Dissolved 400 mg OPA, 7ml ethanol, 1ml of 2-Mercaptoethanol and 2 ml of 30 % w/v Brij-35 in 500 ml OPA buffer.
h. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer.

Sample preparation

100 mg of yeast biomass was taken in a heat stable test tube. Added 10 ml of 6N HCl and heat sealed the tube after filling with pure nitrogen gas. The hydrolysis was carried out at 110 ^{0}C for 24 hours. After hydrolysis, the test tube was opened and the contents were removed quantitatively and filtered into a round bottom flask through Whatman filter paper No.42. The filter paper was washed 2-3 times with distilled water. Flash evaporated the contents of the flask to remove all traces of HCl.

The process was repeated 2-3 times with distilled water. Dissolved the residue and made the volume to 10 ml with 0.05 M HCl.

HPLC Analysis

Filtered the sample prepared through a membrane filter of 0.45 µm and injected 20 µl to an aminoacid analyzer equipped with sulphonated polyvinyl styrene cation exchange column and fluorescence detector. The mobile phase of the system consisted of two buffers, A and B. A gradient system was followed for the effective separation of amino acids. The oven temperature was maintained at 60 ^{0}C. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthalaldehyde. Amino acid standards were run to calculate the concentration of amino acids in the sample.

Estimation of tryptophan

Since tryptophan content could not be obtained through HPLC estimation was done separately.About 200-250 mg of sample was hydrolyzed with 10 ml of 5 % NaOH at 110 ^{0}C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicators. The volume was made up to 100 ml with distilled water. The solution was then filtered through Whatman filter paper No.1 and the filtrate was used for estimation.

To a test tube containing 4ml of 50 % H_2SO_4, 0.1 ml of 2.5 % sucrose and 0.1 ml of 0.6 % thioglycolic acid were added. These tubes were kept for 5 minutes in water bath at 45-50 ^{0}C and cooled. The sample was then added to these test tubes. A set of (0.1 to 0.8) standard was treated similarly. The volume was made up to 5ml with 0.1 N HCl and allowed to stand for 5 minutes. The absorbance was measured using UV-Vis spectrophotometer at 500 nm.

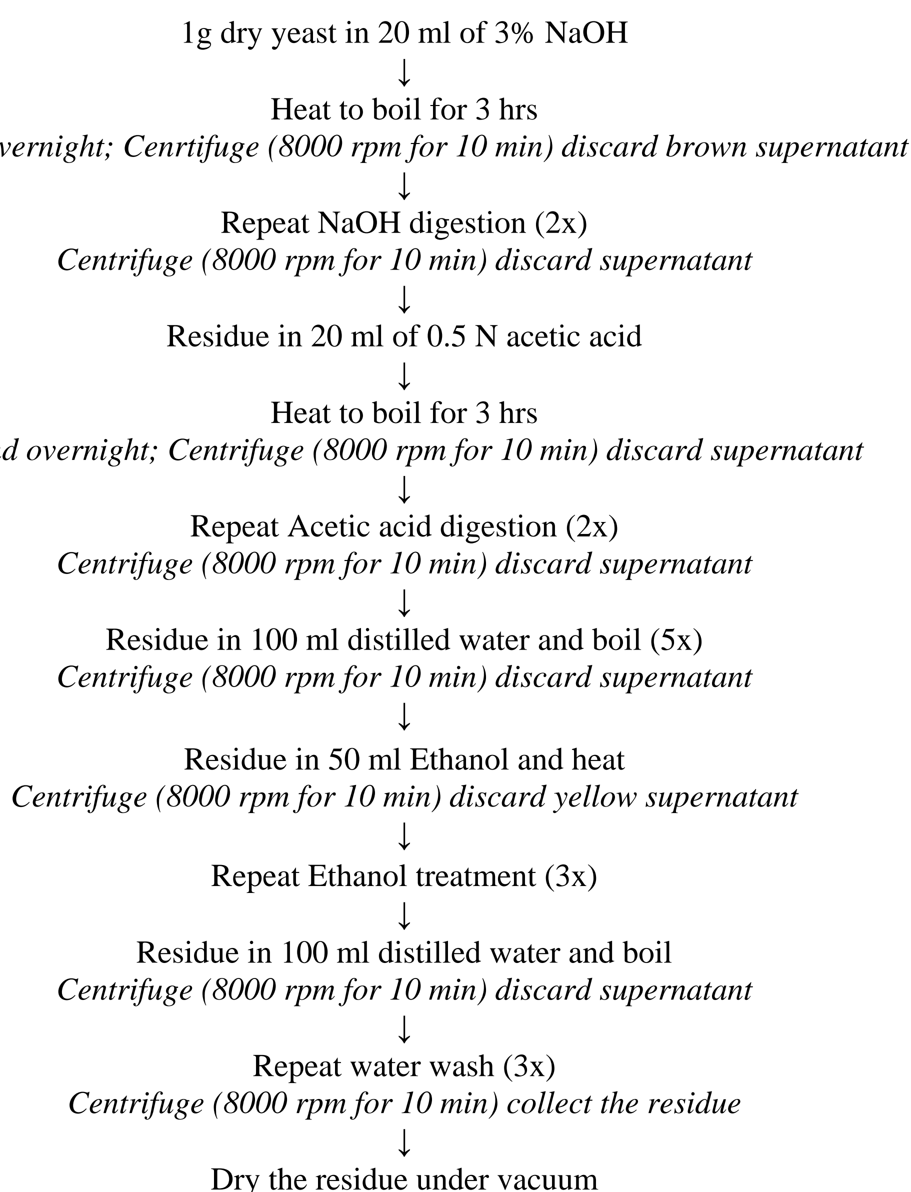
The amount of Tryptophan in the sample was calculated from the equation

$$\text{Tryptophan} = \frac{\mu\text{g of Tryptophan x Volume made up x 100 x 16}}{\text{Sample taken for colour development x Weight of sample x 1000 x \% of } N_2}$$

2.2.4.1.11 Extraction of β- 1,3 glucan from yeast

Alkali insoluble cell wall glucan of marine yeast *Candida* sp. S 27 was extracted as per Williams et al. (1991) and its characterization was done with proton NMR and compared with a standard glucan prepared by Lowman and Williams (2001). Glucan extracted from Baker's yeast *Saccharomyces cerevisiae* (MTCC 36) by the same protocol was also subjected to NMR analysis for comparison. A standard linear glucan was also subjected to NMR as reference.

Flow chart describing the procedure for extraction of water insoluble β-glucan from yeast cell wall (Williams et al., 1991)



NMR spectroscopy

Two-dimensional NMR spectroscopy analysis such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were also done with glucan extracted from marine yeast *Candida* sp. S 27. 1D and 2D gradient-selective double-quantum-filtered homonuclear correlation spectroscopy (COSY) and gradient-selective total correlation spectroscopy (TOCSY) proton NMR spectra were acquired on a JEOL Eclipse+ 600 NMR spectrometer in 5-mm OD NMR tubes operating at 80 °C. Glucan samples were dissolved in DMSO-d_6 with stirring and heating to 80 °C. The residual protons of the perdeuterated DMSO-d_6 provided internal chemical shift reference at 2.50 ppm. A few drops of deuterated trifluoroacetic acid (TFA-d) were added to the solution to shift residual proton resonances downfield. Spectral collection and processing parameters for the 1D proton NMR spectrum was the following: 25 ppm spectral width centered at 7.5 ppm, 65,536 data points, 1024 scans, 11.75 sec relaxation delay, 4.36 sec acquisition time and exponential apodization. Spectral collection and processing parameters for the 2D COSY spectrum were the following: 512 points in f2 and 128 points in f1 zero-filled 4 times for a 512 x 512 data array size, 6 ppm spectral width centered at 3 ppm in both dimensions, 4 prescans, 128 scans and 1.5 sec relaxation delay. Spectral collection and processing parameters for the 2D TOCSY spectrum were the following: 512 points in f2 and 128 points in f1 zero-filled 4 times for a 512 x 512 data array size, 6 ppm spectral width centered at 3 ppm in both dimensions, 4 prescans, 128 scans, 2 sec relaxation delay and a spin lock time of 100 msec.

2.3 Results

2.3.1 Selection of a suitable marine yeast strain for biomass production

Based on optical density measurement for growth (Table 2.1) and the microscopic examination of morphology / size (Fig 2.1) five yeast strains were selected for further screening. Strains showing rapid growth (less generation time) with comparatively large size were segregated. Filamentous forms were not selected, since the measurement of growth by optical density will be difficult. The selected strains were S 8 (*Debaryomyces*), S 87 (*Debaryomyces*), S 27 (*Candida*), S 186 (*Candida*) and S 51 (*Cryptococcus*).

All the strains were found to be potential producers of the enzyme catalase. S 186 exhibited comparatively better production of lipase than S 51 and S 27, while S 8 and S 87 were non producers (Table 2.2). S 51 was found to be antagonistic to *Aeromonas salmonicida.* Absence of clearance zone on prawn blood agar media showed that marine yeasts were non-pathogenic to shrimps (Fig.2.2). The marine yeast strain *Candida* sp. S 27 was selected because it had better growth and biomass production when compared to the four other strains (Table 2.1 and Fig 2.3).

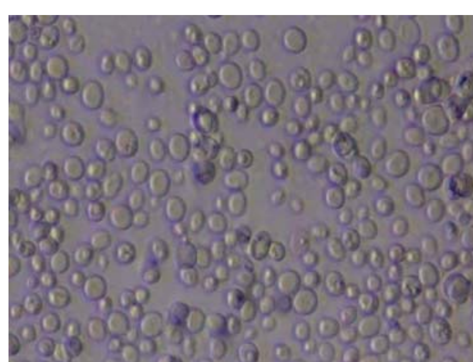
Table 2.1 Screening of marine yeasts based on growth

Sl. No:	Genera	Strain no:	O D at 540 nm
1	*Candida*	**S 27**	**1.348**
		S 165	0.994
		S 186	**1.253**
		S 405	0.717
2	*Williopsis*	S 4	0.240
3	*Filobasidium*	S 200	0.050
		S 258	0.327
		S 434	1.052
4	*Saccharomycopsis*	S 144	0.261
5	*Debaryomyces*	**S 8**	**1.178**
		S 87	**1.280**
		S 100	1.021
		S 169	0.285
		S 290	0.301
		S 294	0.327
6	*Lodderomyces*	S 9	0.357
		S 175	0.469
7	*Leucosporidium*	S 394	0.383
8	*Torulaspora*	S 303	0.518
9	*Aciculoconidia*	S 32	0.529
		S 49	0.696
10	*Mastigomyces*	S 412	0.725
11	*Kluyveromyces*	S 28	1.048
12	*Geotrichum*	S 59	0.878
13	*Dekkera*	S 304	0.890
14	*Hormoascus*	S 71	0.945
		S 75	0.794
15	*Arxioxyma*	S 81	0.974
16	*Cryptococcus*	S 50	1.157
		S 51	**1.223**
17	*Lipomyces*	S 69	0.609
18	*Dipodascus*	S 425	0.469
19	*Oosporidium*	S 34	0.327
20	*Pichia*	S 56	0.528
21	*Rhodotorula*	S 80	0.490
22	*Schizosaccharomyces*	S 45	0.715

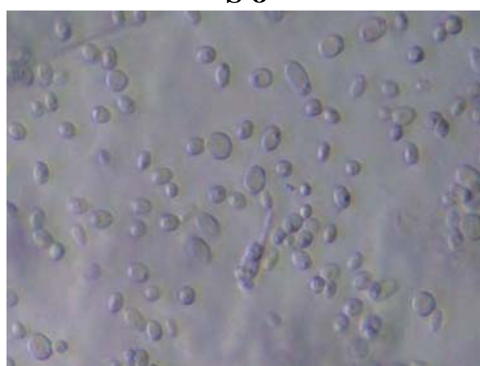
Table 2.2 Characterization of the five selected strains

Strain No:	Enzyme production				Antagonistic activity				
	Amylase	Gelatinase	Lipase	Catalase	V.harveyi	V.alginolyticus	V.paraheamolyticus	A.salmonicida	P.aerUginosa
S 8	-	-	-	+	-	-	-	-	-
S 27	-	-	+	+	-	-	-	-	-
S 51	-	-	+	+	-	-	-	+	-
S 87	-	-	-	+	-	-	-	-	-
S 186	-	-	++	+	-	-	-	-	-

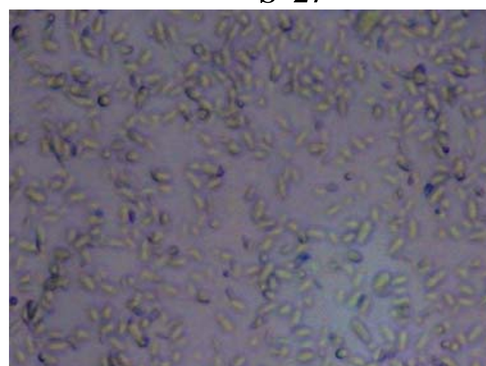
S 8



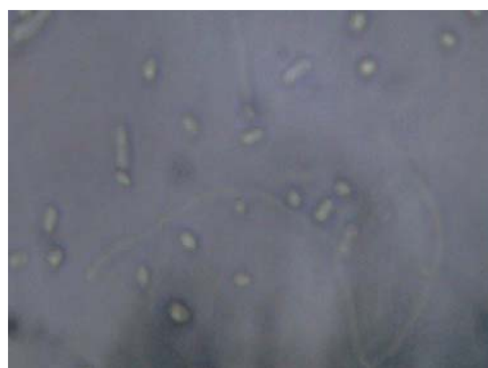
S 27



S 51



S 87



S 186

Fig 2.1 Light Micrograph of 5 yeast strains

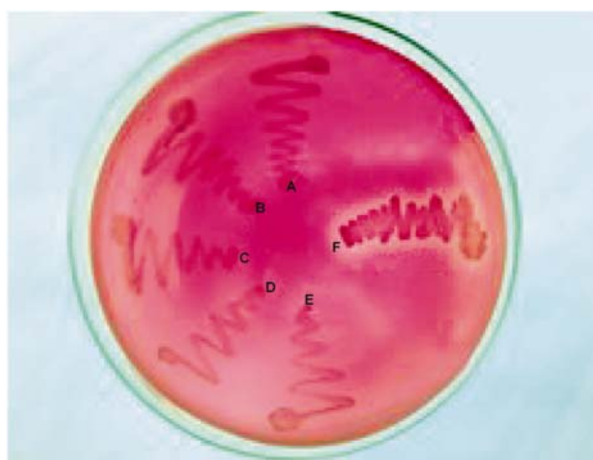


Fig 2.2 Haemolytic assay of marine yeasts using prawn blood agar

A. S 8	B.S 27
C. S 51	D.S 87
E. S 186	F. *Vibrio* sp. MBCS 6 (Positive strain)

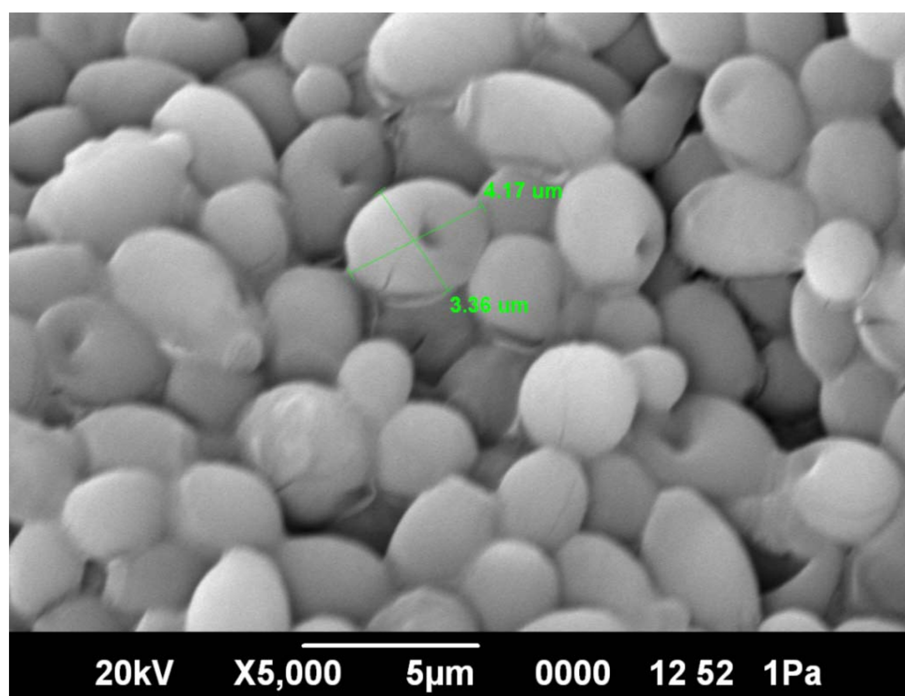


Fig 2.3 Scanning electron micrograph* of marine yeast *Candida* sp. S 27

**An aliquot of 1.5 ml of yeast culture was centrifuged at 8000 RPM in a refrigerated centrifuge for 15 minutes. The pellet was washed with sterile saline of 5 $^{0}/_{00}$ and fixed in 0.5 ml of 2.5 % glutaraldehyde prepared in sterile saline at 4^{0}C overnight. The pellet was washed repeatedly with saline and dehydration was done through an acetone series of 70-100 % and kept overnight in a dessicator. The particle was spread on SEM stubs, dried in critical point dried apparatus, platinum coated and observed under JEOL Analytical Scanning Electron Microscope, JSM 6390 LV, Tokyo, Japan (Sophisticated Test and Instrumentation Centre, CUSAT, Cochin-22).*

2.3.2 Characterization of the selected marine yeast *Candida* sp. S 27

2.3.2.1 Based on phenotypic characteristics

The biochemical characterization done at IMTECH, Chandigarh is presented (Table 2.3).

Table 2.3 Biochemical characteristics of the marine yeast *Candida* sp. S 27 as identified by IMTECH, Chandigarh

S No:		+/-	S No:		+/-
1	D-Galactose growth	+	30	DL-Lactate growth	+
2	L-Sorbose growth	+	31	Succinate growth	+
3	D-Glucosamine growth	+	32	Citrate growth	+
4	D-Ribose growth	-	33	Methanol growth	-
5	D-Xylose growth	+	34	Ethanol growth	+
6	L-Arabinose growth	-	35	Growth at 25 ^{0}C	+
7	D-Arabinose growth	-	36	Growth at 30 ^{0}C	+
8	L-Rhamnose growth	-	37	Growth at 37 ^{0}C	+
9	Sucrose growth	+	38	Growth at 42 ^{0}C	+
10	Maltose growth	+	39	0.01 % Cycloheximide growth	+
11	Alpha,alpha-Trehalose growth	+	40	0.1 % Cycloheximide growth	+
12	Me alpha D-Glucoside growth	+	41	50 % D-Glucose growth	+
13	Cellobiose growth	+	42	60 % D-Glucose growth	+
14	Salicin growth	+	43	Starch formation	-
15	Arbutin growth	-	44	Acetic acid production	-
16	Melibiose growth	-	45	Urea hydrolysis	-
17	Lactose growth	-	46	Pink colonies	-
18	Melezitose growth	+	47	Budding cells	+
19	Glycerol growth	-	48	Splitting cells	-
20	Ribitol growth	+	49	Filamentous	-
21	Xylitol growth	-	50	Pseudohyphae	-
22	L-Arabinitol growth	+	51	Septate hyphae	-
23	D-Glucitol growth	+	52	Arthroconidia	-
24	D-Mannitol growth	+	53	Ballistoconidia	-
25	Myo-Inositol growth	-	54	Symmetric ballistoconidia	-
26	D-Glucono-1,5-lactone growth	+	55	Ascospores	-
27	2-Keto-D-gluconate growth	+	56	Ascospores round, oval, conical, reniform	-
28	D-Gluconate growth	+	57	Ascospores cap, hat, Saturn, walnut-shaped	-
29	D-Glucuronate growth	-	58	Ascospores needle-shaped or whip-like	-

2.3.2.2 DNA based identification

1 tccgtaggtg aatctgcgga aggatcatta ctgatttgct taattgcacc acatgtgttt

61 tttactggac agctgctttg gcggtgggga ctcgtttccg ccgccagagg tcacaactaa

121accaaacttt ttattaccag tcaaccatac gttttaatag tcaaaacttt caacaacgga

181tctcttggtt ctcgcatcga tgaagaacgc agcgaaatgc gatacgtagt atgaattgca

241gatattcgtg aatcatcgaa tctttgaacg cacattgcgc cctttggtat tccaaagggc

301atgcctgttt gagcgtcatt tttccctcaa gcccgcgggt ttggtgttga gcaatacgcc

361aggtttgttt gaaagacgta cgtggagact atattagcga cttaggttct accaaaacgc

421ttgtgcagtc ggcccaccac agcttttcta acttttgacc tcaaatcagg taggactacc

481cgctgaactt aagcatatca ataagcggag ga

DNA sequence (512 bp) of the ITS regions of yeast *Candida* sp. S 27 (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence)

Based on the NCBI blast of the ITS sequence obtained through sequencing the yeast was identified as *Candida* sp. The nucleotide sequence determined in this study has been deposited in GenBank and assigned the Accession No: FJ652102 (Appendix).

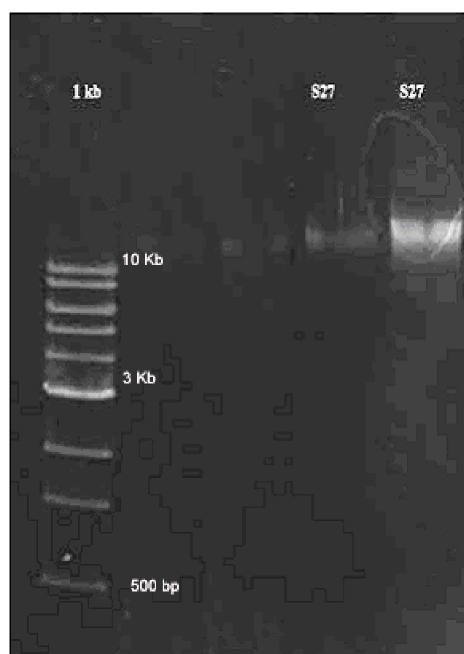


Fig 2.4 Agarose gel electrophoresis of Yeast genomic DNA

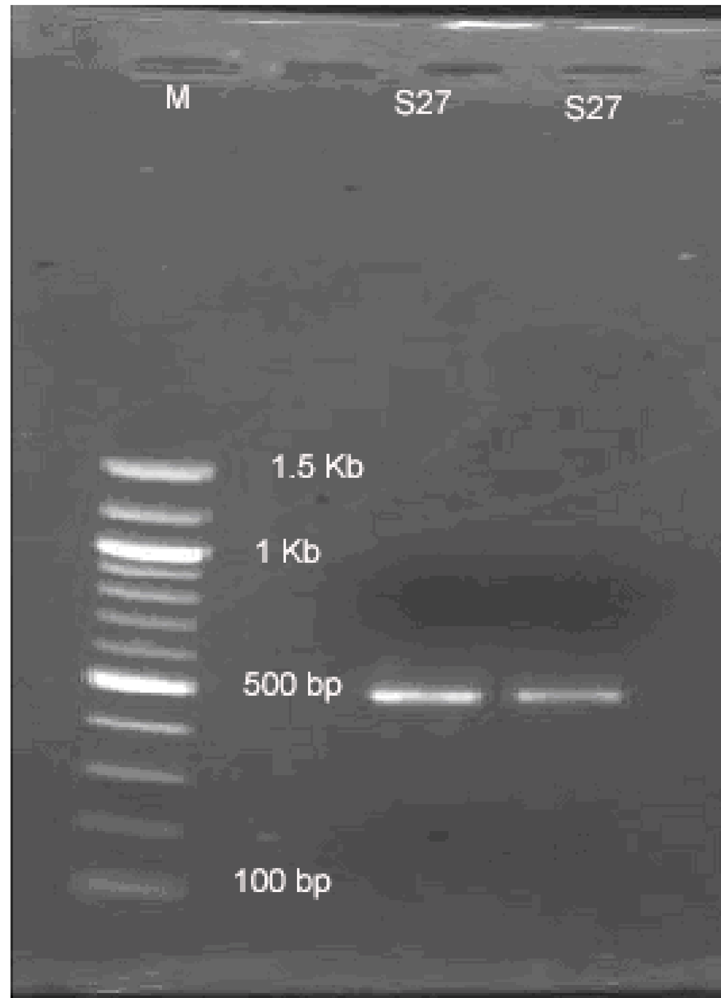


Fig 2.5 PCR products of the ITS-region, amplified from DNA extract

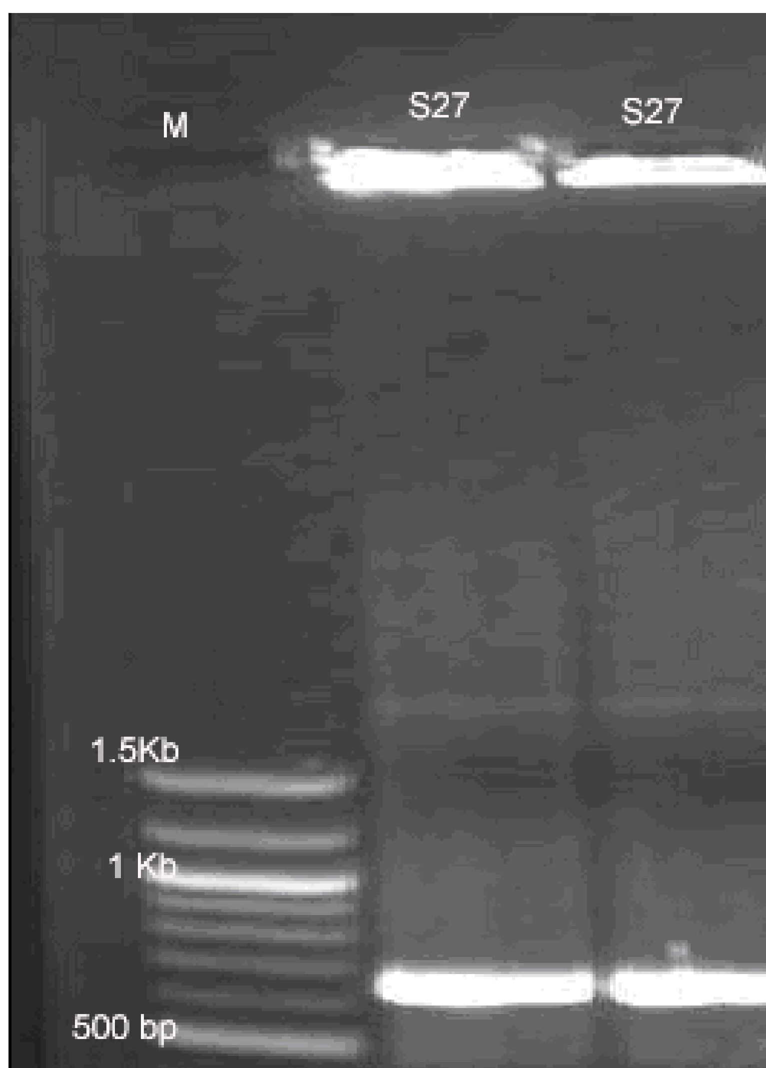


Fig 2.6 Agarose gel electrophoresis of colony PCR using vector primers T7 and SP6

Phylogenetic analysis

The accession numbers of the closest match downloaded from GenBank were DQ249200, DQ249201, DQ 640769, EF658667, EU 288196, FJ623629, and FJ820825. *Candida* sp. S 27 has 92 % similarity with *Candida viswanathii* DQ249201. Though its shows 99 % similarity with *Candida viswanathii* DQ249200, the latter is an incomplete ITS rDNA (5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence) sequence of only 283 bp length (Fig 2.7).

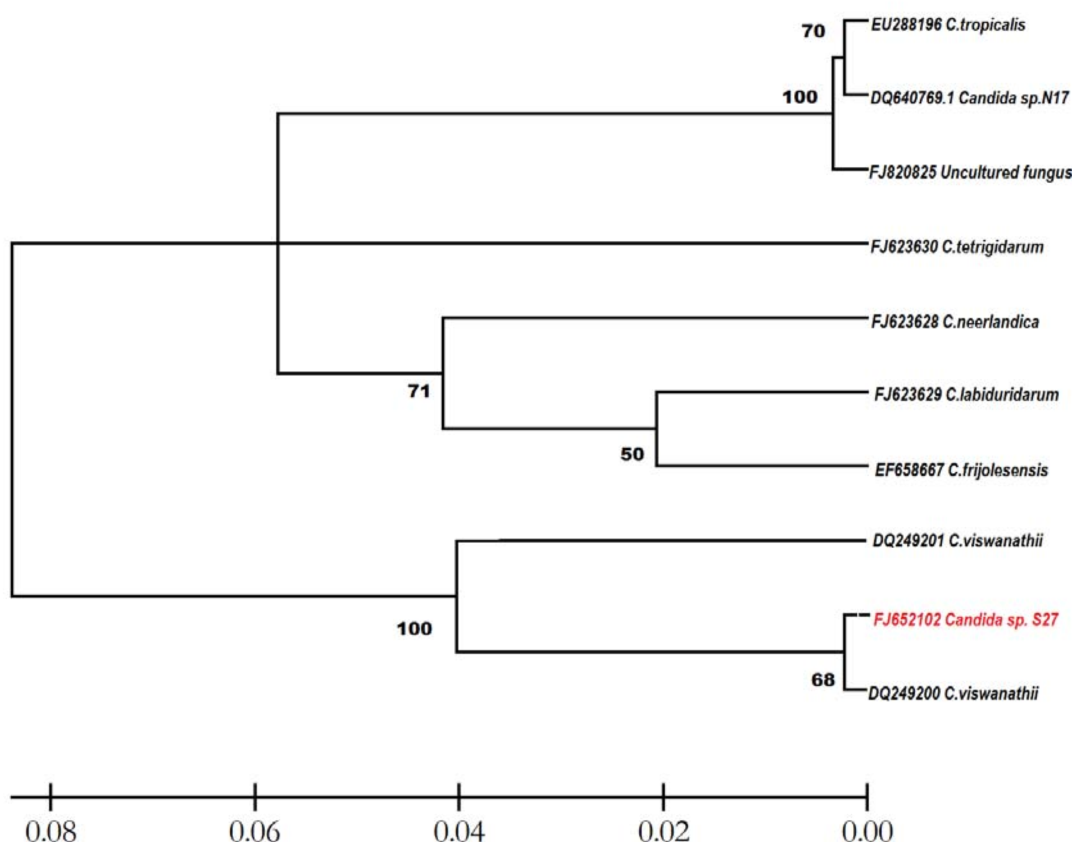


Fig 2.7 A bootstrapped neighbor joining tree obtained using MEGA version 4.0 illustrating the relationship between the ITS sequence of *Candida* sp. S 27 with relevant sequences of some reference strains of *Candida* spp. downloaded from the National Center for Biotechnology Information Database (http:// www.ncbi.nlm.nih.gov/).

2.3.3 Nutritional characterization of the selected marine yeast *Candida* sp. S 27

2.3.3.1Proximate composition of yeast biomass

The protein content of the yeast biomass was estimated to be 32 %. This was verified from the amount of crude nitrogen determination by microKjeldahl method which was 5.12 %. The quantity of lipid in yeast biomass was 2.224 %. The amount of total carbohydrates in the sample was 30.35 % in which the crude fibre portion was up to 11 %. Ash or the mineral content constituted about 6.8 % of the yeast biomass. Nucleic acid content was 11.94 %.Wet biomass contained approximately 72 % moisture (Table 2.4).

Table 2.4Proximate composition of *Candida* sp. S 27

No:	Component	% on dry weight basis
1	Protein	34
2	Fibre	11
3	Nucleic acids	11.94
4	Carbohydrate	30.35
5	Lipid	2.224
6	Ash	6.8

2.3.3.2 Fatty acid profile of *Candida* sp. S 27

Dominant fatty acids in marine yeast *Candida* sp. S 27 were oleic acid (35.91 %) and linoleic acid (25.53 %). Saturated fatty acids like palmitic acid, lauric acid, myristic acid and stearic acid were present in appreciable amounts. None of the highly unsaturated fatty acids such as eicosapentaenoic acid or docosahexaenoic acid were detected in *Candida* sp. S 27 (Table 2.5 and Fig 2.8).

Table 2.5 Fatty acid composition of *Candida* sp. S 27

RT	C atoms	Fatty acid	% of composition
4.984	12:0	Lauric acid	1.09
7.597	14:0	Myristic acid	2.36
10.580	16:1	Palmitoleic acid	12.38
10.889	16:0	Palmitic acid	17.62
12.290	17:1	Margaroic acid	1.49
13.953	18:2	Linoleic acid	25.53
14.042	18:1	Oleic acid	35.91
14.446	18:0	Stearic acid	2.84

```
=================================================================
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Sample Name      :  1459                        Location : Vial 3
Acq. Operator    :                                   Inj :   1
                                              Inj Volume : 2 µl
Method           : C:\HPCHEM\1\METHODS\$MIDI$A.M
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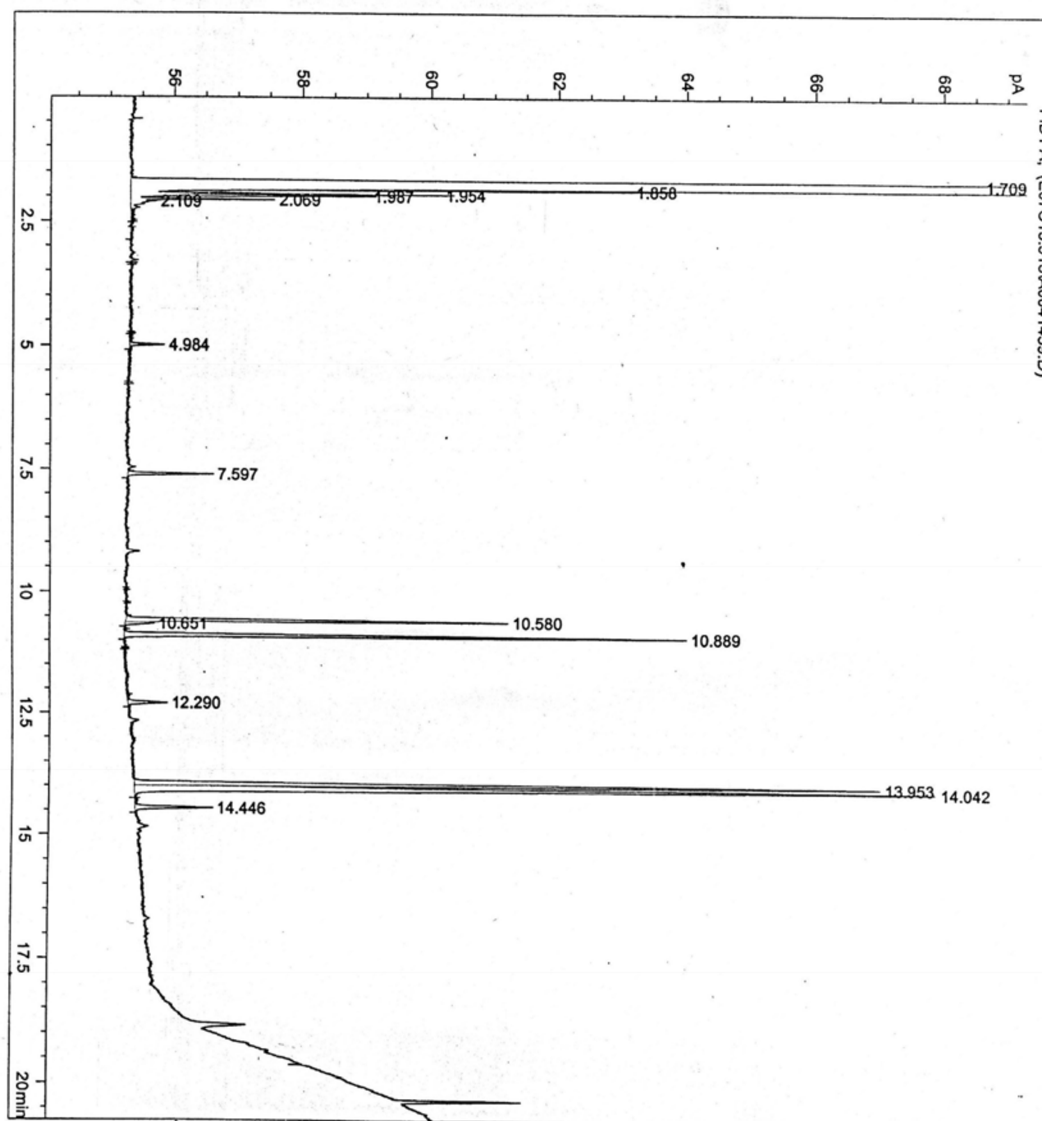

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Fig 2.6 Fatty acid profile of marine yeast *Candida* sp. S 27 – GC chromatogram

2.3.3.3 Amino acid profile of *Candida* sp. S 27

All the 20 amino acids were present in *Candida* sp. S 27. The essential amino acids such as lysine, leucine, tryptophan, valine, threonine, isoleucine and phenylalanine were present in fairly good quantities, whereas methionine, histidine and arginine were in negligible amounts. The non-essential amino acids aspartate and glutamate had the highest concentrations, 10.4 and 18.09 % respectively (Table 2.5 and Fig 2.7).

Table 2.6 Amino acid composition of *Candida* sp. S 27

S No:	RT	Aminoacid	mg/100 mg
1	7.782	Aspartic acid	10.4
2	9.983	Threonine	3.89
3	10.95	Serine	5.17
4	12.642	Glutamic acid	18.09
5	14.195	Proline	4.17
6	19.317	Glycine	5.45
7	20.947	Alanine	7.96
8	24.082	Cysteine	1.14
9	27.062	Valine	7.27
10	29.372	Methionine	1.77
11	31.773	Isoleucine	3.99
12	32.593	Leucine	8.98
13	35.217	Tyrosine	3.13
14	37.017	Phenyl alanine	4.28
15	45.132	Histidine	2.44
16	46.273	Lysine	6.20
17	50.817	Arginine	2.87
18		Tryptophan	6.80

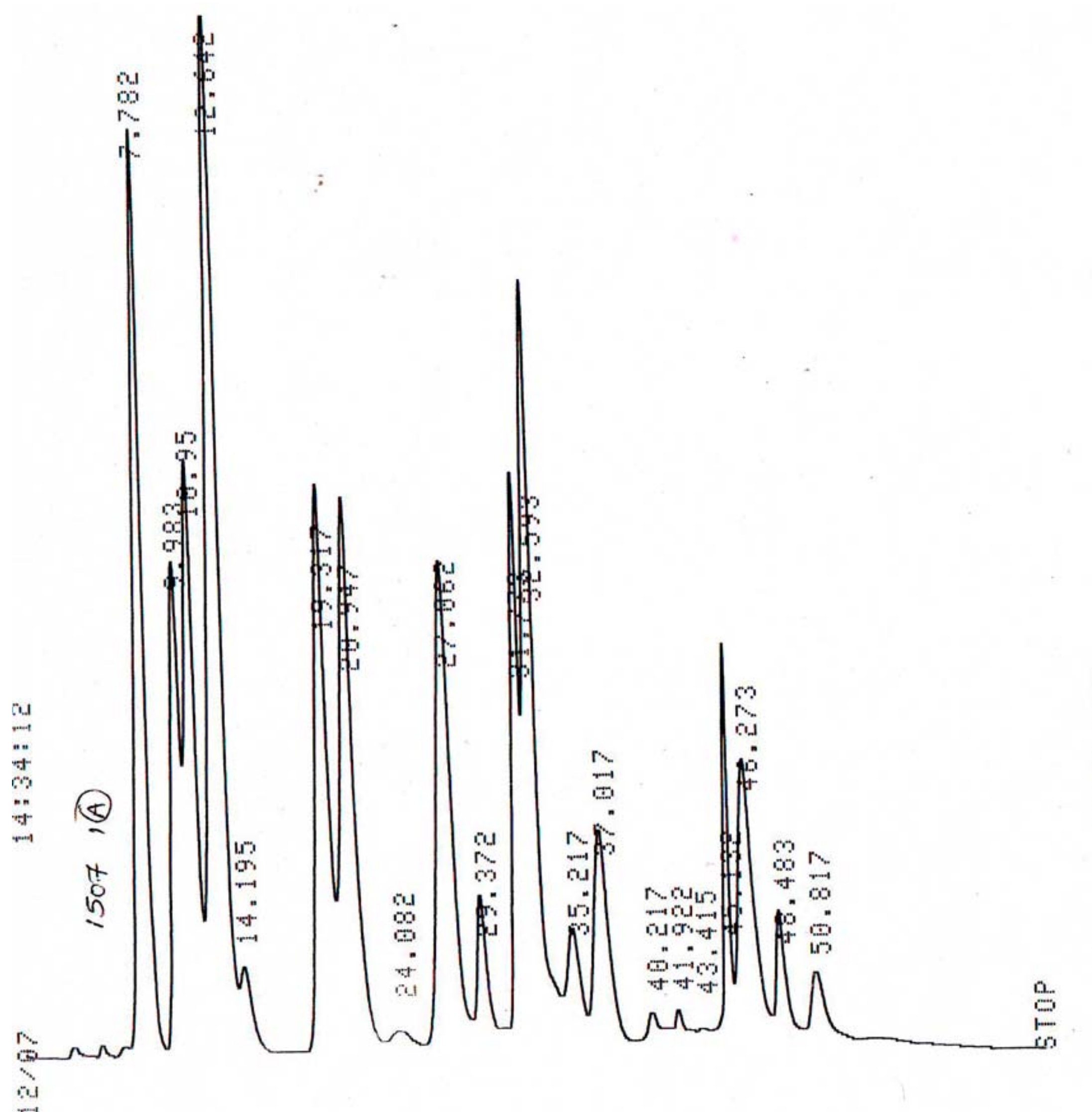

Fig. 2.9 HPLC Chromatogram showing amino acid profile of *Candida* sp. S 27

2.3.3.4 Extraction of β-1,3 glucan from yeast

Percentage yield of glucan

The extraction of glucan from *Candida* sp. S 27 gave a yield of 17.5 % particulate glucan wheras in the case of *Saccharomyces cerevisiae* MTCC 36, it was only 10.11 %.

1D NMR analysis

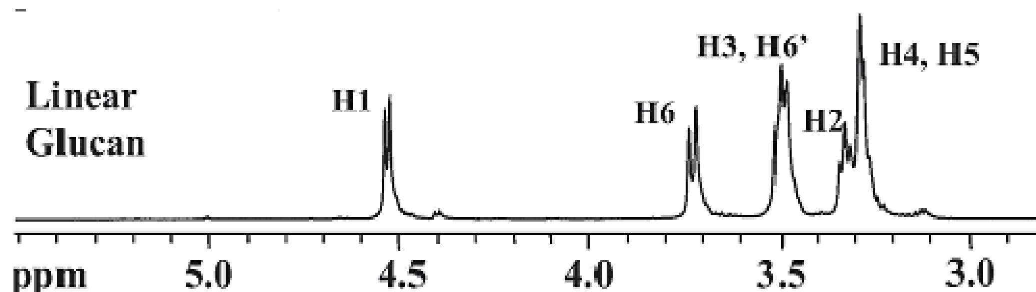

Fig 2.10 The carbohydrate region (from 5.5 to 2.8 ppm) of the proton NMR spectra of the linear glucan for comparison. Resonance labels H1 through H6 refer to glucosyl protons shown in the repeat unit structure.

The 1D proton NMR spectrum of MTCC 36 is shown in Fig 2.11. The carbohydrate and lipid spectral regions of glucan extracted from *Candida* sp. S 27 is shown in Figure 2.12. The carbohydrate spectral region is expanded in Figure 2.13. The 2D COSY and TOCSY 2D NMR spectra, expanded similarly to that in Figure 2.13, are shown in Figures 2.14 and 2.15 respectively. Based upon 1D and 2D COSY and TOCSY NMR experiments, a structure for the glucan isolated from *Candida* sp. S 27 is proposed. This glucan structure (Figure 2.16 b) has a linear (1-3)-linked backbone with poly-(1-6)-linked side chains spaced every 15.3 repeat units on average. The (1-6)-linked side chain has an average length of 5.4 repeat units. There is evidence suggesting other functional group, or groups, which may be attached to the non-reducing terminus of the side chain. The structure of a glucosyl repeat unit with its attached protons numbered is shown below (Fig 2.16 a).

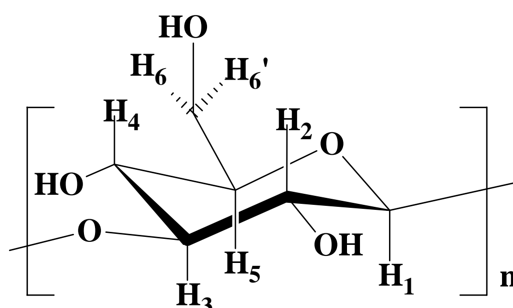

Fig 2.16 a. Structure of a glucosyl repeat unit

Fig 2.16 b. Structure of the (1-3)-β-D-glucan containing a poly-(1-6)-linked side chain. For the average polymer structure, n = 4.4 and m = 14.3.

Fig 2.11 Proton NMR spectrum from *S. cerevisiae* MTCC 36 glucan

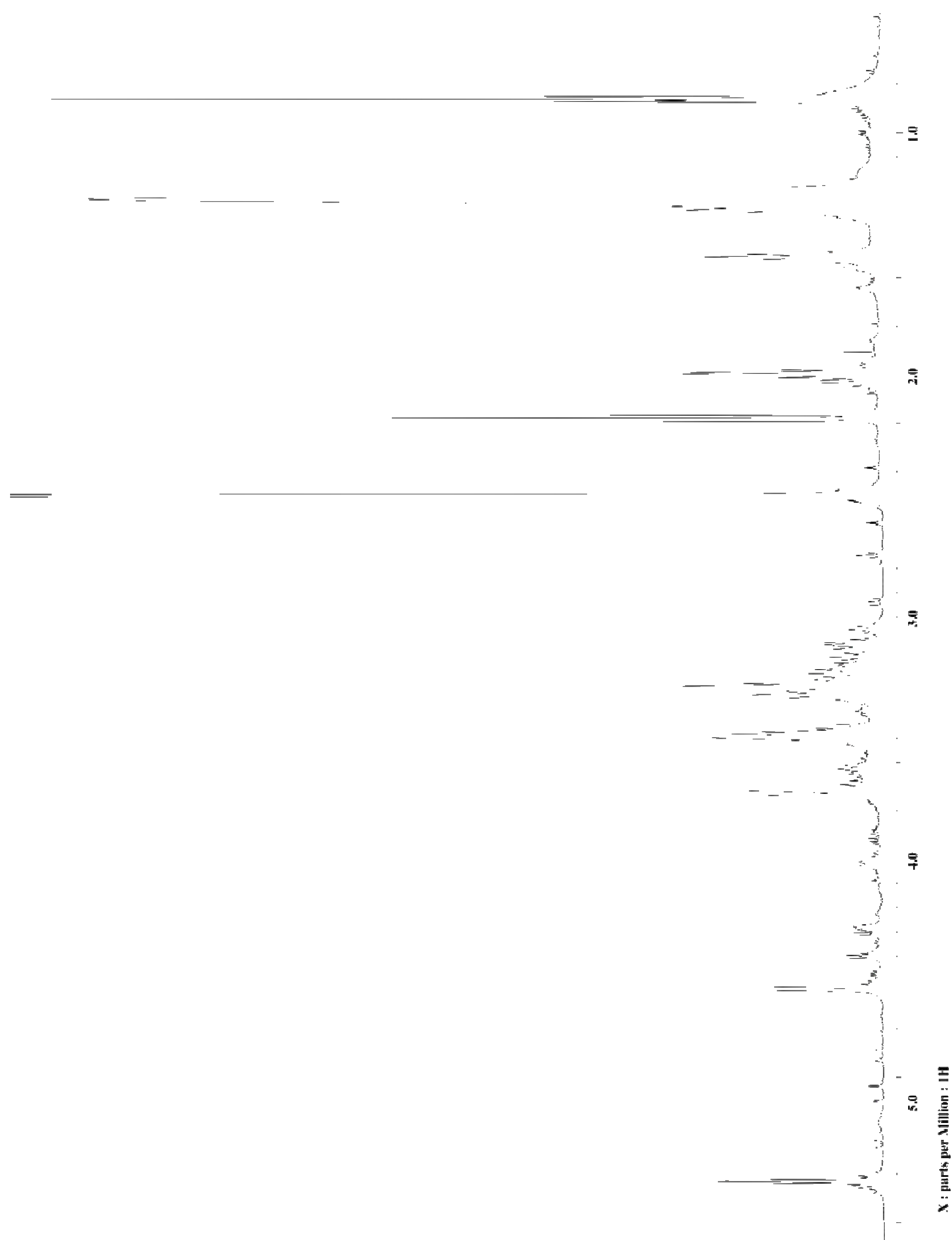

Fig 2.12 The 1D NMR spectrum of the glucan isolate from *Candida* sp. S 27 showing both its carbohydrate glucan content between 5.0 and 2.9 ppm and its lipid content between 2.2 and 0.7 ppm. The large methylene resonance from the lipid at 1.3 ppm is expanded vertically off-scale.

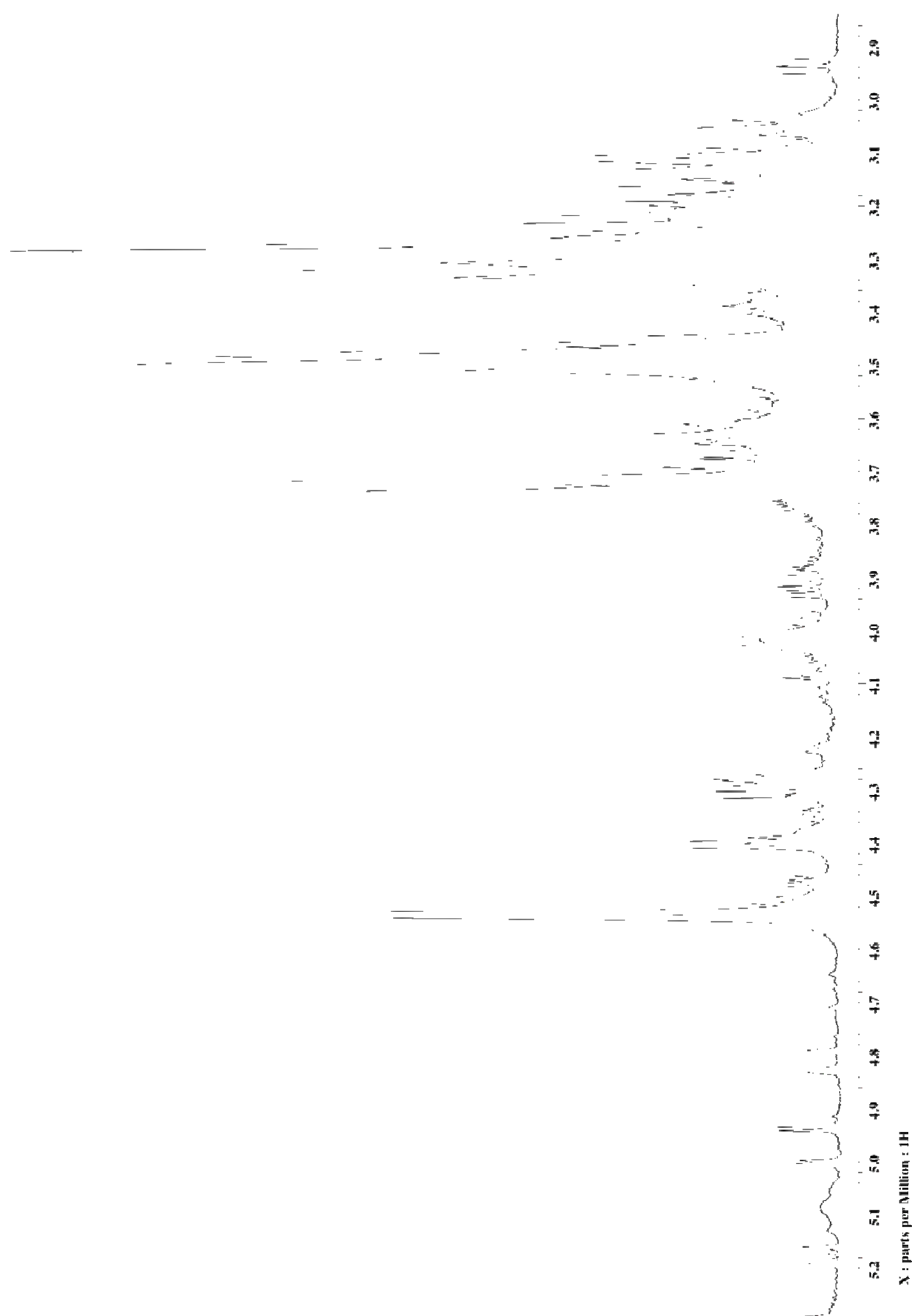

Figure 2.13 The 1D NMR spectrum of the glucan isolate from *Candida* sp. S 27 showing the glucan content between 5.0 and 2.9 ppm.

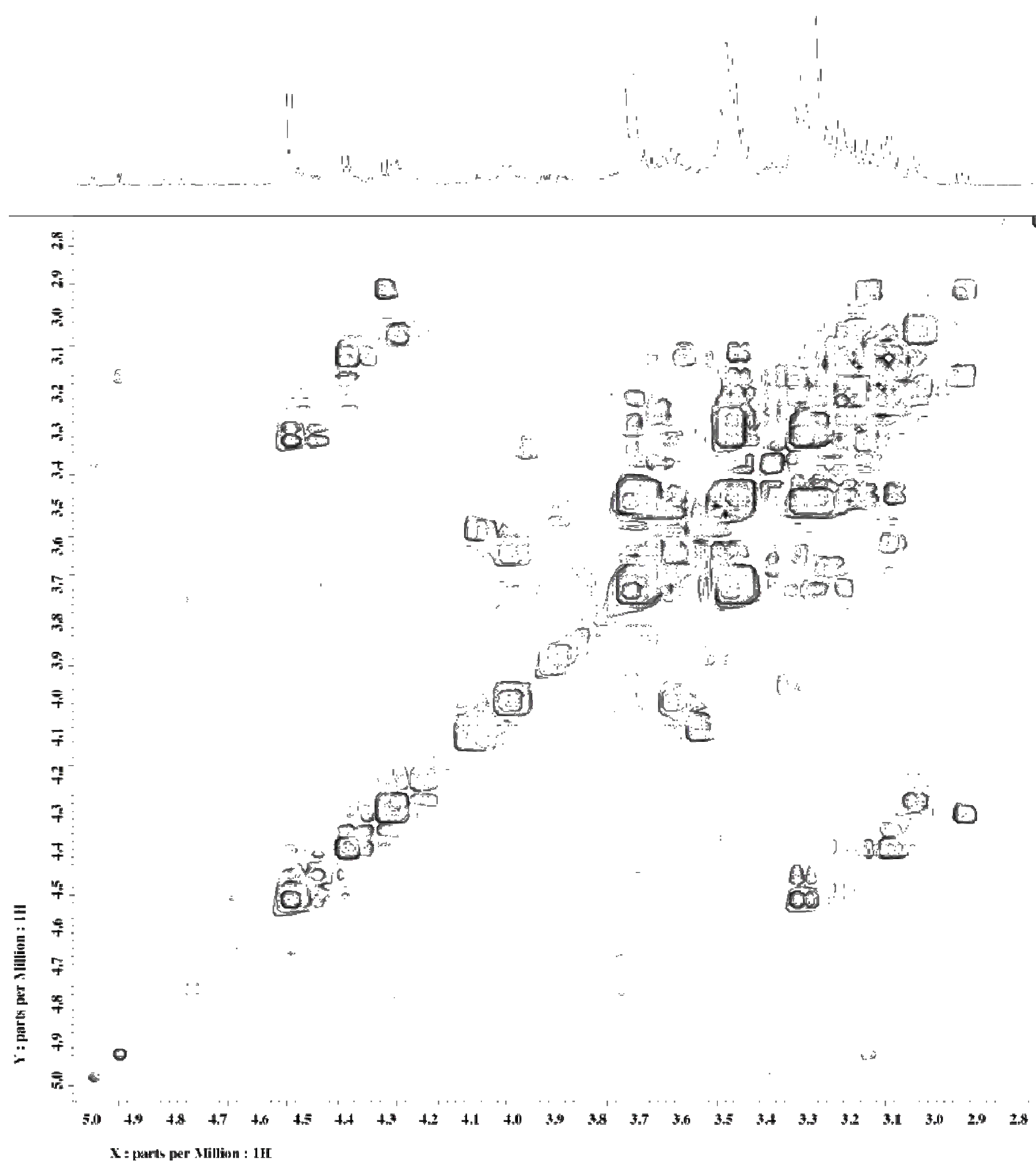

Figure 2.14 COSY 2D NMR spectrum of the glucan isolate from *Candida* sp. S 27

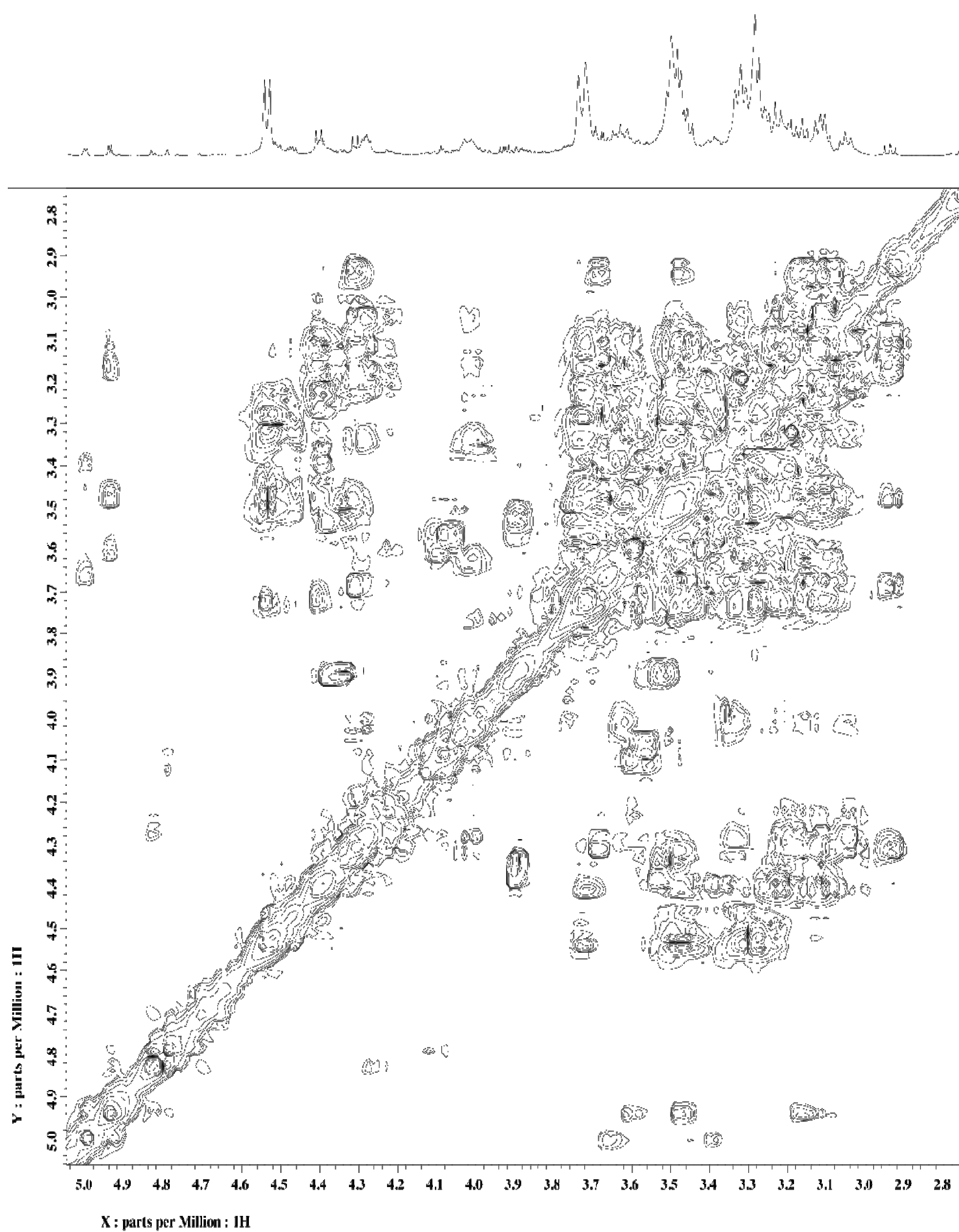

Fig 2.15 TOCSY 2D NMR spectrum of the glucan isolate from *Candida* sp. S 27

Table 2.7 Summary of Proton Chemical Shifts in ppm for Protons Assigned to Backbone Chain and (1-6)-Linked Side Chain Anhydroglucosyl Repeat Units

	Chemical Shift, ppm	
Proton Assignment	**BC**	**(1-6)-Linked Side Chain, SC(1-6)**
H1	4.53	4.27
H2	3.31	3.04
H3	3.49	3.20
H4	3.26	3.17
H5	3.26	3.32
H6	3.49	3.62
H6'	3.73	4.01

Table 2.8 Summary of Proton Chemical Shifts in ppm for glucans from the literature

	Chemical Shift, ppm		
Proton Assignment	**(1-3)-β-D-glucan[1]**	**(1-6)-β-D-glucan[2]**	**Single (1-6)-linked Side Chain[3]**
H1	4.52	4.51	4.245
H2	3.28	3.21	3.043
H3	3.46	3.52	3.228
H4	3.25	3.47	3.118
H5	3.25	3.64	3.169
H6	3.46	3.88	3.700
H6'	3.70	4.21	3.495

[1] Ensley et al. (1994)

[2] Monteiro et al. (2000)

[3] Tada et al. (2009)

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2.4 Discussion

Yeasts are classified according to conventional methods based on their morphological, physiological and biochemical properties. These methods are complex, laborious and time-consuming. Moreover, the reproducibility of these methods has been questioned because they are based on the variable physiological state of the yeasts and also influenced by the culture conditions. Recent progress in molecular biology techniques has led to the development of new methods of yeast identification and characterization, and the specificity of nucleic acid sequences has resulted in several methods for rapid species identification. Comparison of RNA (rRNA) and its template ribosomal DNA (rDNA) has been used extensively in recent years to assess both close and distant relationships among many kinds of organisms including yeast species identification. DNA based methods have the advantage of being independent of gene expression (Ness et al., 1993).

Molecular methods currently provide us with an array of techniques for easy and rapid identification of yeast. Among these techniques, 5.8S-ITS rDNA sequencing have proven to be reliable for identification at species level.

De Hoog et al. (2006) stated that if the similarity between an ITS sequence and its nearest neighbour exceeds 99 %, they are members of a single branch of the phylogenetic tree and are regarded as belonging to single species. But in the present study, the ITS sequence (512 bp) of *Candida* sp. S 27 has only 92 % similarity with its nearest neighbour DQ 292401. So they cannot be regarded as belonging to single species.

In general the topology of a phylogenetic tree depends on the characteristics of DNA sequences and tree construction algorithms utilized (Morrison, 1996). Our ITS based tree displayed generally good bootstrap support at terminal branches, distinguishing different species. Neighbor-joining method produces a unique final tree under the principle of minimum evolution. This method does not necessarily produce the minimum-evolution tree, but computer simulations have shown that it is quite efficient in obtaining the correct tree topology and is applicable to any type of evolutionary distance data (Saitou and Nei, 1987).

As more profiles are added to the database, identification will become increasingly difficult due to no or slight differences between the 5.8S-ITS profiles. Unfortunately, similar or identical 5.8S-ITS patterns do not necessarily belong to related species (Esteve-Zarzoso, 1999). Further more it has to be considered that one single mutation in the 5.8S-ITS region could lead to the loss or gain of a restriction site, resulting in a completely different pattern.

One promising alternative to overcome such an occurrence would be to sequence either the 26S rRNA gene or the 5.8S-ITS region and contrast them with the presently available databases. Both regions, but especially the 26S rRNAgene (Kurtzman and Fell, 1998), have been shown to provide enough variability to distinguish between most yeast species due to their high taxonomic value. The sequencing time requirement and cost are still too high to facilitate use in common quality control labs but may be affordable in the future. Until that time, the use of 5.8S-ITS analysis must be considered as the best method for rapid and accurate identification of yeasts.

Although all the marine yeast strains were found to be potential producers of catalase, none of the strains selected were capable of producing protease or amylase. Similar result has been presented by Chi et al. (2007) where they have suggested that little has been known about protease from marine-derived yeast. But Rishipal and Philip (1998) reported that, out of the 33 marine yeast isolates, a few showed proteolytic activity. Recently Chi et al. (2009) has reported that a few strains of the biotechnologically important yeast *A.pullulans* were capable of producing alkaline protease. These metalloenzymes have proved their potential to produce bioactive peptides from shrimp muscle and spirulina powder that have angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity (Ni et al., 2008). Chi et al. (2009) have suggested that although the commercial alkaline proteases mainly come from *Bacillus* spp. due to their high activity and tolerance to organic compounds, some properties of the alkaline protease and its gene from *A. pullulans* are similar to those of the alkaline proteases and their genes from *Bacillus* spp. Because *A. pullulans* is generally regarded as safe, the alkaline protease and its gene from *A. pullulans* can be more easily accepted than those from bacteria.

In our study, only the marine yeast strains S 186, S 51 and S 27 were lipolytic. But all the 33 marine yeast isolated by Rishipal and Philip (1998) were found to be amylolytic and most of them lipolytic. Under optimal conditions, the marine yeast *A.pullulans* N13d (2E00021) produced 10 U/ml of an extracellular glucoamylase capable of hydrolyzing potato starch granules (Li et al., 2007a).Only glucose was detected during the hydrolysis, indicating that the crude enzyme hydrolyzed both α-1,4 and α-1,6 linkages of starch molecule in the potato starch (Li et al., 2007b). Although amylase activity produced by bacteria is much higher than that produced by *A. pullulans*, the bacteria only can produce α-amylase (Nidhi et al., 2005).

According to Chi et al. (2006) very few studies exist on the lipase produced by yeasts isolated from marine environments. Although a total of 427 yeast strains were screened for lipase activity, only nine strains grown in the medium with olive oil showed lipolytic activity (Wang et al., 2007). They belonged to *Candida intermedia* YA01a (2E00162), *Pichia guilliermondii* N12c (2E00159), *Candida parapsilosis* 3eA2 (2E00087), *Loddermyces elongisporus* YF12c (2E00372), *Candida quercitrusa* JHSb (2E00033), *C. rugosa* wl8 (2E00221), *Y. lipolytica* N9a (2E00152), *Rhodotorula mucilaginosa* L10-2 (2E00168), and *A. pullulans* HN2.3 (2E00148), respectively. Only lipase from *A. pullulans* HN2.3 was extracellular, while other yeast strains tested produced cell-bound lipase. It was also found that although the crude lipase produced by *A. pullulans* HN2.3 has high hydrolytic activity towards olive oil, peanut oil, the purified lipase had the highest hydrolytic activity towards peanut oil (Liu et al., 2008).

The protein content of the biomass of marine yeast *strain Candida* sp. S 27 was 34 %. Yeasts of the genus *Candida* are most commonly used for the production of microbial protein (Bhatfacharjee, 1970; Snyder, 1970). Han et al. (1976) recorded 42 % protein in *Candida utilis*. 100 % yeast diet for bivalve molluscs had a protein content of 53.5 %, followed by 40.6 % carbohydrate, 3.3 % lipid and 2.6 % ash (Epifanio, 1979). Brown et al. (1996) noticed that protein was a major constituent in all the six marine yeast strains with the values ranging from 25 % (*Debaryomyces hansenii*) to 37 % (*Candida utilis*) dry weight. In *Torula* yeast 46.11 % protein was recorded by Olvera-Novoa et al. (2002). *Saccharomyces* sp. was found to contain

larger quantity of protein of about 48-83 % by Kamel and Kawano (1986) whereas Brown et al. (1996) noticed only 29 % protein. A marine yeast strain YA03a isolated from the surface of Sargassum from Pengalai coast in Shandong province had 56.38 % of crude protein content on the basis of dry cell weight (Chi et al., 2006). Chi et al. (2007) isolated *Yarrowia lipolytica* strains with high protein content from different marine environments. Seven *Y. lipolytica* strains contained more than 41.0g protein per 100g of cell dry weight when they were grown in the medium with 20 g/L glucose. The highest crude protein content of 47.6 % was reported for the strain SWJ-1b.

The selected marine yeast *Candida* sp. S 27 contained on dry weight basis 30.35 % of carbohydrates. Brown et al. (1996) observed that marine yeasts were also rich in carbohydrate, ranging from 21 % (*D. hansenii*) to 39 % (*S. cerevisiae*) of dry weight. Lipid content of the biomass of marine yeast *Candida* sp. S 27 was found to be 2.224 %. Lipid being a minor component was in the range 2.5- 7.7 in the six marine yeast strains isolated by Brown et al. (1996). However, Sanderson and Jolly (1994) have reported a very high content of fat (23 %) in *Phaffia*. Nucleic acid ranged from 3.88 to 8.02 % with RNA being the major constituent (80-99%) and ash ranged from 4.7 to 13 % in yeast (Brown et al., 1996). The marine yeast *Candida* sp. S 27 has higher content of nucleic acid of about 11.94 % and ash content of 6.8 %. Ash content of *Torula* yeast estimated by Olver-Novoa et al. (2002) accounted for about 4.37 % only. Nucleic acid content and ash content of *Candida utilis* were 7.1 % and 7 % of dry matter respectively (Han et al., 1976).

All the aminoacids were present in marine yeast *Candida* sp. S 27. On acid hydrolysis asparagine and glutamine were converted to aspartic acid and glutamic acid correspondingly, this was indicated by the highest concentration of aspartate and glutamate (10.4 and 18.09 % respectively) in the yeast biomass. Cysteine, methionine, histidine and arginine had the lowest concentrations (1.14- 2.87 %). Other amino acids ranged from 3.13- 8.98 %. This is found to be almost similar with the earlier report of Brown et al. (1996) where aspartate and glutamate had the highest concentrations (8.2-20 %) whereas cystine, methionine, tryptophan and histidine had the lowest (0.7-3.2 %). The other 12 amino acids ranged from 3.6- 8.4

%. Kang et al. (2006) evaluated the amino acid composition of two marine yeasts *Debaryomyces hansenii* Yeast-14 and *Candida austromarina* Yeast-16) as diet for *Moina macrocopa*. Alanine, histidine and leucine were observed to be the highest (8-10.4 %), while cysteine, serine, proline, tyrosine and methionine were lowest (0.4-3.9 %). Essential amino acids as well as total amino acid concentration was significantly higher in yeasts than the commercial diet (*Erythrobacter* sp.S п-I).

Dominant fatty acids in marine yeast *Candida* sp. S 27 were 18:1 (oleic acid), 18:2 (linoleic acid) and 16:0 (palmitic acid). It had also minute quantities of other unsaturated fatty acids such as 16: 1 (palmitoleic acid) and 17: 1 (margaroic acid) and saturated fatty acids like stearic acid, lauric acid, myristic acid etc. Highly unsaturated fatty acids were not detected in *Candida* sp. S 27. This is in agreement with the result of Brown et al. (1996) where the major fatty acids were 16:0 (13-20 % of total fatty acids) and 18:1 n-9 (12-54 %). All the six marine yeast strains contained reasonable amount of PUFA such as 18:2n-6 (16.1-64.1 %) and 18:3n-3 (0.5-9.3%). All strains lacked the highly unsaturated fatty acids like 20:5n-3 and 22:6n-3 which restrain their application as a complete larval diet. Similar results have been presented by Kang et al. (2006) where the dominant fatty acids in two marine yeast strains were 16:0 (15.8-22.9% of the total fatty acids), 18: 1n-9 (39.1-39.4%) and 18:1n-7 (10.4-17.7%). Higher proportions were also observed for 16:1n-7 (1.4-11.2%), 18:0 (4.3-10.1 %) and 18:2n-6 (2.1-3.5 %). Though low, n-3 polyunsaturated fatty acids such as 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) were detected in *Debaryomyces hansenii* Yeast-14 and *Candida austromarina* Yeast-16). For cold-adapted basidiomycetous yaests, Libkind et al. (2008) reported that linoleic, oleic, palmitic and α-linolenic acids were the major fatty acid constituents, which accounted for about 40%, 34%, 13% and 9% of total fatty acids respectively.

The marine yeast *Candida* sp. S 27 had a glucan content of 17.5 %. Sajeevan (2006) reported that the percentage yield of glucan from marine yeast *Candida sake* S 165 was 12.31 %. Vrinda (2002) extracted glucan from marine yeast strains *Debaryomyces hansenii* (S 8), *Debaryomyces hansenii* (S 169) and *Candida*

tropicalis (S 186) and found that the yield on dry weight basis were 16.732 %, 13.70 % and 16.234 % respectively.

The COSY spectrum demonstrates scalar connectivities between neighboring protons in the glucosyl ring systems while the TOCSY spectrum shows all protons that are scalar coupled within a particular spin system. The diagonal of the COSY spectrum is basically the 1D NMR spectrum while the off-diagonal peaks, or cross peaks, indicate scalar connectivities.

The anomeric proton (H1) resonance at 4.53 ppm is coupled to H2 at 3.31ppm, and then H2 is coupled to H3 at 3.49 ppm. H3 is coupled to H4 at 3.26 ppm, which overlaps the resonance for H5 at 3.26 ppm. The methylene protons of H6 are geminally coupled to each other, H6 at 3.49 ppm and H6' at 3.73 ppm, and vicinally coupled to H5. By comparison with the assignments for a linear (1-3)-β-D-glucan in Table 2.7 (Ensley et al., 1994) these resonances can be assigned to the (1-3)-β-D-glucan backbone structure. That the protons assigned to these resonances all belong to the same spin system is confirmed by examination of the cross peaks in the TOCSY spectrum (Figure 2.12). For example, consider the cross peaks associated with H1 at 4.53 ppm. Both vertical and horizontal lines from the diagonal resonance at 4.53 ppm of the TOCSY intersect with three groups of off-diagonal, or cross, peaks corresponding to the chemical shifts of the resonances assigned to the other protons in this (1-3)-linked glucosyl repeat unit.

Tada and coworkers (Tada et al., 2009) reported chemical shift assignments for single glucosyl groups attached to a (1-3)-linked polymer backbone through a (1-6)-linkage for a glucan, referred to as grifolan, isolated from the edible/medicinal mushroom *Grifola frondosa*. Of particular interest in this work by Tada and coworkers are the chemical shift assignments for H1 of the side chain AGRU (4.24 ppm) and H6 of the branch point repeat unit (4.08 and 3.59 ppm). These shifts agree very well with resonances observed at 4.27 ppm assigned to H1 and 3.62 and 4.01 ppm assigned to H6 and H6', respectively (Table 2.6) in the glucan from this study based upon cross peak correlations in the COSY and TOCSY spectra. Also resonances assigned by Tada and coworkers for H2, H3 and H4 agree well with H2 at 3.04 ppm, H3 at 3.20 ppm and H4 at 3.17 ppm observed in this work. Resonances

for H5, H6 and H6' from grifolan (Table 2.7) are not expected to agree with resonances for H5 at 3.32 ppm, H6 at 3.62 ppm and H6' at 4.01 ppm from this work since they are in a side chain containing a single repeat unit. Resonances assigned to H6 and H6' from the isolate from *Candida* sp. S 27, are deshielded to about the same degree as similar resonances assigned to H1 and H6 in a (1-6)-linked glucan isolated from *Actinobacillus suis* as shown in Table 2.7 (Monteiro and coworkers, 2000). These chemical shifts suggest that the (1-6)-linked side chain in this work is composed of several (1-6)-linked repeat units. In addition, the peak shape of the resonance for H6 at 4.02 ppm suggests several overlapping multiplet resonances. Størseth and coworkers (Størseth et al., 2006) suggested that this peak shape results from several overlapping methylene groups resulting from (1-6)-linked side chains of different, non-uniform lengths.

For the single (1-6)-linked AGRU side chain, the integral areas of H1 from the side chain AGRU at 4.24 ppm and H6 from the backbone branch point AGRU at 4.08 ppm are of equal areas. For the isolate from *Candida* sp. S 27, the integral area of the resonance at 4.02 ppm for H6 is about 23% larger than for the resonance at 4.29 ppm for H1. If the difference in areas represents the average area of one repeat unit in the side chain, then the average length of the (1-6)-linked side chain is 5.4 repeat units. By comparison to the area of the anomeric proton resonance of the backbone chain, each (1-6)-linked side chain is separated along the backbone chain by 16.3 backbone repeat units on average, including the branchpoint AGRU. We speculate that the difference in areas of these two resonances arises from the anomeric proton of the non-reducing terminus of the (1-6)-linked side chain having a different chemical shift from H1 of the other repeat units in this side chain. Reasons for this difference in chemical shift are not understood at present but may result from a different functional group being attached to the non-reducing terminal AGRU. For example, a phosphate group or one or more (1-3)-linked AGRUs could be attached to the end of the side chain. Further work will be required to describe the structure of these (1-6)-linked side chains in greater detail.

Because of its high nutritional value, feasibility of large scale production at low cost and consumer acceptability, yeast SCPs are getting wide attention currently in the feed industry especially as an aquaculture feed supplement. Six new strains of yeast were examined and evaluated as food for bivalves by Brown et al. (1996). Agh and Sorgeloos (2005) reported that the yeasts *Saccharomyces cerevisiae, Rhodotorula, Zygosaccharomyces marina, Torulopsis candida* var. *marina, Torulopsis larvae, and Saccharomyces acidosaccharophill* can be used as rotifer food. However to date, yeast is still limited in terms of its use as a partial substitute for algae as food for marine organisms because of the deficiency of certain compositional components in yeast products, particularly the lack of highly unsaturated fatty acids (HUFA), which are essential for the successful growth and development of many marine organisms.

Chapter 3

Optimization of process parameters salinity, temperature and pH for the growth of *Candida* sp. S 27

3.1 Introduction

The growth of aquatic microorganisms rely on physico-chemical factors such as incubation temperature, pH and salt concentrations, which may influence their metabolism, cell morphology and reproduction (Rheinheimer, 1980). For biomass production high specific growth rate during initial stages of cultivation is utmost important for process performance and efficiency. The specific growth rate of yeast depends strictly on the strain, its physiological state and environmental conditions such as pH, temperature and salt concentrations.

3.1.1 Culture conditions

Salinity

Salinity determines to a particularly large extent the living communities in waters. At high salt concentrations, the hypertonic environment tends to dehydrate non halotolerant microorganisms. In addition to affecting osmotic pressure, high salt concentrations tend to denature proteins, i.e. they disrupt the tertiary protein structure which is essential for enzymatic activity. Besides denaturing proteins, high concentrations of salt dehydrate cells. The deviation of salinity to some degree from the optimum prolongs the generation time in microorganisms and often causes morphological and physiological changes in them.

Studies on the growth of several marine occurring yeasts related to graded NaC1 concentrations have revealed a high salt tolerance for members of the genera *Debaryomyces, Pichia,* and *Candida,* isolated from marine fish (Ross and Morris, 1962) or sea water (Norkrans, 1966b). Although *Debaryomyces hansenii* (Zopf) van Rij, was found to be strongly salt tolerant, with turbidometrically measurable growth even at 24 % (w/v) NaCl, the lag phase was prolonged and growth rate as well as maximum total growth decreased when compared with lower NaC1 concentrations. This is because the solubility of oxygen decreased with increasing salinity, resulting in reduced oxygen supply leading to an increase in fermentation and lower total yield. *D. hansenii* is very salt tolerant and it has been reported that some strains are able to grow in the presence of 20% (w/v) NaC1 (Besancon et al., 1992).

Smittle (1977) reported that increasing concentrations of salt shifted the pH optimum for growth of *Zygosaccharomyces bailii* and *Z.acidifaciens* to lower values. For most yeasts, tolerance of NaCl decreased at the extremes of pH. Increase in salt concentration resulted in increased lag phase of *Debaryomyces hansenii* in YM (Yeast-Malt extract) broth (Sorensen and Jakobsen, 1997). The effects of pH, NaCl, sucrose, sorbic and benzoic acids on growth were examined for 30 strains of food spoilage yeasts (Praphailong and Fleet, 1997). *S. cerevisiae* and *K. marxianus* were the least salt tolerant, showing no growth at 7.5% NaCl and 10% NaCl. Medium pH influenced growth in the presence of NaCl.

It was found that maximal fusel alcohol production from branched-chain amino acids by the salt-tolerant yeast *Zygosaccharomyces rouxii* occurred at low pH (3.0–4.0) and low NaCl concentrations (0–4%) at 25°C (Jansen et al., 2003).

Higher concentration of NaCl in the cultivation medium caused changes in the composition of extracellular yeast glycoproteins (Brejerova, 1997a). Significant changes in the fatty acid composition of the cell lipids and extracellular glycoproteins of the yeast-like species *Dipodascus australiensis* grown under NaCl stress or in salt-free conditions were observed in the different growth phases (Brejerova, 1997b). High NaCl concentrations impose both ionic and hyperosmotic stress on yeast cells (Blomberg, 2000). The plasma-membrane fluidity in relation to NaCl concentrations in yeasts and yeast-like fungi isolated from either subglacial ice or hypersaline waters was studied by Turk et al. (2007). When salinity exceeded their optimal range, the ubiquitous stress-tolerant species (*A. pullulans, R. mucilaginosa*) showed increased plasma-membrane fluidity, whereas in the extremophiles (*H. werneckii, C. liquefaciens*), it decreased.

Temperature

The life processes of all microorganisms are affected by temperature. The range of temperature within which individual kinds of microorganisms live differs a great deal. Within this range, the temperature affects the growth rate, nutritional requirements and enzymatic and chemical composition of the cells (Inagraham, 1962; Precht et al., 1973). All microorganisms have a characteristic optimal growth

temperature at which they exhibit their highest growth and reproduction rates. Microorganisms also have minimal growth temperatures below which they are metabolically inactive and upper temperature limits beyond which they fail to grow. A particular challenge for microorganisms is to maintain the integrity and fluidity of their cell membranes under varying temperature conditions. High temperature can disintegrate the cell membranes, and low temperature can freeze or gel them, interrupting their vital functions.

Yeasts usually have optimum growth temperature from 25- 30 ^{0}C (Barnett et al., 1990). Kamel and Kawano (1986) noted that optimum temperature for the growth of *Candida* sp. was 37 ^{0}C and the growth declined with increase or decrease in temperature. Charoenchai et al. (1998) reported that the growth rates of *S. cerevisiae, P. anomala, K. apiculata,* and *T. delbrueckii* increased with temperature up to 25 °C. They also mentioned that the variation of medium pH between 3.0 and 4.0 did not affect the growth rate and cell biomass of wine yeasts significantly.

The pH, temperature and inoculum ratio for the production of β-galactosidase by *Kluyveromyces marxianus* CDB 002 were optimized using sugar-cane molasses (100g/L) in a lactose-free medium. Maximum cell growth was observed at 28 to 30 ^{0}C and decreased as the temperature increased. Little influence of pH was noted on the cell growth, production of ethanol and the rate of substrate consumption (Furlan et al., 2001). Elevated temperature caused significant reduction in overall biomass and ethanol yield by a thermotolerant strain of *Kluyveromyces marxianus* (Hughes et al., 1984). Optimization of physicochemical parameters for the enhancement of carbonyl reductase production by *Candida viswanathii* has been done by the classical 'one factor at a time' approach (Soni et al., 2006). The strain showed maximum growth and enzyme activity at 25 ^{0}C and the enzyme activity at 40 ^{0}C was five times lower as compared to that at 25 ^{0}C.

Kang et al. (2006) tested the optimal growth condition of two marine yeast strains *D. hansenii* (Yeast-14) and *C. austromarina* (Yeast-16) had maximal growth in the temperature range of 20-30 and 20-25 ^{0}C respectively .The marine yeasts displayed a maximum optical density in the pH range of 3-8 and showed an abrupt decline in growth with increased NaCl concentration over 5%.

The effect of temperature, pH, and salinity on the growth and dimorphism of *Penicillium marneffei* was studied by Cao et al. (2007). They compared 11 isolates of *P. marneffei* and found that all could grow at a wide range of temperature (8.0-39.8°C), but growth was dramatically inhibited at 40°C. The morphological switch from hyphae to yeast growth was initiated at 32°C. However, the sensitivity to elevated temperatures during this transition varied among isolates.

The effect of physicochemical conditions (pH, pO_2, temperature, salinity) and substrate (ethanol, propanol, tryptone and yeast extract) on the specific growth rate as well as ethanol and glycerol production rate of *Saccharomyces cerevisiae* S288C was mapped during the fermentative growth in aerobic auxoaccelerostat cultures (Kasemets et al., 2007). Due to the increase in temperature, the specific growth rate as well as the specific ATP production rate started to increase and followed the Arrhenius equation up to a temperature of 34 ^{0}C. The maximum specific growth rate obtained was 0.57 /h at a temperature range of 34–35 ^{0}C and started to decrease quickly as the temperature exceeded 38 ^{0}C.

Moeller et al. (2007) studied the influence of pH and temperature on the growth and citric acid production of *Yarrowia lipolytica* H222 using glucose as a substrate and found that the best fermentation conditions were pH 6.0 and temperature 30 °C.

pH

The pH of the culture medium affects cell growth and its influence may vary considerably among yeast strains. Apart from affecting cell membrane permeability, pH also determines the solubility of some components of the medium; thus a modification in the pH might cause some micronutrient to precipitate and so become impossible to be assimilated (Sanchez et al., 1997). The influence of the aqueous medium pH on the growth of *Candida guilliermondii* in media containing diesel oil as the main carbon source was studied by Hiss et al. (1977). The maximum values for specific growth rate and productivity were obtained at pH 6.0 and 7.0 respectively. Du Preez et al. (1984) reported that in their experiments, the most adequate pH for the growth of *C.shehatae* was between 3.5-4.5. Du Preez et al.

(1986) noted that the optimum pH was in the range of 4-5.5, and temperature 30 ^{0}C. Most yeasts prefer a pH range of 3.0-7.0 (Walker, 1977; Miller, 1979; Deak, 1991). Yeast cells are capable of accumulation of various heavy metals at lower pH values without losing their biological activities (Donmez and Aksu, 1999). The effect of temperature and salts on the growth of halotolerant yeast *Debaryomyces nepalensis* NCYC 3413 was studied by Gummadi and Kumar (2008). Specific growth rate increased with increase in temperature from 15 to 35 ^{0}C and then decreased with increase in temperature beyond 35 ^{0}C when grown in the presence or absence of different salts. At 40 ^{0}C, both NaCl and KCl at 1.0 M concentration enhanced the specific growth rate. *Debaryomyces nepalensis*, a halotolerant food spoiling yeast could grow in complex (YEPD) medium at different pH ranging between 3.0 and 11.0 in the absence of salt and at pH 3.0–9.0 in the presence of different concentrations of NaCl and KCl (Kumar and Gummadi, 2008).

3. 1. 2 Bioprocess Design

Over the years, considerable progress has been made in designing media to facilitate the commercial production of several microorganisms. In bioprocess development, medium designing is of critical importance because the composition of the medium can significantly affect product concentration, yield and volumetric productivity. It can also affect the ease and cost of downstream processes as well as overall economics. Often medium-screening exercises involve the ‘one factor at a time’ technique, i.e by varying one parameter while keeping all other parameters at constant levels. This strategy has the advantage that it is simple and easy. Most significantly, the individual effects of the medium components can be seen on a graph, without the need to revert to statistical analysis. The technique has the following major flaws such as interactions between components are ignored and the optimum can be missed completely. But because of its ease and convenience, one-at-a-time has historically been one of the most popular choices for improving medium composition (Panda et al., 1983; Shaker et al., 1985; Al-Obaidi et al., 1987).

Response Surface Methodology

Since the classical method of media optimization has been found inadequate for a full understanding of the response, optimization studies are now done using Response Surface Methodology (RSM) or Box Wilson Methodology (Box and Wilson, 1951) which is a combination of statistical and mathematical techniques widely used to determine the effects of several variables that influence the responses by varying them simultaneously in limited number of experiments. Fisher (1926) developed the basic theory of experimental design which shows that changing more than one factor at a time can be more efficient than changing only one factor at a time. Applications to medium improvement date from the 1970s and many studies claim substantial improvements over media obtained using 'one-at-a-time' (Silveria et al., 1991; Elibol, 2004; Iyer and Singhal, 2008). The statistical optimization techniques make possible the optimization of state variables such as temperature, pH, agitation etc together with quantitative variables such as medium components (Kennedy and Krouse, 1999).

The procedure involved in statistical experimental design or design of experiments (DOE) for the optimization of media can be subdivided into four steps : (i) identification of the most important media components ('screening'), (ii) identification of the optimum variable ranges ('narrowing'), (iii) identification of the optimum ('optimum search'), and (iv) experimental verification of the identified optimum (Weuster- Botz, 2000). For the identification of the most important media components, Plackett-Burman experimental designs are used, where it is assumed that no interactions between the different media components occur in the range of variables under consideration. Moreover in this design, the significance levels are determined and non-significant media components are screened out. For the identification of the optimum variable ranges (partial) factorial experimental designs are often used and to identify an optimum within the optimal variable range identified, the response surface method (RSM) is well established, where it is assumed that the estimated response surface 'Y' can be described with the aid of a second-order polynomial:

$Y = b_0+b_1X_1+ b_2X_2 +b_3 X_3+b_{12} X_1 X_2 +b_{13} X_1 X_3+b_{23} X_2 X_3+b_{11} X_1^2+b_{22} X_2^2+b_{33} X_3^2$ where Y is the estimated response, b_0 is a constant, b_i are coefficients for each term and X_i is the experimental factor and 'i' in coded units.

Response Surface Methodology (RSM) is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously (Sen and Swaminathan, 1997). This statistical technique has been extensively applied in many areas of biotechnology such as optimization of media (Farrera et al., 1998; Chakravarti and Sahai, 2002; Lai et al., 2003; Elibol, 2004; Soni et al., 2007), cultivation conditions (Sen and Swaminathan, 1997; Hujanen et al., 2001), biomass production (Lhomme and Roux, 1991), xylitol production (Silva and Roberto, 2001) and lipase production (He and Tan, 2006). The 3D response surface and the 2D contour plots are the graphical representations of the regression equations, which can be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response. Each contour curve in a 2D plot represents an infinite number of combinations of two test variables with all the others at fixed levels. The yield values for different concentrations of the variables can also be predicted from the respective contour plots (Box and Wilson, 1951; Box et al., 1958; Khuri and Cornell, 1987). The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Elibol, 2004). The shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not (Manimekalai and Swaminathan, 1999). If it is circular, the interactions between the variables are negligible and if it is elliptical the interaction between the variables are significant (Liu et al., 2003). The only disadvantage with the response surfaces is that the plotting is limited to two variables at a time.

The central composite design (CCD) is the most frequently and extensively used RSM design. Central composite design is a well established widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments (Soni et al., 2007).

A CCD can be broken down into three parts:

1) Two – level full (**2^n** where "n" is the no: of factors) or fractional factorial design
2) Axial points (**2n** where "n" is the no: of factors)
3) Center points (**n_0**)

Two–level factorial part (the core) of the design consists of all possible combinations of the plus or minus ("– 1" or "+ 1") levels of the factors. Axial points (outside the core), often represented by stars, emanate from the center point, with all but one of the factors set to 0. The coded distance of the axial points is represented as a plus or minus alpha ("– α" or "+ α"). The "α" is set default at 1.68179 in coded units, is the axial distance from the center point and makes the design rotatable and provides equally good predictions at points equally distant from the center. Center points are usually repeated to get an estimate of experimental error. Thus the central composite design requires five coded levels of each factor: "– 1" or "+ 1" (factorial points), "– α" or "+ α" (axial points), and the all zero level (center point). The variables are coded according to the following equation:

$$\text{Coded value} = \frac{[\text{Actual value} - 1/2(\text{high level} + \text{low level})]}{[1/2(\text{high level} - \text{low level})]}$$

Central Composite Designs are intended to estimate the coefficients of a quadratic model.

Response surface modeling was applied to determine the optimum temperature and pH for the growth of *Pachysolen tannophilus* in shake flasks using a two-level factorial design (Roebuck et al., 1995). The response surface analysis of the experimental results clearly revealed the interrelationship between pH and temperature. Sorensen and Jakobsen (1997) analyzed the combined effects of temperature, pH and NaCl on growth of *Debaryomyces hansenii* by flow cytometry and predictive microbiology. The effects of temperature (10, 20, 25, 30°C), pH (4.7, 5.3, 6.0) and NaCl (1, 6, 12% w/v) on the growth kinetics in YM broth were examined using a complete factorial design experiment (4 x 3 x 3 = 36). An increase

in pH had little effect on the maximum specific growth rate at low temperatures. At higher temperatures the maximum specific growth rate increased with increasing pH. An increase in temperature in the range 10-25°C increased the maximum specific growth rate with optimal growth in the range 25-30°C. The influence of pH and dilution rate on continous production of xylitol from sugarcane bagasse hemicellulosic hydrolysate by *Candida guilliermondii* using response surface methodology was done by Martinez et al. (2003). It was found that pH was significant at 90 % confidence limit.

Statistical experimental designs were applied for the optimization of phytase production by a marine yeast *Kodamaea ohmeri* BG3 in a cost-effective oats medium (Li et al., 2008). The optimum variables that supported maximum enzyme activity were oats 1.0%, ammonium sulfate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3. The validity of the optimized variables was verified at shake-flask level. An overall 9-fold enhancement in phytase activity (62.0→ 575.5 U/ml) was attained due to the optimization.

The optimization of process parameters for high inulinase production by the marine yeast strain *Cryptococcus aureus* G7a in solid-state fermentation (SSF) was carried out using central composite design (Sheng et al., 2008). They found that moisture, inoculation size, the amount ratio of wheat bran to rice husk, temperature and pH had great influence on inulinase production by strain G7a. The optimal parameters obtained with the RSM were the initial moisture 61.5%, inoculum 2.75%, the ratio of wheat bran to rice husk 0.42, temperature 29 °C, and pH 5.5. The inulinase activity produced by the yeast strain (436.2 U/g) was found to be the highest reported.

The interaction of salt (NaCl and KCl), initial pH, and temperature and their effects on the specific growth rate and lag phase of food spoiling halotolerant yeast, *Debaryomyces nepalensis* NCYC 3413 was carried out using response surface methodology based on central composite design (Lal et al., 2009). The mathematical model showed that salt has a significant effect on specific growth rate and lag phase of *D. nepalensis*. The optimal conditions of salt concentration, pH, and temperature of growth were found to be 0.3 M NaCl, 7.1, 26 °C and 0.6 M KCl, 5.6, 25°C,

respectively. Under these conditions, a maximum specific growth rate of 0.41/h and 0.5/h was observed in medium containing NaCl and KCl, respectively.

The effects of temperature, pH and sugar concentration (50% glucose + 50% fructose) on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid were studied by means of response surface methodology based on a central composite circumscribed design (Arroyo-Lopez et al., 2009).

3.2 Materials and methods

3.2.1 Organism used

The selected marine yeast *Candida* sp.S 27 was used for the study.

3.2.2 Medium used

Malt extract agar was used as the culture medium.

3.2.3 Preparation of Inoculum

Malt extract agar slants were prepared and sterilized at 121.5 ^{0}C for 15 minutes in an autoclave. The selected yeast strain was streaked on to malt extract agar slants. Incubation was done at room temperature (28 ± 2 ^{0}C) for 24 hours. The cells were harvested at the logarithmic phase using 30 $^0/_{00}$ sterile sea water. Optical density of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601). OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

3.2.4 Inoculation and Incubation

10 µl of 1 OD cell suspension was inoculated into the tubes so that the initial OD of the culture medium was 0.001. The tubes were incubated at room temperature (28 ± 2^{0}C) for 48 hours.

3.2.5 Estimation of growth

Growth was determined by measuring the optical density of the culture suspension at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601).

3.2.6 Optimization of culture conditions

Optimization of process parameters for maximum growth of the selected marine yeast *Candida* sp. S27 was tested using malt extract medium.

3.2.6.1 Experimental Design

In the present study, the process parameters salinity, pH and temperature were screened by conventional 'one-variable-at-a-time method' and further optimized statistically by a full factorial central composite design (CCD) of the RSM. . Initially the effect of each of these variables was independently observed keeping other variables constant in order to strike off the variables which did not have observable effect on yeast biomass production. The software Design expert (Version 6.0.9, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and the quadratic model building. The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using the same software.

3.2.6.1.1 First step optimization

One dimensional screening was done initially to find out the range that has to be selected for further optimization experiment.

3.2.6.1.1.1 Parameters

Salinity and pH of the culture medium and temperature for growth were the parameters subjected to study.

3.2.6.1.1.2 Preparation of medium

Malt extract broth was the culture medium used for the optimization of process parameters.

Optimum salinity

Malt extract broth in triplicate was prepared using sea water of different salinities (0, 5, 10, 15, 20, 25, 30, 35 and 40 $^{0}/_{00}$).

Optimum pH

Culture medium was prepared at different pH 2, 3, 4, 5, 6, 7, 8 and 9 using 1 N HCl and 1N NaOH.

Optimum temperature

Following inoculation the growth of the selected strains in malt extract broth was studied at different incubation temperatures (25, 30, 35, 40 and 45 ^{0}C).

3. 2.6.1.1.3 Inoculation, incubation and estimation of growth

10 µl of 1 OD cell suspension was inoculated into the malt extract tubes (10 ml) prepared in triplicate so that the initial OD of the culture medium was 0.001. Incubation was done at room temperature (28 ± 2 ^{0}C) for 48 hours. Yeast growth was estimated by measuring the optical density at 540 nm using Shimadzu UV-1601 spectrophotometer.

3.2.6.2 Second step optimization by RSM

The effect of the selected process parameters on biomass production by marine yeast *Candida* sp. S 27 has been first optimized by 'one variable at a time' technique. The minimum and maximum ranges of these parameters that had significant effect on yeast growth were considered. The most popular RSM design, the central composite design (CCD) was used to find out the optimum biomass production by marine yeast *Candida* sp. S 27 at different combinations of salinity, pH and temperature. The software, Design Expert (version 6.0.9, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and the quadratic model building. The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using the same software. A 2^3-full factorial CCD, with six axial points (a=1.682) and six replications at the centre points (n_0=6) leading to a total number of 20 experiments was employed for the optimization of the three chosen process variables. The lower and higher limits of these parameters that supported significant growth was taken into consideration as -1 and +1 respectively in Central Composite Design of RSM. Each of the three process parameters were varied over five different levels. The actual values of the variables and the combinations are presented (Table 3.1 and Table 3.2).

Table 3.1Levels of the variables tested in CCD

Variables	Coded levels				
	-α	-1	0	+1	+ α
Salinity ($^0/_{00}$) (X_1)	-11.93	0	17.5	35	46.93
Temperature (^{0}C) (X_2)	19.89	25	32.5	40	45.11
pH(X_3)	2.64	4	6	8	9.36

Table 3.2 Central composite design matrix of the three process variables

Run Order	Factors		
	Salinity ($^0/_{00}$) A (X_1)	Temperature (^{0}C) B (X_2)	pH C (X_3)
1	0 (17.5)	0 (32.5)	0 (6)
2	0 (17.5)	0 (32.5)	0 (6)
3	0 (17.5)	0 (32.5)	-α (2.64)
4	-1 (0)	-1 (25)	+1 (8)
5	0 (17.5)	0 (32.5)	0 (6)
6	+1 (35)	-1 (25)	+1 (8)
7	+1 (35)	+1 (40)	-1 (4)
8	-1 (0)	+1 (40)	+1 (8)
9	+1 (35)	+1 (40)	+1 (8)
10	0 (17.5)	0 (32.5)	0 (6)
11	+1 (35)	-1 (25)	-1 (4)
12	0 (17.5)	0 (32.5)	0 (6)
13	-1 (0)	+1 (40)	-1 (4)
14	-1 (0)	-1 (25)	-1 (4)
15	-α (-11.93)	0 (32.5)	0 (6)
16	0 (17.5)	+α (45.11)	0 (6)
17	0 (17.5)	0 (32.5)	+α (9.36)
18	+α (+ 46.93)	0 (32.5)	0 (6)
19	0 (17.5)	-α (19.89)	0 (6)
20	0 (17.5)	0 (32.5)	0 (6)

Media were prepared in all the above combinations of salinity and pH. After inoculation with yeast suspension, incubation was done at respective temperatures and the growth was measured by recording the OD at 540 nm.

3.2.6.3 Experimental verification

The optimum values of the process parameters salinity, temperature and pH were found out from the model (Table 3.7). Experiments were conducted at the above mentioned optimum levels. For this malt extract medium was prepared with 0.468 % NaCl and the pH was adjusted to 5.97. After inoculation with yeast suspension, incubation was done at 32.72 ^{0}C for 48 hours and OD was measured at 540 nm in a UV-VIS spectrophotometer. The experimental values were subsequently compared with predicted values obtained from the model equations.

**Biomass estimation (Corresponding to OD)*

For dry cell weight estimation, centrifuged known volume of 24 hour yeast culture at 7000 rpm for 15 minutes in a refrigerated centrifuge (Remi C-30, Mumbai).Washed repeatedly with 0.5% saline and transferred to a pre-weighed filter paper. The cells were dried to constant weight at 80 ^{0}C in a vacuum oven. Dry cell weight (DCW) was plotted against OD_{540} of the samples in the range of linearity (0-1 OD). Dry cell weight of 1 OD cells corresponded to 0.829 g/L DCW.

3.3 Results

3.3.1 First step optimization

3.3.1.1 Optimization of salinity

Yeast growth was found to be maximum at 30 $^0/_{00}$ salinity (OD at 540 nm = 1.649) but there was no significant change in growth with increase or decrease of salinity over the range 5 - 40 $^0/_{00}$ (OD values at 5 $^0/_{00}$ = 1.280 and at 40 $^0/_{00}$ = 1.369) (Fig.3.1a)

3.3.1.2 Optimization of pH

Maximum biomass production by *Candida* sp. S27 was found at pH 6 (OD = 1.692) and there was drastic reduction in growth with increase or decrease of pH of the culture medium (OD values at pH 2 = 0.59 and at pH 9 = 0.356) (Fig 3.1 b)

3.3.1.3 Optimization of temperature

Candida sp. S 27 exhibited fairly good growth at incubation temperatures 30-40 ^{0}C with maximum at 40^{0}C (OD = 1.634). However considerable reduction in growth could be observed beyond this range (OD at 45 ^{0}C = 1.037) (Fig 3.1 c).

The screening experiments showed that salinity, pH and incubation temperature have critical roles in the yeast biomass production. Regression analysis (Table 3.3) was done using biomass as X(dependent factor) for salinity, temperature and pH and wherever regression was found to be highly significant, the corresponding factor levels were taken for further analysis, such as 0- 35 $^{0}/_{00}$ for salinity, 4-8 for pH and 25-40 ^{0}C for temperature.

3.3.2 Second step optimization

The range for the 3 process parameters *viz.* salinity, temperature and pH was selected based on the results of optimization of 'one factor at a time' i.e. regression analysis. CCD of RSM was used to maximize the biomass production by marine yeast *Candida* sp. S 27 and to determine the interaction between the process parameters such as salinity, temperature and pH.

The coded values of the independent variables are given in Table 3.4 along with the experimental and predicted values of biomass. The experiments were performed in triplicate with the mean values taken for analysis. The matrix were analysed by standard analysis of variance (ANOVA) as approximate to the experimental design used (Table 3.5). The ANOVA of the quadratic regression model demonstrated that the model was highly significant (Table 3.6) for the concerned response i.e. Biomass as evident from the Fisher's *F*-test with a very low probability value [(P model>F) =0.0001]. In this case, linear coefficient A, quadratic coefficients such as A^2, B^2 and C^2 were significant model terms, where "A" is salinity, "B" is temperature and "C" stands for pH. The interaction coefficients were found to be not significant in determining the response. But since it was a hierarchical model, the insignificant coefficients were not omitted from the final regression equation.

The goodness of fit of the model was checked by coefficient of determination (R^2). R^2 gives a measure of how much variability in the observed response value can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. R^2 can be expressed in percentage also and then it is interpreted as the percent variability in response in the given model. The closer the value to one, the stronger the model and better will be the response predicted by the model. As per the model R^2 for biomass =0.9270. It indicated that sample variation of 92.70 % for biomass was attributed to the independent variables and only 7.3 % of the total variation was not explained by the model. Lack of fit is a measure of how well the model fits the data. If the model has a significant lack of fit, it is not a good predictor of the response and should not be used. A higher value of correlation coefficient (R) indicates an excellent correlation between independent variables but a relatively low value of coefficient of variation (CV) shows the reliability and improved precision of experiments. For biomass R is equal to 0.9628 and the coefficient of variation is 10.83 %. The purpose of statistical analysis to determine which experimental factors generate signals, which are large in comparison to noise. Adequate precision measures the signal to noise ratio. (A comparison of the influence of controllable factors (*signal*) to the influence of uncontrollable factors (*noise*).). A ratio greater than 4 is desirable. As per the model, the adequate precision ratio obtained was 10.531 for biomass, indicating an adequate signal. In the case of biomass, the "Pred R-Squared" of 0.5909 is not as close, but still in agreement to the "Adj R-Squared" of 0.8612 which indicates that the current model is still a good predictor of our concerned response ie. Biomass

The RSM gave the following regression equation for the biomass Y_1 as a function of salinity (X_1), temperature (X_2) and pH (X_3).

Final equation in terms of coded factors is:

Y_1 = -5.10811 + 7.75559E-003 X_1 + 0.34164 X_2 + 0.36424 X_3 -2.59825E-004X_1^2– 5.62500E-003X_2^2 -0.042188 X_3^2+ 3.15048 E-005 X_1 X_2 -1.06329E-003 X_1X_3+ 4.41067E-003 X_2 X_3

Based on the data generated, the software Design-Expert suggested a single solution as the optimum values of the physical parameters. (Table 3.7).

Table 3.7 Optimum values of the physical parameters recommended by the software

Solution No:	Salinity ($^0/_0$)	Temperature (^{0}C)	pH	Biomass	Desirability	
1	4.68	32.72	5.97	1.58647	0.999	Selected

The 3D response surface and the 2D contour plots are the graphical representation of the regression equation. Response surface and contour plots of the above mentioned model was drawn using the software Design Expert and they provided a method to visualize the relation between the response and experimental levels of each variable and the type of interaction between the test variables.

The circular contour plots indicated that the interactions between the process parameters salinity, temperature and pH were not significant (P>0.05). The two-dimensional contour plot and its respective response surface plot (Fig 3.2) on biomass show the effect of salinity and temperature on yeast biomass production at the optimum pH (5.97). The yeast growth was found to be maximum at salinity 4.68 $^0/_{00}$ (1.59g/L) and decreased with increase of salinity. At lower salinity, the biomass yield increased gradually from 1.10 g/L to 1.59 g/L with increase in temperature up to 32.72^{0}C but decreased slowly beyond the range. The Fig 3.3 shows similar plots at various salinities and pH at optimum temperature (32.72^{0}C). With increase in pH up to 6, the biomass production was enhanced and thereafter it decreased. The effect of pH and temperature on yeast growth at fixed level of salinity (4.68 $^0/_{00}$) is presented in Fig 3.4. It is evident that at low temperature the biomass yield steadily increased with increase in pH up to 6, but the yield decreased at higher temperature and pH (1.10g/L).

3.3.3 Experimental verification of identified optimum from the model

The validation was carried out under optimum process conditions predicted by the model. The experimental value of biomass (1.63) was closer to the predicted

value (1.58647) validating the model thus proving Response Surface Methodology as a powerful tool for optimization of growth conditions.

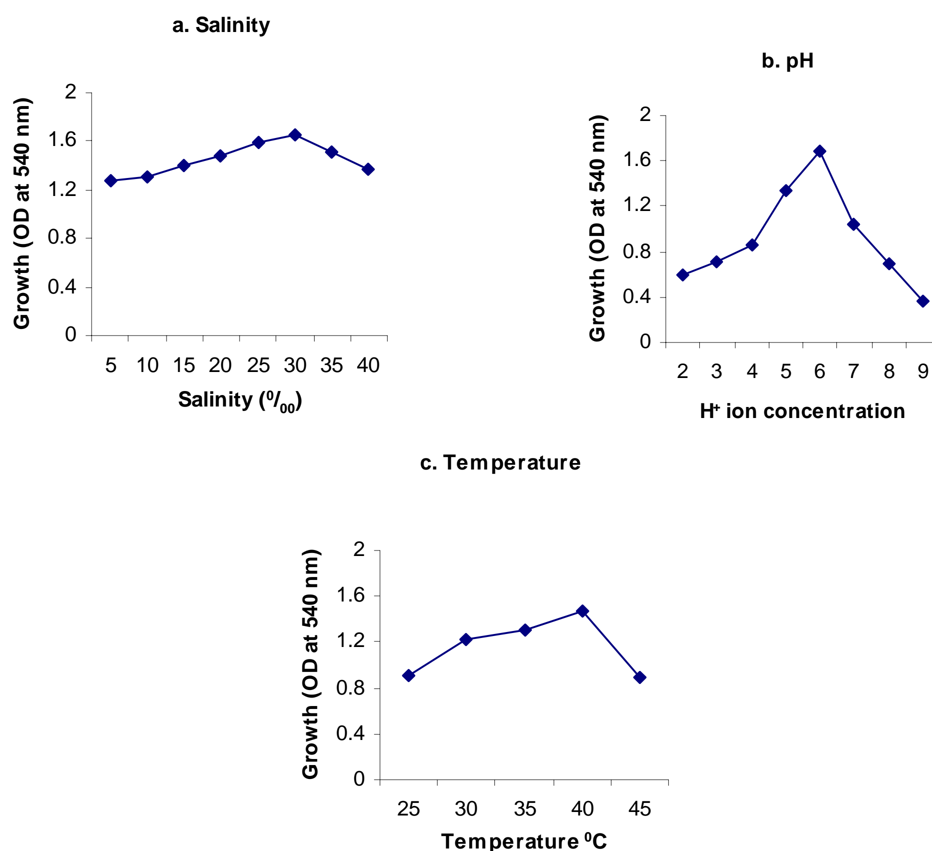


Fig 3.1 a-c. Growth of *Candida* sp. S 27 in malt extract broth at different salinity, pH and temperature

Table 3.3. Factor levels used for regression analysis and their significance in the first step optimization

Salinity	Significance F	pH	Significance F	Temperature	Significance F
0 $^{0}/_{00}$	0.06825	2	0.06508	25 ^{0}C	0.00346
5 $^{0}/_{00}$	0.00292	3	0.09836	30 ^{0}C	0.00196
10 $^{0}/_{00}$	0.00858	4	0.03672	35 ^{0}C	0.01984
15 $^{0}/_{00}$	0.01090	5	0.01245	40 ^{0}C	0.00895
20 $^{0}/_{00}$	0.01785	6	0.00168	45 ^{0}C	0.2157
25 $^{0}/_{00}$	0.03154	7	0.02450		
30 $^{0}/_{00}$	0.00364	8	0.03689		
35 $^{0}/_{00}$	0.04919	9	0.08154		
40 $^{0}/_{00}$	0.05483				

Table 3.4 Central composite design matrix of the three variables along with the experimental and predicted values of biomass.

Run Order	Factors			Biomass (g/L)	
	Salinity ($^0/_{00}$) A (X_1)	Temperature (^{0}C) B (X_2)	pH C (X_3)	Experimental	Predicted
1	0 (17.5)	0 (32.5)	0 (6)	1.34	1.54
2	0 (17.5)	0 (32.5)	0 (6)	1.59	1.54
3	0 (17.5)	0 (32.5)	-α (2.64)	1.20	1.12
4	-1 (0)	-1 (25)	+1 (8)	1.13	1.01
5	0 (17.5)	0 (32.5)	0 (6)	1.59	1.54
6	+1 (35)	-1 (25)	+1 (8)	0.79	0.70
7	+1 (35)	+1 (40)	-1 (4)	0.86	0.89
8	-1 (0)	+1 (40)	+1 (8)	1.25	1.18
9	+1 (35)	+1 (40)	+1 (8)	1.00	0.88
10	0 (17.5)	0 (32.5)	0 (6)	1.59	1.54
11	+1 (35)	-1 (25)	-1 (4)	0.99	0.97
12	0 (17.5)	0 (32.5)	0 (6)	1.59	1.54
13	-1 (0)	+1 (40)	-1 (4)	1.03	1.05
14	-1 (0)	-1 (25)	-1 (4)	1.11	1.14
15	-α (-11.93)	0 (32.5)	0 (6)	1.47	1.51
16	0 (17.5)	+α (45.11)	0 (6)	0.65	0.69
17	0 (17.5)	0 (32.5)	+α (9.36)	0.81	1.01
18	+α (+ 46.93)	0 (32.5)	0 (6)	1.04	1.12
19	0 (17.5)	-α (19.89)	0 (6)	0.53	0.61
20	0 (17.5)	0 (32.5)	0 (6)	1.59	1.54

Table 3.5 ANOVA for Response Surface Quadratic Model (as obtained from the software Design-Expert)

Coefficient	F value	P value
A	11.93	0.0062
B	0.45	0.5177
C	1.04	0.3324
A^2	5.81	0.0367
B^2	91.87	<0.0001
C^2	26.13	0.0005
AB	8.71E-003	0.9275
BC	0.71	0.4206
AC	2.23	0.1662

Significant coefficients are highlighted in red

Table 3.6 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	F-value	Probability P>F
Model	1.99	9	0.22	14.10	0.0001
Residual error	0.16	10	0.016		
Lack of fit	0.11	5	0.021	2.060	0.2231
Pure error	0.051	5	0.010		
Cor total	2.15	19			

SS- sum of squares; DF- degrees of freedom; MS- mean square.

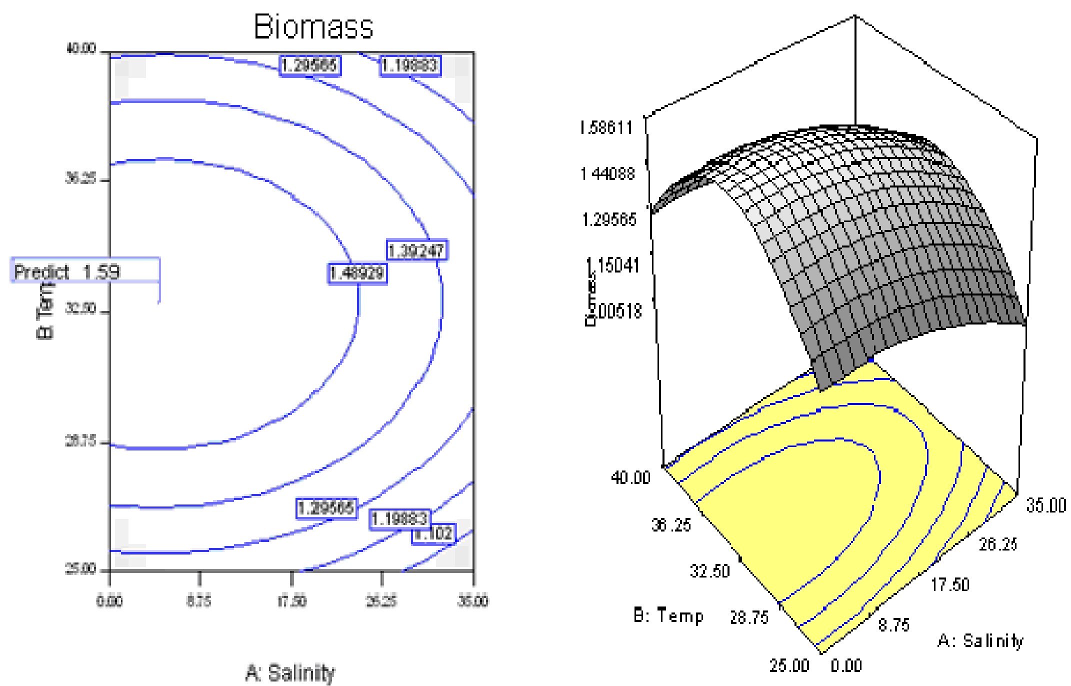


Fig 3.2 Contour plot and the corresponding response surface plot of biomass (g/L) vs. X=A: salinity and Y=B: temperature at constant pH (6)

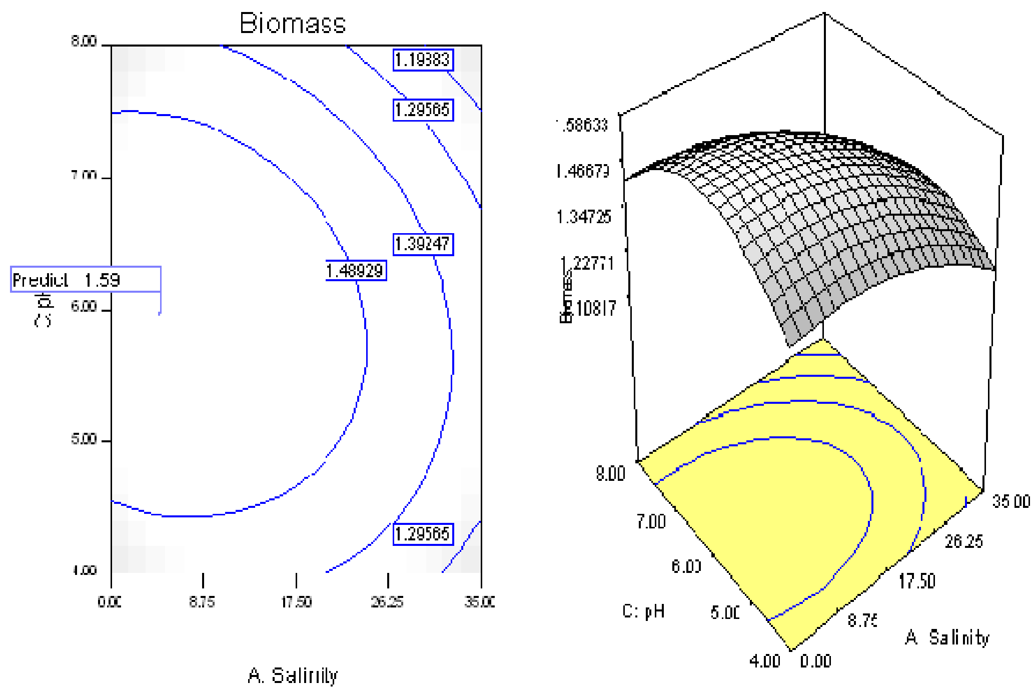


Fig 3.3 Contour plot and its corresponding response surface plot of biomass (g/L) vs. X = A: salinity and Y= C: pH at constant temperature (32.72 ^{0}C)

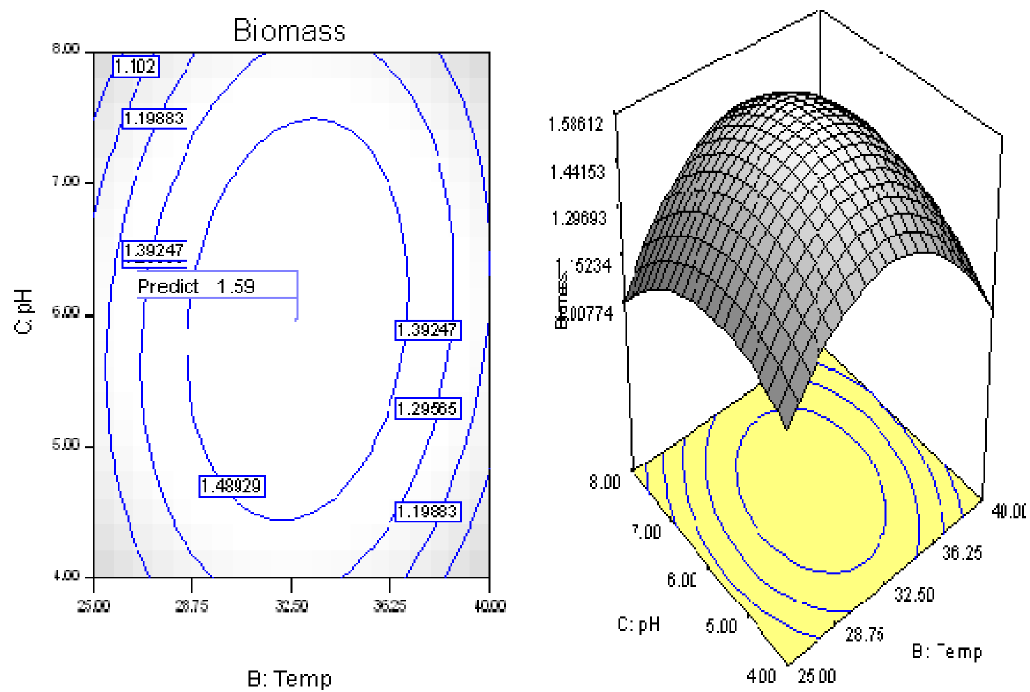


Fig 3.4 Contour plot and its response surface plot of biomass (g/L) vs. X=B: temperature and Y=C: pH at constant salinity (4.68 $^{0}/_{00}$)

3. 4 Discussion

Many environmental factors affect the growth of yeasts but the response to any particular condition varies with the species (Rose, 1987). The marine yeast *Candida* sp. S 27 has a euryhaline characteristic with ability to grow at salinities 5-40 $^{0}/_{00}$. Most aquatic yeasts are known to be capable of growing at a concentration of NaCl exceeding the range normally found in seawater (Hagler and Ahearn, 1987). Kang et al. (2006) reported that the cell growth of two marine yeast strains (*D. hansenii* Yeast-14) and *C. austromarina* (Yeast-16) varied depending on the gradients of NaCl concentration, pH and temperature. The optical density of *D. hansenii* was dramatically decreased at the concentration of more than 5% NaCl, without any significant difference from 0% to 4% NaCl. The optical density of *C. austromarina* exponentially increased with increasing NaCl from 0% to 2.5% NaCl and rapidly decreased at 3% NaCl, being significantly higher at the concentration from 1.5% to 3%NaCl with maximum at 2.5%. The growth of *C. austromarina* was extended even up to pH 9. *D. hansenii* grew well at 20, 25 and 30 ^{0}C and *C. austromarina* did well at 20 and 25 ^{0}C. For both species, low growth was observed for the other tested levels.

Environmental pH is particularly significant in determining the growth of yeasts (Pitt, 1974; Cole and Keenan, 1986). Yeasts are known to grow in a broad pH range from below 2.0 to over 9.0 (Hagler and Ahearn, 1987). They prefer an acidic pH for growth, thus preventing bacterial contamination during mass production. Although yeasts do not generally grow well at alkaline pH, certain marine yeasts are specially adapted to growing in slightly alkaline seawater (Walker, 1998).The marine yeast *Candida* sp. S 27 preferred pH 5.97 for maximum biomass production. Anas and Singh (2003) reported that the yeast *Acremonium dyosporii* preferred pH 4 for higher cell yield. pH of the media, between 3 and 8 supported the growth of marine yeasts *D. hansenii* (Yeast-14) and *C. austromarina* (Yeast-16) (Kang et al., 2006).

In the present work, the optimum temperature for the growth of marine yeast *Candida* sp. S 27 determined by RSM was 32.72 °C. In terms of temperature

preference, this yeast species would be suitable for mass cultivation in a tropical country like India were the ambient atmospheric temperature is closer to this value.

The statistical optimization of process parameters for high inulinase production by the marine yeast strain *Cryptococcus aureus* G7a in solid-state fermentation (SSF) using central composite design showed that temperature and pH had great influence on inulinase production the optimal values being temperature 29 °C and pH 5.5 (Sheng et al., 2008).Response surface optimization of temperature and pH for the growth of *Pachysolen tannophilus* revealed the interrelationship between pH and temperature (Roebuck et al., 1995). The maximum concentration of *P. tannophilus* corresponded to a point defined by pH 3.7 and a temperature of 31.5 °C. The least favourable growth occurred at higher temperatures, particularly at lower pH values below the optimum.

Sorensen and Jakobsen (1997) analyzed the combined effects of temperature, pH and NaCl on growth of *Debaryomyces hansenii* using a complete factorial design experiment. They found that an increase in pH had little effect on the maximum specific growth rate at low temperatures. At higher temperatures the maximum specific growth rate increased with increasing pH. An increase in temperature in the range 10-25°C increased the maximum specific growth rate with optimal growth in the range 25-30°C.

The influence of pH and dilution rate on continous production of xylitol from sugarcane bagasse hemicellulosic hydrolysate by *Candida guilliermondii* using response surface methodology was determined by Martinez et al. (2003).The statistical results clearly indicated that the interaction effects of pH and dilution rate were significant. The optimum process variables that supported maximum phytase production by a marine yeast *Kodamaea ohmeri* BG3 were statistically determined as 2.0% NaCl and initial pH 6.3 (Li et al., 2008).

Lal et al. (2009) noticed that the growth of the food spoilage yeast *D. nepalensis* in NaCl and KCl was completely inhibited when temperature was maintained at extreme conditions (-1 and +1). At extreme pH of 3.6, specific growth was 0.29 and 0.37 /h for NaCl and KCl, respectively exhibiting the yeast's preference

for acidic condition for growth. Optimization of the process parameters using RSM clearly showed the strong interaction effect between the pH, temperature, and salt concentration on the growth of food spoilage yeast *D. nepalensis*.

Though there was no significant interaction between the process parameters, the linear coefficient of salinity and quadratic coefficients of salinity, temperature and pH were significant in determining maximum yeast biomass production. After the optimization by using response surface methodology, the optimal parameters for the mass production of marine yeast *Candida* sp. S 27 were salinity 4.68 $^0/_{00}$, temperature 32.72 ^{0}C and pH 5.97.Under the optimized condition, biomass production increased notably indicating that the chosen method of optimization of process parameters was efficient, relatively simple and time and material saving.

Chapter 4

Optimization of a chemically defined medium for biomass production of *Candida* sp. S 27

4.1 Introduction

Yeast growth is affected not only by temperature, pH, NaCl concentration but also by the intrinsic properties of the investigated system, namely, type of strain, culture medium, and physiological state of the inoculum. The microbial environment is largely determined by the composition of the growth medium. The cellular functions, in particular, phenotypes of microbial strains are modulated by concentration of ions in the culture medium and differences in elemental levels. Perhaps this may be the reason for phenotypic variability observed when microbial strains are grown on synthetic versus complex media (Abelovska et al., 2007). Although complex media are often convenient since they contain all nutrients, they suffer from the disadvantage of being undefined and sometimes variable in composition, which may mask important nutritional effects. For this reason, studies are difficult to interpret in detail because of inadequate characterization of growth-limiting factors in the media. Thus, in order to elucidate the nature of nutritional effects as far as possible, synthetic or chemically defined media (CDM) must be used. Though in certain complex media, growth is often faster than in chemically defined media, the latter is preferred for repeatability.

Although carbon, nitrogen, oxygen, phosphorus and sulphur are major constituents of living cells, additional components are required for various cellular activities. For example, sodium and potassium are the principle ions involved in generation of electrochemical gradients across cell membranes; calcium is required for signal transduction; magnesium, copper, iron and manganese are necessary for various enzymatic activities; and zinc is a required cofactor for structural integrity of various proteins. Cells employ various strategies to regulate intracellular distribution and concentration of these elements to prevent their potential toxic effects.

In defined medium, the carbon and nitrogen sources play a critical role as a source of precursors and cofactors for synthesis of biomass building blocks. Basic nutritional requirements of heterotrophs for growth in defined medium are

sources of glucose, NH_4^+, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}, PO_4^{3-} (Moat, 1979). Since biomass is typically 50 % carbon on a dry weight basis, carbon sources are present in the media in concentrations higher than other nutrients and are used in the range 0.2-20 %. Majority of microbes are able to assimilate ammonium salts, ammonium sulphate being the least expensive and most commonly used inorganic nitrogen source (Solomons, 1969). Trace amounts of potassium, calcium, iron, molybdenum, cobalt, zinc, manganese and copper required by the microbes are to be supplied in the culture medium.

Metal ions of potassium, magnesium, calcium, and zinc have been reported to influence the rate of sugar conversion and biomass accumulation by yeasts (Chandrasena and Walker, 1997). Amount of SCP production can be improved with the addition of ammonium sulphate as nitrogen supplement (Cristiani-Urbina et al., 2000; Moeini et al., 2004).

A cultivation medium is designed to reflect the elemental composition and the biosynthetic capacity of a given microbial cell. The design of CDM is quite laborious, time-consuming, costly, and less reliable because, although the cellular compounds of microorganisms are very similar, the specific nutrient requirements differ widely among microbial species. Many methodologies, like the single-omission technique, elemental composition analysis of biological materials, and statistically designed experiments, have been applied to formulate chemically defined media for various microorganisms (Spaargaren, 1996; Mantha et al., 1998; Zang and Greasham, 1999; Shi et al., 2006; Preetha et al., 2007).

Supplementation of defined media with exogenous nitrogen sources improved biomass formation by *S. cerevisiae*, especially during fully respiratory growth on ethanol (Thomas and Ingledew, 1990, 1992; Gu et al., 1991; Chen et al., 1993). Nitrogen sources most strongly preferred by *S. cerevisiae* include glutamine, asparagine and ammonium. These compounds are utilized first from mixtures of nitrogen sources and support higher growth rates than less preferred nitrogen sources (Cooper, 1982; Wiame et al., 1985; Dubois and Messenguy, 1997; Ter Schure et al., 2000). Exogenous amino acids can be incorporated directly into biomass during consumption (Albers et al., 1996), whilst amino

acids have been known to improve the production of heterologous proteins by *S. cerevisiae* in defined media (Blechl et al., 1992; Mendoza-Vega et al., 1994; Wittrup and Benig, 1994; Toman et al., 2000).

The influence of ammonium, phosphate and citrate on astaxanthin production by the yeast *Phaffia rhodozyma* in a chemically defined medium was investigated by Flores-Cotera (2001). The astaxanthin content in cells and the total pigment concentration in the broth increased upon reduction of ammonium and phosphate from 61 mM to 12.9 mM and from 4.8 mM to 0.65 mM respectively.

A chemically defined medium was developed in order to investigate the influence of carbon and nitrogen sources on lovastatin biosynthesis by *Aspergillus terreus* (Hajjaj et al., 2001). Among several organic and inorganic nitrogen sources metabolized by *A. terreus*, glutamate and histidine gave the highest lovastatin production. A threefold-higher specific productivity was found with the defined medium on glucose and glutamate, compared to growth on complex medium with glucose, peptonized milk, and yeast extract.

A chemically defined medium that included urea (5 g /L) as a nitrogen source and various vitamins was substituted for a complex medium containing yeast extract (10 g/L) in the production of xylitol by *Candida tropicalis* (Kim and Oh, 2003). In a fed-batch culture with the chemically defined medium, 237 g xylitol /L was produced from 270 g xylose /L after 120 h. The volumetric rate of xylitol production and the xylitol yield from xylose were 2 g /L/h and 89%, respectively indicating that xylitol could be produced effectively in a chemically defined medium.

Supplementation of a chemically defined medium with amino acids or succinate to improve heterologous xylanase production by a prototrophic *Saccharomyces cerevisiae* transformant showed that alanine, arginine, asparagine, glutamic acid, glutamine and glycine improved both biomass and xylanase production, whereas several other individual amino acids inhibited biomass and/or xylanase production (Gorgens et al., 2005).

Isolates of *Dekkera* and *Brettanomyces* when cultured in a defined medium supplemented with hydroxycinnamic acids and vinylphenols exhibited reduced growth with no significant conversion to vinyl or ethyl derivatives. The growth rate and substrate utilisation rates of *Dekkera anomala* and *Dekkera bruxellensis* differed depending on strain and the acid precursor present. Growth of *D. bruxellensis* slowed in the presence of ferulic acid and 1 mM ferulic acid inhibitied the growth completely (Harris et al., 2008).

The impact of medium composition on ethanol tolerance of the self-flocculating yeast SPSC01 was investigated by Xue et al. (2008) using a chemically defined medium. The highest cell viability (90.2%) of SPSC01 against ethanol shock treatment was observed in the optimized medium containing $(NH_4)_2SO_4$ 9 g/L, K_2HPO_4 2 g/L, vitamin mixtures 9 x base (original vitamin level in the starting defined medium before optimization) and the optimal concentrations of magnesium sulfate, calcium chloride and zinc sulfate were 0.4, 0.2, and 0.01 g/L, respectively.

Media development through statistical experimental design

The optimal design of the culture medium is a very important aspect in the development of a bioprocess. Classical experimental design requires that only one variable be changed at a time to determine its effect. Since chemically defined medium contains a fairly good number of components, it is not feasible to study independently each component for its effect on microbial growth. Moreover, such experiments would not account for the interactions between the components. The real optimum response may not be found if these interactions are significant. To reduce the number of experiments despite the large number of variables, methods of statistical experimental design are followed. These are very useful tools as they can provide statistical models which help in understanding the interactions among the process parameters at varying levels and in calculating the optimal level of each parameter for a given target.

A rapid and effective method of approach for media optimization is the Response Surface Methodology. It is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and

searching for the optimum conditions (Kalil et al., 2000). It is a statistically designed experimental protocol in which several factors are simultaneously varied.

The Central Composite Design (CCD) is an effective RSM design that is used for sequential experimentation and provides reasonable amount of information for testing the goodness of fit and does not require unusually large number of design points thereby reducing the overall cost associated with the experiment. CCD has following three set of experimental runs: (1) Fractional factorial runs in which factors are studied at +1, −1 levels. (2) Center points with all factors at their center points that help in understanding the curvature and replication helps to estimate pure error. (3) Axial points which are similar to center point, but one factor takes values above and below the median of two factorial levels typically both outside their range and axial points make the design rotatable.

Response surfaces, the graphical representations of the regression equations provide a method to visualize the relation between the response and experimental levels of each variable and the type of interaction between the test variables in order to deduce the optimum conditions. The response surfaces are inverted paraboloids that can be used to predict the biomass yields for different values of the test variables and to identify whether the major interactions between the test variables are significant or not from the elliptical or circular nature of contour.

Response surface methodology was applied to optimize the fermentation medium for stereoinversion of (S)-1-phenyl-1,2-ethanediol by *Candida parapsilosis* CCTCC M203011 (Mu et al., 2005). The optimal value for concentration of $MgSO_4$, Zn SO_4 and KH_2PO_4 were 1.17 g/L, 0.10 g/L and 9.41 g/L, respectively. Using this statistical optimization method, the enantioselective conversion ratio increased from 75.2% to 96.0%, while the enantiomerically excess increased from 84.2% enantiomeric excess (e.e.) to 96.7% e.e.

Factorial designs and second order response surface methodology (RSM) were employed for the optimization of medium composition for biomass

production of recombinant *Escherichia coli* cells (Nikerel et al., 2006). The concentrations of carbon source (glucose), inorganic nitrogen ($(NH_4)_2HPO_4$), potassium (KH_2PO_4) and magnesium ($MgSO_4$. $7H_2O$) sources in medium were changed according to the central composite rotatable design consisting of 29 experiments, and the biomass yield was calculated. The optimum medium composition was found to be 15 g /L glucose, 6.6 g/L $(NH_4)_2HPO_4$, 20.1 g/L KH_2PO_4 and 1.7 g/L $MgSO_4$. $7H_2O$. The model prediction of 2.72g dry cell weight (DCW)/L biomass at optimum conditions was verified experimentally as 2.68g DCW/L .

A chemically defined medium was optimized for the maximum biomass production of recombinant *Pichia pastoris* in the fermentor cultures using glycerol as the sole carbon source and $(NH_4)_2SO_4$ as the nitrogen source (Ghosalkar et al., 2008). Optimization was done using the statistical methods for getting the optimal level of salts, trace metals and vitamins for the growth of recombinant *P. pastoris*. The response surface methodology was effective in optimizing nutritional requirements using the limited number of experiments. The optimum medium composition was found to be 20 g/L glycerol, 7.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4$. $7H_2O$, 8.5 g/L KH_2PO_4, 1.5 mL/L vitamin solution and 20 mL/L trace metal solution. Using the optimized medium the biomass produced was 11.25 g dry cell weight/L giving an yield coefficient of 0.55 g biomass/g of glycerol in a batch culture. Thus a dynamic approach to bioprocess optimization could be carried out by the employment of a mathematical model developed to describe yeast growth.

4. 2 Materials and methods

4.2.1 Organism used

The marine yeast *Candida* sp. S 27 was used for the study.

4.2.2. Culture conditions

The optimal culture conditions obtained as per the Response Surface Methodology (RSM) studies in Chapter 3 were used in this study .The optimal conditions were salinity 4.68 $^0/_{00}$, pH 5.97 and temperature 32.72 ^{0}C.

4.2.3 Medium used

The basal medium selected for yeast biomass production was the one formulated by Barnett and Ingram (1955). The composition is given in Table 4.1

Table 4.1 Composition of the basal medium (per Litre)

Nitrogen sources	$(NH_4)_2 SO_4$	3.5 g
	L- Asparagine	1.5 g
Carbon source	D-Glucose	10 g
Amino acids	L-Histidine	10 mg
	DL- Methionine	20 mg
	DL-Tryptophan	20 mg
Growth factors (Vitamin mix)	p-Aminobenzoic acid	200 µg
	Biotin	20 µg
	Folic acid	2 µg
	Myo-inositol	10 mg
	Nicotinic acid	400 µg
	Pantothenate (Ca)	2 mg
	Pyridoxine HCl	400 µg
	Riboflavin	200 µg
	Thiamin HCl	400 µg
Trace element sources	H_3BO_3	500 µg
	$CuSO_4.5 H_2O$	40 µg
	KI	100 µg
	$FeCl_3. 6H_2O$	200 µg
	$MnSO_4.4H_2O$	400 µg
	$Na_2MoO_4.2H_2O$	200 µg
	$ZnSO_4.7H_2O$	400 µg
Salts	KH_2PO_4	850 mg
	K_2HPO_4	150 mg
	$MgSO_4.7H_2O$	500 mg
	NaCl	100 mg
	$CaCl_2.6 H_2O$	100 mg

Preparation of medium

The media components (glucose, $(NH_4)_2SO_4$, L- Asparagine, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.6\ H_2O$) were weighed and transferred to distilled water containing NaCl (4.68g/L) and sterilized at 10 lbs for 10 minutes. Stock solutions of growth factors, amino acids and trace element sources were prepared, filter sterilized and the required quantities were added to the cooled sterile medium and pH was adjusted to 5.97.

4.2.4 Experimental Design

4.2.4.1 First step optimization

The concentration range of media components were screened using the traditional 'one-variable at a time' (OVAT) strategy. The concentration of one ingredient was varied over the given range (Table 4.2), while, all the other ingredients were kept at their standard levels as per Barnett and Ingram (1955). Likewise, all the ingredients were tested over their respective concentration ranges, while keeping all others at standard levels. All the experiments were done in triplicate. Biomass was determined. Regression analysis was done using biomass and media component and wherever regression was found to be highly significant, the corresponding factor level was taken for further analysis.

Table 4.2 Media components and the concentrations selected for first step optimization

	Ingredient	Concentration (g/L)
1	Glucose	5, 10, 20, 30, 40 and 50
2	$(NH_4)_2SO_4$	3, 4, 5, 6, 7, 8, 9 and 10
3	KH_2PO_4	2, 3, 4, 5, 6, 7 and 8
4	K_2HPO_4	0.05, 0.01, 0.2, 0.3, 0.4 and 0.5
5	$MgSO_4.\ 7H_2O$	0.5, 1, 1.5, 2, 2.5 and 3
6	$CaCl_2.\ 2H_2O$	0.1, 0.2, 0.3, 0.4 and 0.5
7	Asparagine	0, 1, 2 and 3
8	Amino acid mix*	Nil, HS, SS, DS,TS
9	Trace metal mix*	Nil, HS, SS, DS,TS
10	Vitamin mix*	Nil, HS, SS, DS,TS

**Stock solutions of amino acid mix, trace metal mix and vitamin mix were prepared. Concentration as per Barnett and Ingram (1955) was taken as single strength. 5 different levels of each of these factors [nil (0), half strength (HS), single strength (SS), double strength (DS) and triple strength (TS)] were assessed to find out the optimum amounts required.*

Optimization of carbon source

Different concentrations of D-glucose (5, 10, 20, 30, 40 and 50 g/L) were incorporated into the Barnett and Ingram's medium as source of carbon to find out the range for second level optimization.

Optimization of nitrogen source

As inorganic source of nitrogen, $(NH_4)_2SO_4$ was tested over the range 3,4,5,6,7,8,9 and 10 g/L in Barnett and Ingram's medium

Asparagine was screened as organic nitrogen source at 0, 1, 2 and 3 g/L concentrations.

Optimization of KH_2PO_4

Various concentrations of KH_2PO_4 (2, 3, 4, 5, 6, 7 and 8 g/L) were tested to find out the optimum range required for yeast biomass production.

Optimization of K_2HPO_4

Various concentrations of K_2HPO_4 (0.05, 0.01, 0.2, 0.3, 0.4 and 0.5 g/L) were tested to find out the optimum range required for yeast biomass production.

Optimization of magnesium

As source of magnesium, $MgSO_4$. $7H_2O$ at varying concentrations (0.5, 1, 1.5, 2, 2.5 and 3 g/L) were tested.

Optimization of calcium

Different concentrations of $CaCl_2$. $2H_2O$ (0.1, 0.2, 0.3, 0.4 and 0.5 g/L) were incorporated into the medium to find out the optimal range for second step optimization.

Optimization of amino acids

Different concentrations of amino acid mix (0, half strength, single strength, double strength and triple strength) were tested in the medium

Optimization of trace metal

Different concentrations of trace metal mix (0, half strength, single strength, double strength and triple strength) were tested

Optimization of vitamin mix

Different concentrations of vitamin mix (0, half strength, single strength, double strength and triple strength) were tested to find out the range for second level optimization.

4.2.4.1.1. Preparation of inoculum

Yeast cell suspension was prepared by harvesting young culture (24 hours old) of marine yeast *Candida* sp. S 27 on malt extract agar slant using sterile saline of 4.68 $^{0}/_{00}$. Optical density of the culture suspension was measured at 540 nm and adjusted to 1 OD by appropriate dilution.

4.2.4.1.2 Inoculation and incubation

Ten µl of the yeast suspension (1OD) was used as inoculum for 10 ml culture medium so that the initial O D of the culture broth was 0.001. The tubes were incubated at 32.72 ^{0}C for 48 hours.

4.2.4.1.3 Determination of growth

Growth was determined by measuring the optical density of the culture suspension at 540 nm in a UV-VIS spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan).

4.2.4.2 Second step optimization

4.2.4.2.1. Design of experiment

The initial test range of each ingredient for approaching the optimal conditions was selected based on the experimental results of the traditional 'one variable- at-a-time' (OVAT) method. A central composite design (CCD) for finding the optimum medium composition was employed to maximize the biomass production and to determine the interaction between the ten parameters, The experiments were carried out with the aid of the statistical software Design-Expert

(version 6.0.9, Stat-Ease, Minneapolis, MN, USA) The software suggested 158 combinations (***1/8 fractional factorial design* i.e. *Here $2^n = 2^{10} = 1024$; 1/8 of 1024= 128*; 20 axial points for the 10 different factors i.e. –α and + α for each factor and 10 center points**) for the different ranges of the 10 different media components. (*Design-Expert offers full factorial design for up to seven factors, above which the number of runs becomes excessive. Hence fractional factorial designs with much efficiency and no loss of information is suggested*). The actual values of the variables and the combinations are presented. (Table 4. 3 and Table 4.4)

Table 4.3 Levels of variables tested in CCD

Designation	Variables	Coded levels				
		-α	-1	0	+1	+α
A	$(NH_4)_2SO_4$ (g/L)	0.45	4	5.5	7	10.55
B	Glucose (g/L)	-25.45	10	25	40	75.45
C	KH_2PO_4 (g/L)	-1.55	2	3.5	5	8.55
D	K_2HPO_4 (g/L)	-0.25	0.05	0.17	0.3	0.60
E	$MgSO_4.7H_2O$ (g/L)	0.82	2	2.5	3	4.18
F	$CaCl_2.2H_2O$ (g/L)	-0.25	0.1	0.25	0.4	0.75
G	Amino acid mix	-5 X	Nil	HS	DS	5 X
H	Trace metal mix	-5 X	Nil	HS	DS	5 X
*J	Vitamin mix	-5 X	Nil	HS	DS	5 X
K	Asparagine (g/L)	-1.36	1	2	3	5.36

* *The alphabet* I *is absent in Design-Expert*

Table 4.4 Central composite design matrix of the ten variables

Run order	A	B	C	D	E	F	G	H	J	K
1	0	0	0	0	0	0	0	0	0	0
2	-1	+1	-1	-1	+1	-1	-1	-1	+1	+1
3	0	0	0	0	0	0	-α	0	0	0
4	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1
5	+1	+1	-1	+1	+1	+1	-1	+1	-1	-1
6	-α	0	0	0	0	0	0	0	0	0
7	+1	+1	+1	+1	+1	-1	-1	-1	+1	-1
8	+1	-1	-1	+1	+1	+1	-1	-1	+1	-1
9	-1	-1	-1	-1	+1	-1	-1	+1	-1	+1

10	+1	-1	-1	+1	+1	-1	+1	+1	+1	+1
11	-1	+1	+1	-1	+1	-1	-1	+1	-1	-1
12	+1	-1	+1	-1	-1	-1	-1	+1	-1	+1
13	+1	-1	-1	-1	-1	+1	-1	-1	+1	+1
14	-1	+1	+1	-1	-1	+1	-1	+1	+1	+1
15	0	0	0	0	0	0	0	0	0	0
16	+1	+1	-1	-1	-1	-1	-1	+1	-1	-1
17	-1	-1	-1	+1	+1	-1	+1	-1	+1	-1
18	-1	-1	-1	-1	+1	+1	+1	-1	-1	-1
19	0	0	0	0	0	0	0	0	0	+α
20	+1	+1	+1	-1	+1	-1	-1	-1	-1	+1
21	0	0	0	0	0	0	+α	0	0	0
22	-1	+1	-1	-1	+1	-1	+1	+1	+1	+1
23	+1	-1	+1	+1	-1	+1	-1	+1	+1	+1
24	+α	0	0	0	0	0	0	0	0	0
25	-1	-1	+1	-1	-1	+1	+1	+1	-1	+1
26	-1	+1	-1	+1	-1	+1	-1	-1	+1	+1
27	-1	-1	+1	+1	+1	-1	-1	-1	-1	+1
28	-1	+1	+1	-1	+1	+1	+1	-1	-1	+1
29	0	0	0	0	0	0	0	0	0	0
30	+1	+1	+1	+1	-1	+1	-1	-1	-1	+1
31	+1	-1	+1	-1	-1	-1	+1	-1	-1	+1
32	+1	-1	+1	-1	+1	+1	+1	-1	+1	-1
33	+!	-1	-1	+1	+1	+1	+1	+1	+1	-1
34	+1	-1	+1	-1	-1	+1	-1	+1	-1	-1
35	-1	+1	-1	+1	+1	+1	+1	+1	-1	+1
36	+1	+1	+1	-1	-1	-1	+1	+1	+1	+1
37	-1	-1	-1	+1	+1	+1	-1	+1	+1	+1
38	+1	+1	-1	+1	+1	-1	+1	-1	-1	+1
39	-1	+1	+1	-1	-1	-1	-1	+1	+1	-1
40	-1	+1	-1	-1	+1	+1	-1	-1	+1	-1
41	-1	-1	+1	-1	+1	+1	-1	-1	+1	+1
42	-1	+1	+1	-1	-1	+1	+1	-1	+1	+1
43	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1
44	0	0	0	0	0	0	0	0	0	0
45	-1	+1	+1	+1	-1	+1	+1	-1	-1	-1
46	-1	+1	+1	-1	-1	-1	+1	-1	+1	-1
47	0	0	0	0	0	0	0	0	+α	0
48	+1	-1	+1	-1	+1	-1	-1	+1	+1	+1
49	0	0	0	0	0	0	0	0	0	0

50	+1	-1	+1	-1	-1	+1	+1	-1	-1	-1
51	0	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	-α	0	0	0	0
53	-1	-1	-1	+1	-1	+1	-1	+1	-1	+1
54	-1	+1	+1	+1	+1	+1	-1	+1	+1	-1
55	+1	-1	+1	+1	-1	-1	-1	+1	+1	-1
56	-1	-1	+1	-1	+1	+1	+1	+1	+1	+1
57	+1	-1	+1	-1	+1	+1	-1	+1	+1	-1
58	-1	-1	+1	-1	-1	-1	+1	+1	-1	-1
59	0	0	0	0	-α	0	0	0	0	0
60	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1
61	-1	+1	-1	+1	+1	-1	+1	+1	-1	-1
62	-1	-1	-1	-1	-1	-1	+1	-1	+1	+1
63	+1	+1	+1	-1	+1	-1	+1	+1	-1	+1
64	+1	-1	+1	+1	+1	+1	-1	+1	-1	+1
65	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1
66	+1	+1	-1	+1	-1	+1	+1	-1	+1	-1
67	+1	+1	+1	-1	+1	+1	-1	-1	-1	-1
68	0	0	0	0	0	0	0	0	0	0
69	+1	+1	+1	+1	+1	-1	+1	+1	+1	-1
70	-1	-1	+1	+1	-1	-1	+1	+1	+1	+1
71	0	0	0	0	0	0	0	0	0	0
72	-1	-1	-1	+1	+1	+1	+1	-1	+1	+1
73	0	0	0	0	0	0	0	+α	0	0
74	+1	+1	-1	+1	-1	-1	+1	-1	+1	+1
75	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1
76	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1
77	0	0	0	0	0	0	0	0	0	0
78	+1	+1	-1	-1	+1	-1	-1	+1	+1	-1
79	-1	-1	-1	+1	-1	-1	-1	+1	-1	-1
80	+1	-1	-1	-1	-1	-1	+1	+1	+1	-1
81	+1	-1	-1	+1	+1	-1	-1	-1	+1	+1
82	+1	+1	-1	+1	+1	-1	-1	+1	-1	+1
83	-1	-1	+1	-1	+1	-1	+1	+1	+1	-1
84	0	0	0	0	0	0	0	0	0	0
85	+1	+1	+1	-1	-1	+1	-1	-1	+1	-1
86	-1	+1	+1	+1	-1	-1	+1	-1	-1	+1
87	+1	-1	-1	+1	-1	-1	-1	-1	-1	+1
88	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
89	0	0	0	0	0	0	0	0	0	-α

90	+1	+1	+1	+1	-1	-1	+1	+1	-1	-1
91	-1	+1	-1	+1	-1	+1	+1	+1	+1	+1
92	+1	-1	+1	-1	+1	-1	+1	-1	+1	+1
93	-1	+1	+1	+1	-1	+1	-1	+1	-1	-1
94	+1	-1	+1	+1	-1	-1	+1	-1	+1	-1
95	-1	+1	-1	-1	-1	+1	+1	+1	-1	-1
96	0	0	0	0	+α	0	0	0	0	0
97	-1	-1	-1	+1	-1	+1	+1	-1	-1	+1
98	-1	+1	+1	-1	+1	+1	-1	+1	-1	+1
99	-1	+1	+1	+1	+1	-1	-1	+1	+1	+1
100	+1	-1	-1	-1	+1	-1	+1	+1	-1	-1
101	-1	-1	+1	+1	+1	+1	+1	+1	-1	-1
102	-1	-1	+1	+1	+1	+1	-1	-1	-1	-1
103	-1	+1	-1	-1	+1	+1	+1	+1	+1	-1
104	-1	-1	+1	-1	-1	-1	-1	-1	-1	-1
105	-1	-1	-1	+1	+1	-1	-1	+1	+1	-1
106	0	0	0	-α	0	0	0	0	0	0
107	+1	-1	-1	+1	-1	-1	+1	+1	-1	+1
108	-1	+1	-1	-1	-1	+1	-1	-1	-1	-1
109	0	0	0	0	0	+α	0	0	0	0
110	-1	-1	+1	+1	-1	-1	-1	-1	+1	+1
111	-1	-1	-1	-1	+1	-1	+1	-1	-1	+1
112	0	0	0	+α	0	0	0	0	0	0
113	+1	+1	-1	-1	+1	+1	+1	-1	+1	+1
114	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1
115	+1	+1	+1	+1	-1	+1	+1	+1	-1	+1
116	0	0	0	0	0	0	0	0	-α	0
117	0	0	-α	0	0	0	0	0	0	0
118	+1	+1	+1	-1	-1	-1	-1	-1	+1	+1
119	+1	+1	+1	+1	-1	-1	-1	-1	-1	-1
120	+1	-1	+1	+1	+1	-1	-1	+1	-1	-1
121	+1	-1	+1	+1	+1	+1	+1	-1	-1	+1
122	+1	+1	-1	-1	-1	+1	-1	+1	-1	+1
123	-1	+1	-1	+1	-1	-1	+1	+1	+1	-1
124	-1	-1	-1	+1	-1	-1	+1	-1	-1	-1
125	-1	-1	+1	+1	-1	+1	-1	-1	+1	-1
126	+1	+1	-1	-1	-1	-1	+1	-1	-1	-1
127	+1	+1	-1	+1	-1	+1	-1	+1	+1	-1
128	-1	-1	+1	-1	-1	+1	-1	-1	-1	+1
129	+1	+1	+1	+1	+1	+1	-1	-1	+1	+1

130	-1	+1	+1	-1	+1	-1	+1	-1	-1	-1
131	-1	+1	-1	+1	-1	-1	-1	-1	+1	-1
132	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1
133	-1	+1	+1	+1	+1	+1	+1	-1	+1	-1
134	-1	-1	-1	-1	-1	+1	-1	+1	+1	-1
135	+1	+1	+1	-1	-1	+1	+1	+1	+1	-1
136	+1	-1	-1	-1	-1	-1	-1	-1	+1	-1
137	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1
138	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1
139	0	0	+α	0	0	0	0	0	0	0
140	0	-α	0	0	0	0	0	0	0	0
141	+1	-1	+1	+1	+1	-1	+1	-1	-1	-1
142	-1	-1	-1	-1	-1	+1	+1	-1	+1	-1
143	+1	-1	-1	+1	-1	+1	+1	+1	-1	-1
144	+1	-1	-1	-1	+1	+1	-1	-1	-1	+1
145	0	0	0	0	0	0	0	-α	0	0
146	+1	-1	+1	+1	-1	+1	+1	-1	+1	+1
147	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1
148	+1	+1	-1	-1	+1	-1	+1	-1	+1	-1
149	+1	-1	-1	-1	+1	-1	-1	-1	-1	-1
150	-1	-1	+1	-1	+1	-1	-1	-1	+1	-1
151	-1	+1	+1	+1	+1	-1	+1	-1	+1	+1
152	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
153	0	+α	0	0	0	0	0	0	0	0
154	-1	+1	-1	+1	+1	-1	-1	-1	-1	-1
155	-1	-1	-1	-1	+1	+1	-1	+1	-1	-1
156	+1	+1	-1	-1	-1	+1	+1	-1	-1	+1
157	-1	-1	-1	-1	-1	-1	-1	+1	+1	+1
158	-1	+1	-1	-1	-1	-1	-1	-1	-1	+1

The inoculation, incubation and measurement of growth were done as given in session 4.2.4.1.1. All the experiments were carried out in triplicate and the result (response) was expressed as Biomass in g/L. The effects of the medium components on biomass production were statistically analysed with response surfaces and the medium composition was optimized using mathematical equations and response surface plots.

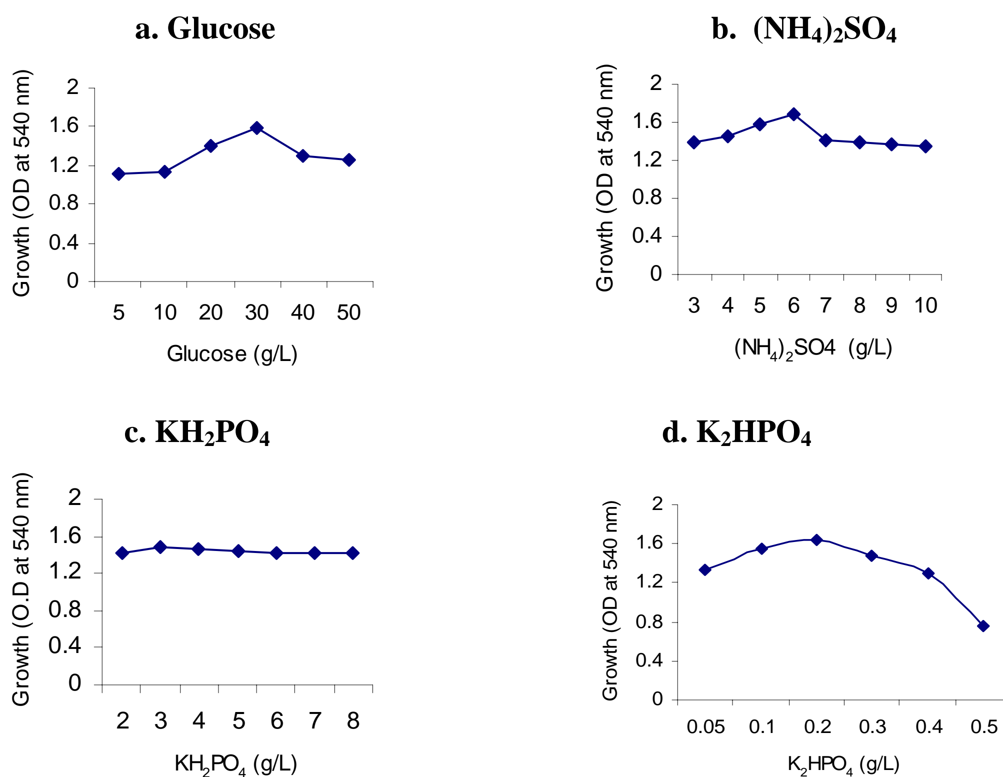
4.2.4.3. Experimental verification of the identified optimum

The optimum values of the 10 media ingredients were found out from the regression equation for the concerned response. Biomass production was evaluated at optimum levels (Solution no: 1, Table 4.9) and compared with the values predicted by the model. For this the medium was prepared with the optimum concentration of all the 10 ingredients in distilled water with 0.468 % NaCl and the pH was adjusted to 5.97. After inoculation of yeast suspension incubation was done at 32.72 ^{0}C for 48 hours and the growth was measured at 540 nm.

4. 3 Results

4.3.1 First step optimization

Regression analysis was done using biomass and the ten different media components and wherever regression was found to be highly significant (P<0.05), the corresponding factor level were taken for further analysis. The one-dimensional screening of the 10 different media components showed that they were significant for growth of the selected yeast *Candida* sp. S 27 in the following ranges (Table 4.5). Fig 4.1 a-j.



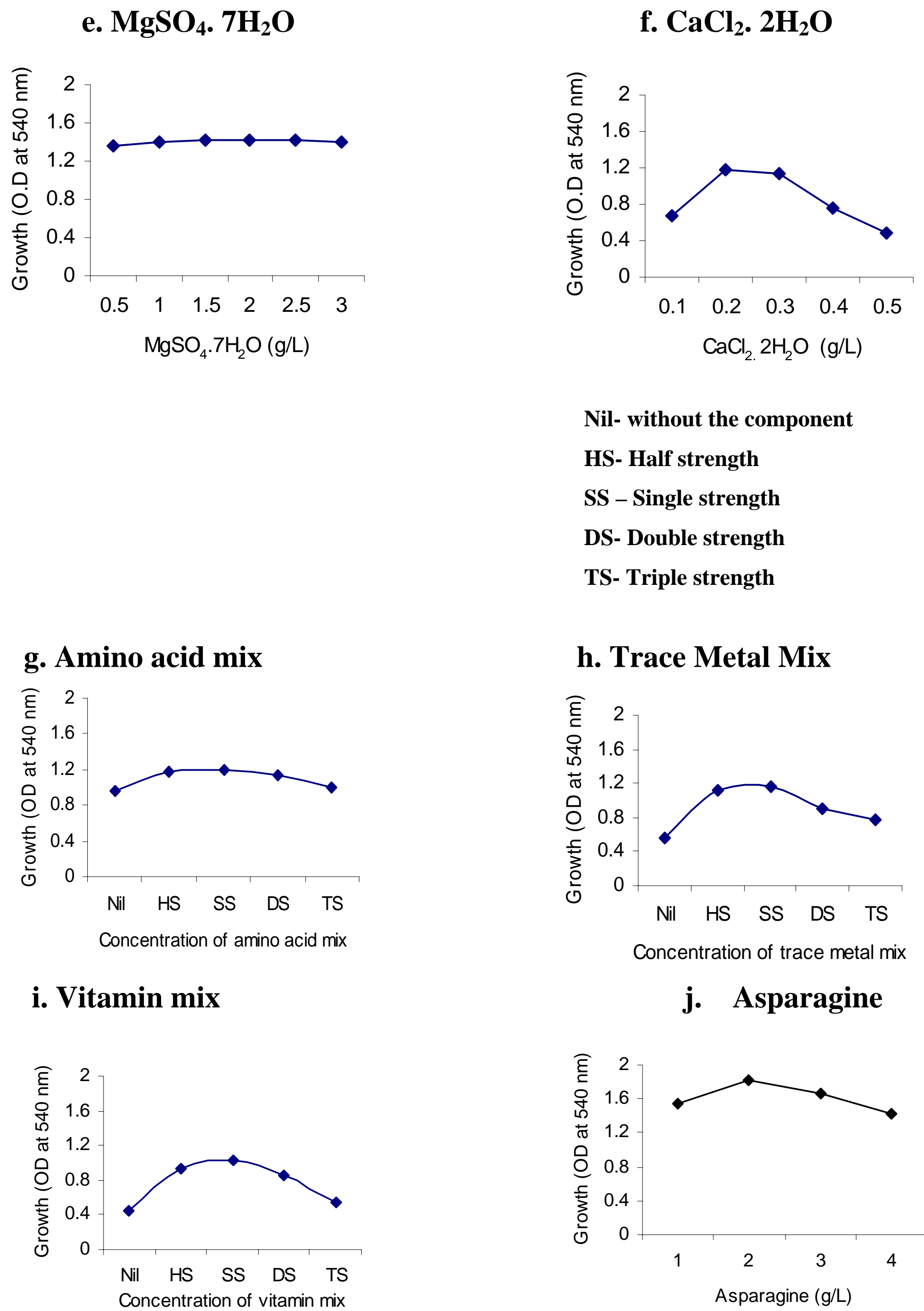


Fig 4.1 a-j One-Dimensional analyses of the 10 different components of chemically defined medium

Table 4.5 Numerical factors and their respective ranges selected for second level optimization based on regression analysis

S No:	Designation	Component	Range g/L
1	**A**	$(NH_4)_2SO_4$	4-7
2	**B**	Glucose	10- 40
3	**C**	KH_2PO_4	2-5
4	**D**	K_2HPO_4	0.05-0.3
5	**E**	$MgSO_4$. $7H_2O$	2-3
6	**F**	$CaCl_2$. $2H_2O$	0.1-0.4
7	**G**	Amino acid mix	(Nil-Double strength)
8	**H**	Trace Metal Mix	(Nil-Double strength)
9	***J**	Vitamin mix	(Nil-Double strength)
10	**K**	Asparagine	1-3

** The alphabet* **I** *is absent in Design-Expert*

4.3.2 Second step optimization

The coded values of the variables along with experimental and predicted values of biomass are given in the table 4.6.The CCD matrix was analyzed by standard analysis of variance (ANOVA) (Table 4.7). The model F value of 41.85 for biomass implied that the model was significant. The value of the determination coefficient (R^2 = 0.9673) indicated that only 3.27 % of the total variation was not explained by the model. The value of multiple correlation coefficient (R= 0.9835) indicated a high degree of correlation between the observed and predicted values. The adjusted R^2 (0.9442) value was also very high, making the model very significant. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of the experiment. Here, a lower value of CV (7.28 %) indicated a better precision and greater reliability of the experiments performed (Table 4.8).

Table 4.6 Central composite design matrix of the ten variables along with the experimental and predicted values of biomass

Run order	A	B	C	D	E	F	G	H	J	K	Biomass (g/L)	
											Experimental value	Predicted value
1	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.23	1.18
2	4	40	2	0.05	3	0.1	Nil	Nil	DS	3	0.87	0.88
3	5.5	25	3.5	0.17	2.5	0.25	-5X	HS	HS	2	0.89	0.91
4	7	10	2	0.3	2	0.4	Nil	Nil	Nil	1	0.49	0.49
5	7	40	2	0.3	3	0.4	Nil	DS	Nil	1	0.59	0.61
6	0.45	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	0.43	0.47
7	7	40	5	0.3	3	0.1	Nil	Nil	DS	1	0.87	0.96
8	7	10	2	0.3	3	0.4	Nil	Nil	DS	1	1.10	1.11
9	4	10	2	0.05	3	0.1	Nil	DS	Nil	3	0.94	0.94
10	7	10	2	0.3	3	0.1	DS	DS	DS	3	1.13	1.16
11	4	40	5	0.05	3	0.1	Nil	DS	Nil	1	0.71	0.72
12	7	10	5	0.05	2	0.1	Nil	DS	Nil	3	0.53	0.51
13	7	10	2	0.05	2	0.4	Nil	Nil	DS	3	1.02	0.99
14	4	40	5	0.05	2	+1	Nil	DS	DS	3	1.26	1.26
15	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.22	1.18
16	7	40	2	0.05	2	0.1	Nil	DS	Nil	1	0.65	0.68
17	4	10	2	0.3	3	0.1	DS	Nil	DS	1	0.82	0.91
18	4	10	2	0.05	3	0.4	DS	Nil	Nil	1	0.65	0.65
19	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	5.36	1.00	1.03
20	7	40	5	0.05	3	0.1	Nil	Nil	Nil	3	0.37	0.40
21	5.5	25	3.5	0.17	2.5	0.25	5X	HS	HS	2	1.24	1.14
22	4	40	2	0.05	3	0.1	DS	DS	DS	3	1.00	0.98
23	7	10	5	0.3	2	0.4	Nil	DS	DS	3	1.06	1.06
24	10.55	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	0.56	0.45
25	4	10	5	0.05	2	0.4	DS	DS	Nil	3	0.74	0.71
26	4	40	2	0.3	2	0.4	Nil	Nil	DS	3	0.83	0.83
27	4	10	5	0.3	3	0.1	Nil	Nil	Nil	3	0.39	0.42
28	4	40	5	0.05	3	0.4	DS	Nil	Nil	3	0.76	0.76
29	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.22	1.18
30	7	40	5	0.3	2	0.4	Nil	Nil	Nil	3	0.29	0.34
31	7	10	5	0.05	2	0.1	DS	Nil	Nil	3	0.46	0.55
32	7	10	5	0.05	3	0.4	DS	Nil	DS	1	1.05	1.08
33	7	10	2	0.3	3	0.4	DS	DS	DS	1	1.11	1.12
34	7	10	5	0.05	2	0.4	Nil	DS	Nil	1	0.47	0.51
35	4	40	2	0.3	3	0.4	DS	DS	Nil	3	0.68	0.70
36	7	40	5	0.05	2	0.1	DS	DS	DS	3	1.03	1.00
37	4	10	2	0.3	3	0.4	Nil	DS	DS	3	1.17	1.14
38	7	40	2	0.3	2	0.1	DS	Nil	Nil	3	0.72	0.75
39	4	40	5	0.05	2	0.1	Nil	DS	DS	1	1.23	1.28
40	4	40	2	0.05	3	0.4	Nil	Nil	DS	1	0.97	0.96
41	4	10	5	0.05	3	0.4	Nil	Nil	DS	3	1.03	1.07
42	4	40	5	0.05	2	0.4	DS	Nil	DS	3	1.23	1.27
43	4	10	5	0.3	3	0.1	DS	DS	Nil	3	0.62	0.66
44	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.22	1.18
45	4	40	5	0.3	2	0.4	DS	Nil	Nil	1	0.69	0.67
46	4	40	5	0.05	2	0.1	DS	Nil	DS	1	1.25	1.31
47	5.5	25	3.5	0.17	2.5	0.25	HS	HS	5X	2	1.47	1.47
48	7	10	5	0.05	3	0.1	Nil	DS	DS	3	0.98	1.03
49	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.19	1.18
50	7	10	5	0.05	2	0.4	DS	Nil	Nil	1	0.53	0.56

51	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.24	1.18
52	5.5	25	3.5	0.17	2.5	-0.25	HS	HS	HS	2	0.88	0.82
53	4	10	2	0.3	2	0.4	Nil	DS	Nil	3	0.60	0.63
54	4	40	5	0.3	3	0.4	Nil	DS	DS	1	1.02	0.99
55	7	10	5	0.3	2	0.1	Nil	DS	DS	1	1.05	1.07
56	4	10	5	0.05	3	0.4	DS	DS	DS	3	1.14	1.13
57	7	10	5	0.05	3	0.4	Nil	DS	DS	1	1.09	1.08
58	4	10	5	0.05	2	0.1	DS	DS	Nil	1	0.66	0.68
59	5.5	25	3.5	0.17	0.82	0.25	HS	HS	HS	2	0.81	0.80
60	7	40	2	0.3	3	0.4	DS	Nil	Nil	1	0.72	0.73
61	4	40	2	0.3	3	0.1	DS	DS	Nil	1	0.66	0.67
62	4	10	2	0.05	2	0.1	DS	Nil	DS	3	1.00	0.97
63	7	40	5	0.05	3	0.1	DS	DS	Nil	3	0.60	0.62
64	7	10	5	0.3	3	0.4	Nil	DS	Nil	3	0.63	0.62
65	4	40	2	0.3	3	0.4	Nil	Nil	Nil	3	0.54	0.51
66	7	40	2	0.3	2	0.4	DS	Nil	DS	1	1.13	1.12
67	7	40	5	0.05	3	0.4	Nil	Nil	Nil	1	0.39	0.47
68	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	0.84	1.18
69	7	40	5	0.3	3	0.1	DS	DS	DS	1	1.08	1.09
70	4	10	5	0.3	2	0.1	DS	DS	DS	3	1.01	1.02
71	5.5	25	0	0.17	2.5	0.25	HS	HS	HS	2	1.22	1.18
72	4	10	2	0.3	3	0.4	DS	Nil	DS	3	1.09	1.02
73	5.5	25	3.5	0.17	2.5	0.25	HS	5X	HS	2	1.12	1.09
74	7	40	2	0.3	2	0.1	DS	Nil	DS	3	0.93	0.94
75	4	40	2	0.05	2	0.1	DS	DS	Nil	3	0.77	0.78
76	7	10	2	0.05	3	0.4	DS	DS	Nil	3	0.86	0.86
77	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.20	1.18
78	7	40	2	0.05	3	0.1	Nil	DS	DS	1	1.08	1.04
79	4	10	2	0.3	2	0.1	Nil	DS	Nil	1	0.63	0.61
80	7	10	2	0.05	2	0.1	DS	DS	DS	1	1.05	1.05
81	7	10	2	0.3	3	0.1	Nil	Nil	DS	3	1.04	1.02
82	7	40	2	0.3	3	0.1	Nil	DS	Nil	3	0.66	0.64
83	4	10	5	0.05	3	0.1	Nil	DS	DS	1	0.96	0.98
84	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	2	1.28	1.18
85	7	40	5	0.05	2	0.4	Nil	Nil	DS	1	1.18	1.11
86	4	40	5	0.3	2	0.1	DS	Nil	Nil	3	0.69	0.69
87	7	10	2	0.3	2	0.1	Nil	Nil	Nil	3	0.47	0.46
88	4	40	5	0.3	2	0.1	Nil	DS	Nil	3	0.63	0.61
89	5.5	25	3.5	0.17	2.5	0.25	HS	HS	HS	-1.36	1.16	1.05
90	7	40	5	0.3	2	0.1	DS	DS	Nil	1	0.65	0.70
91	4	40	2	0.3	2	0.4	DS	DS	DS	3	0.96	0.96
92	7	10	5	0.05	3	0.1	DS	Nil	DS	3	1.03	1.02
93	4	40	5	0.3	2	0.4	Nil	DS	Nil	1	0.58	0.58
94	7	10	5	0.3	2	0.1	DS	Nil	DS	1	1.12	1.13
95	4	40	2	0.05	2	0.4	DS	DS	Nil	1	0.68	0.70
96	5.5	25	3.5	0.17	4.18	0.25	HS	HS	HS	2	0.93	0.86
97	4	10	2	0.3	2	0.4	DS	Nil	Nil	3	0.55	0.56
98	4	40	5	0.05	3	0.4	Nil	DS	Nil	3	0.74	0.74
99	4	40	5	0.3	3	0.4	Nil	DS	DS	3	0.95	0.98
100	4	10	2	0.05	3	0.1	DS	DS	Nil	1	0.77	0.83
101	4	10	5	0.3	3	0.4	DS	DS	Nil	1	0.36	0.43
102	4	10	5	0.3	3	0.4	Nil	Nil	Nil	1	0.28	0.31
103	4	40	2	0.05	3	0.4	DS	DS	DS	1	0.95	0.95
104	4	10	5	0.05	2	0.1	Nil	Nil	Nil	1	0.59	0.58
105	4	10	2	0.3	3	0.1	Nil	DS	DS	1	1.03	1.01
106	5.5	25	3.5	-0.25	2.5	0.25	HS	HS	HS	2	1.08	1.03
107	7	10	2	0.3	2	0.1	DS	DS	Nil	3	0.69	0.67
108	4	40	2	0.05	2	0.4	Nil	Nil	Nil	1	0.62	0.64

109	5.5	25	3.5	0.17	2.5	0.4	HS	HS	HS	2	0.85	0.83
110	4	10	5	0.3	2	0.1	Nil	Nil	DS	3	0.88	0.85
111	4	10	2	0.05	3	0.1	DS	Nil	Nil	3	0.82	0.81
112	5.5	25	3.5	0.6	2.5	0.25	HS	HS	HS	2	0.84	0.81
113	7	40	2	0.05	3	0.4	DS	Nil	DS	3	0.99	1.05
114	7	40	5	0.05	3	0.4	DS	DS	Nil	1	0.56	0.56
115	7	40	5	0.3	2	0.4	DS	DS	Nil	3	0.58	0.59
116	5.5	25	3.5	0.17	2.5	0.25	HS	HS	-5X	2	0.17	0.10
117	5.5	25	-1.55	0.17	2.5	0.25	HS	HS	HS	2	0.70	0.76
118	7	40	5	0.05	2	0.1	Nil	Nil	DS	3	0.88	0.85
119	7	40	5	0.3	2	0.1	Nil	Nil	Nil	1	0.48	0.49
120	7	10	5	0.3	3	0.1	Nil	DS	Nil	1	0.58	0.58
121	7	10	5	0.3	3	0.4	DS	Nil	Nil	3	0.66	0.67
122	7	40	2	0.05	2	0.4	Nil	DS	Nil	3	0.60	0.54
123	4	40	2	0.3	2	0.1	DS	DS	DS	1	1.03	0.99
124	4	10	2	0.3	2	0.1	DS	Nil	Nil	1	0.55	0.56
125	4	10	5	0.3	2	0.4	Nil	Nil	+1	1	0.90	0.93
126	7	40	2	0.05	2	0.1	DS	Nil	Nil	1	0.79	0.79
127	7	40	2	0.3	2	0.4	Nil	DS	DS	1	0.95	0.99
128	4	10	5	0.05	2	0.4	Nil	Nil	Nil	3	0.60	0.58
129	7	40	5	0.3	3	0.4	Nil	Nil	DS	3	0.97	0.93
130	4	40	5	0.05	3	0.1	DS	Nil	Nil	1	0.75	0.74
131	4	40	2	0.3	2	0.1	Nil	Nil	DS	1	0.92	0.89
132	7	10	2	0.05	2	0.4	DS	DS	DS	3	1.02	1.03
133	4	40	5	0.3	3	0.4	DS	Nil	DS	1	0.98	1.03
134	4	10	2	0.05	2	0.4	Nil	DS	DS	1	1.12	1.12
135	7	40	5	0.05	2	0.4	DS	DS	DS	1	1.09	1.14
136	7	10	2	0.05	2	0.1	Nil	Nil	DS	1	1.00	1.05
137	7	40	2	0.3	2	0.1	Nil	DS	DS	3	0.82	0.83
138	7	10	5	0.05	2	0.4	DS	DS	DS	1	1.03	0.99
139	5.5	25	8.55	0.17	2.5	0.25	HS	HS	HS	2	0.82	0.69
140	5.5	-25.45	3.5	0.17	2.5	0.25	HS	HS	HS	2	0.26	0.24
141	7	10	5	0.3	3	0.1	DS	Nil	Nil	1	0.69	0.64
142	4	10	2	0.05	2	0.4	DS	Nil	DS	1	1.00	1.00
143	7	10	2	0.3	2	0.4	DS	DS	Nil	1	0.60	0.59
144	7	10	2	0.05	3	0.4	Nil	Nil	Nil	3	0.75	0.76
145	5.5	25	3.5	0.17	2.5	0.25	HS	-5X	HS	2	0.97	0.92
146	7	10	5	0.3	2	0.4	DS	Nil	DS	3	1.06	1.11
147	7	40	2	0.05	3	0.4	Nil	DS	DS	3	0.98	1.01
148	7	40	2	0.05	3	0.1	DS	Nil	DS	1	1.10	1.09
149	7	10	2	0.05	3	0.1	Nil	Nil	Nil	1	0.75	0.76
150	4	10	5	0.05	3	0.1	Nil	Nil	DS	1	0.98	0.95
151	4	40	5	0.3	3	0.1	DS	Nil	DS	3	1.03	1.00
152	7	40	5	0.3	3	0.4	DS	DS	DS	3	1.09	1.10
153	5.5	-75.45	3.5	0.17	2.5	0.25	HS	HS	HS	2	0.28	0.22
154	4	40	2	0.3	3	0.1	Nil	Nil	Nil	1	0.51	0.51
155	4	10	2	0.05	3	0.4	Nil	DS	Nil	1	0.79	0.77
156	7	40	2	0.05	2	0.4	DS	Nil	Nil	3	0.66	0.64
157	4	10	2	0.05	2	0.1	Nil	DS	DS	3	1.10	1.10
158	4	40	2	0.05	2	0.1	Nil	Nil	Nil	3	0.59	0.60

The significance of each coefficient was determined by P-values. The smaller the magnitude of P-value, the more significant is the corresponding coefficient. In the case of Biomass, linear coefficients such as D, G, H, J, quadratic coefficients like A^2, B^2,C^2, D^2, E^2, F^2, G^2, H^2, J^2, K^2, and interaction coefficients AB, AC, AD, AE,

AG, AH, AJ, AK, BC, BE, BG, BK, CE, CJ, DG, EJ, EK, FJ, FK,GH, GJ, HK and JK were significant model terms (Table 4.8) . Although the linear coefficients of $(NH_4)_2SO_4$, Glucose, KH_2PO_4, $MgSO_4$. $7H_2O$, $CaCl_2$. $2H_2O$ and Asparagine were not significant; they showed significant quadratic effects and interaction effects, which justify their inclusion in the medium for biomass production.

Table 4.7 ANOVA for Response Surface Quadratic Model (from the software Design- Expert)

	F value	P value		F value	P value		F value	P value
A	0.28	0.5960	AD	26.09	<0.0001	DE	1.67	0.2000
B	0.11	0.7365	AE	12.33	0.0007	DF	0.098	0.7548
C	3.87	0.0521	AF	0.15	0.7016	DG	5.62	0.0199
D	44.29	<0.0001	AG	5.75	0.0185	DH	0.31	0.5801
E	3.21	0.0764	AH	4.42	0.0383	DJ	2.88	0.0932
F	8.511E-003	0.9267	AJ	4.59	0.0348	DK	0.23	0.6312
G	44.63	<0.0001	AK	13.23	0.0005	EF	0.12	0.7352
H	24.60	<0.0001	BC	21.45	<0.0001	EG	0.63	0.4301
J	1634.25	<0.0001	BD	4.560E-004	0.9830	EH	0.24	0.6245
K	0.36	0.5514	BE	12.94	0.0005	EJ	7.29	0.0083
A^2	236.94	<0.0001	BF	0.063	0.8020	EK	12.90	0.0005
B^2	409.61	<0.0001	BG	10.24	0.0019	FG	0.98	0.3239
C^2	94.59	<0.0001	BH	2.31	0.1323	FH	0.77	0.3810
D^2	30.71	<0.0001	BJ	1.01	0.3182	FJ	12.36	0.0007
E^2	54.69	<0.0001	BK	12.38	0.0007	FK	16.08	<0.0001
F^2	56.27	<0.0001	CD	0.13	0.7192	GH	31.78	<0.0001
G^2	10.37	0.0018	CE	19.96	<0.0001	GJ	4.96	0.0283
H^2	13.41	0.0004	CF	0.39	0.5342	GK	2.27	0.1353
J^2	70.00	<0.0001	CG	3.17	0.0785	HJ	1.05	0.3081
K^2	8.37	0.0048	CH	3.518E-003	0.9528	HK	4.09	0.0461
AB	12.31	0.0007	CJ	36.89	<0.0001	JK	4.31	0.0407
AC	12.07	0.0008	CK	1.103E-006	0.9736			

Significant coefficients are highlighted in red

Table 4.8 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	F-value	Probability P>F
Model	10.33	65	0.16	41.85	<0.0001
Residual error	0.35	92	3.797E-003		
Lack of fit	0.21	83	2.541E-003	0.17	1.0000
Pure error	0.14	9	0.015		
Cor total	10.68	157			

SS, sum of squares; DF, degrees of freedom; MS, mean square; CV= 7.28 %; R^2 = 0.9673; R = 0.9835

The application of RSM yielded the following regression equation which is the empirical relationship between biomass production (Y_1) and the test variables in coded unit designated as A, B, C, D, E, F, G, H, J, and K where 'A' is **$(NH_4)_2SO_4$** ; 'B' is **Glucose**; 'C' is **KH_2PO_4** ;'D' is **K_2HPO_4**; 'E' is **$MgSO_4$. $7H_2O$**; 'F' is **$CaCl_2$. $2H_2O$**; 'G'is **Amino acid mix**; 'H' is **Trace metal mix**; 'J' is **Vitamin mix** and 'K' is **Asparagine**.

Y_1 = -1.50586 +0.34582 A+ 0.034144 B +0.22234 C-1.34143 D +0.72265 E +0.25146 F -0.034238 G +9.38240 H +18.57215 J +3.96722E-003 K- 0.034252 A^2 – 4.50356E-004 B^2- 0.021642 C^2- 1.77579 D^2- 0.14811 E^2- 1.66924 F^2-71.66094 G^2- 81.48187 H^2 -186.17295 J^2-0.014489 K^2-1.02708E-003 AB –0.010167 AC+ 0.17942 AD+ 0.030833 AE+0.011250 AF+ 0.70208 AG – 0.61528 AH+ 0.62708 AJ - 0.015969AK+ 1.35556E-003 BC+ 7.50000E-005 BD -3.15833E-003 BE- 7.36111E-004 BF+0.093681 BG -0.044444 BH -0.029375 BJ- 1.54479E-003BK+ 0.012667CD- 0.039229 CE+0.018264 CF +0.52083 CG- 0.017361 CH +1.77778 CJ +1.45833E-004 CK+ 0.13600 DE -0.11000 DF+ 8.32500 DG + 1.95000 DH + 5.95833 DJ + 0.025375 DK+ 0.029792 EF -0.69583 EG +0.43125 EH -2.37083 EJ+ 0.047313 EK -2.90278 FG -2.57639 FH +10.29167 FJ+ 0.17604 FK -165.00000 GH- 65.20833 GJ +0.66146 GK-30.00000 HJ +0.88750 HK- 0.91146 JK.

Based on the data generated, the software suggested 10 solutions for the optimum concentration of the 10 different medium components (Table 4.9) of which the solution with maximum desirability was selected.

Table 4.9 Optimum values of the 10 media ingredients suggested by the software

No:	A	B	C	D	E	F	G	H	J	K	Biomass	Desirability
1	5.42	32.17	5	0.15	2.07	0.25	DS	HS	DS	1.00	1.356	0.936
2	5.25	29.79	4.56	0.11	2.16	0.30	DS	HS	DS	1.71	1.328	0.930
3	5.64	28.49	3.96	0.05	2.00	0.23	SS	HS	DS	1.00	1.316	0.915
4	5.75	23.46	4.10	0.11	2.25	0.15	SS	SS	DS	1.07	1.299	0.904
5	5.97	23.12	3.06	0.13	2.82	0.31	SS	SS	DS	1.68	1.240	0.892
6	5.01	18.90	2.48	0.05	2.43	0.36	HS	DS	DS	3.00	1.207	0.890
7	6.44	26.05	3.15	0.25	2.71	0.33	DS	HS	DS	1.25	1.200	0.889
8	5.51	23.33	3.17	0.19	2.71	0.23	DS	HS	DS	2.91	1.189	0.885
9	5.95	21.30	3.87	0.25	2.79	0.30	DS	NIL	DS	2.35	1.162	0.874
10	5.47	10.00	2.20	0.05	3.00	0.25	NIL	SS	SS	2.85	1.148	0.815

The location of optimum obtained by the differentiation of the quadratic model for achieving maximum biomass production was: $(NH_4)_2SO_4$ = 5.42 g/l; Glucose = 32.17 g/l; KH_2PO_4 = 5g/l; K_2HPO_4 = 0.15g/l; $MgSO_4$. $7H_2O$ = 2.07 g/l; $CaCl_2$. $2H_2O$ = 0.25 g/l; Amino acid mix = DS; Trace metal mix = HS; Vitamin mix = DS; Asparagine = 1g/l (Table 4.10)

The two-dimensional contour plots and its respective three dimensional response surface plots on biomass showed the interactive effect of varying concentrations of $(NH_4)_2SO_4$ & Glucose, $(NH_4)_2SO_4$ & KH_2PO_4, $(NH_4)_2SO_4$ & K_2HPO_4, $(NH_4)_2SO_4$ & $MgSO_4$. $7H_2O$, $(NH_4)_2SO_4$ & Amino acid mix, $(NH_4)_2SO_4$ & Trace metal mix, $(NH_4)_2SO_4$ & Vitamin mix, $(NH_4)_2SO_4$ & Asparagine, Glucose & KH_2PO_4, Glucose & $MgSO_4$. $7H_2O$, Glucose & Amino acid mix, Glucose & Asparagine, KH_2PO_4 & $MgSO_4$. $7H_2O$, KH_2PO_4 & Vitamin mix, K_2HPO_4 & Amino acid mix, $MgSO_4$. $7H_2O$ & Vitamin mix, $MgSO_4$. $7H_2O$ & Asparagine, $CaCl_2$. $2H_2O$ & Vitamin mix, $CaCl_2$. $2H_2O$ &

Asparagine, Amino acid mix & Trace metal mix, Amino acid mix & Vitamin mix, Trace metal mix & Asparagine, Vitamin mix & Asparagine on biomass. (Fig 4.2 a-w).

Three dimensional plot and its respective contour plot showed the effect of $(NH_4)_2SO_4$ and Glucose on the response (Biomass), while the other 8 variables, i.e, KH_2PO_4, K_2HPO_4, $MgSO_4$. $7H_2O$, $CaCl_2$. $2H_2O$, Amino acid mix, Trace metal mix, Vitamin mix and Asparagine were fixed at their optimal concentration (Fig 4.2 a). At low concentration of $(NH_4)_2SO_4$, the biomass yield increased gradually from 1.19g/l to 1.35 g/l with increase in concentration of glucose up to 32.72g/l but decreased slowly beyond the range. With increase in concentration of $(NH_4)_2SO_4$, the biomass yield did not vary much with concentration of glucose.

The effect of $(NH_4)_2SO_4$ and KH_2PO_4 on yeast biomass production at fixed levels of 8 other variables are presented in Fig 4.2 b. It is evident that at low $(NH_4)_2SO_4$ concentration, the biomass yield steadily increased with increasing concentration of KH_2PO_4 up to 5 g/l. but with increase in concentration of $(NH_4)_2SO_4$, the yield decreased.

A comparison of media ingredients in Barnett and Ingram's medium and the modified medium through RSM is shown in Table 4.10.

A notable increase in Nitrogen source and Carbon source could be observed in the optimized medium developed through RSM. Amino acid and vitamin mix were required in double the amount. Salt composition (KH_2PO_4, $MgSO_4$. $7H_2O$ and $CaCl_2$. $2H_2O$) was also higher. However the trace metal concentration was found to be lower than that of original medium.

Table 4.10 Concentration of ingredients in the Barnett and Ingram's medium and Barnett and Ingram's modified medium (per Litre). * Medium developed through RSM in the present study

Ingredients		Barnett and Ingram's medium	Barnett and Ingram's modified medium (RSM)*
Nitrogen sources	$(NH_4)_2SO_4$	3.5 g	5.42 g
	L- Asparagine	1.5 g	1 g
Carbon source	D-Glucose	10 g	32.17 g
Amino acids	L-Histidine	10 mg	20 mg
	DL- Methionine	20 mg	40 mg
	DL-Tryptophan	20 mg	40 mg
Growth factors (Vitamin mix)	p-Aminobenzoic acid	200 µg	400 µg
	Biotin	20 µg	40 µg
	Folic acid	2 µg	4 µg
	Myo-inositol	10 mg	20 mg
	Nicotinic acid	400 µg	800 µg
	Pantothenate (Ca)	2 mg	4 mg
	Pyridoxine HCl	400 µg	800 µg
	Riboflavin	200 µg	400 µg
	Thiamin HCl	400 µg	800 µg
Trace element sources	H_3BO_3	500 µg	250 µg
	$CuSO_4.5H_2O$	40 µg	20 µg
	KI	100 µg	50 µg
	$FeCl_3.6H_2O$	200 µg	100 µg
	$MnSO_4.4H_2O$	400 µg	200 µg
	$Na_2MoO_4.2H_2O$	200 µg	100 µg
	$ZnSO_4.7H_2O$	400 µg	200 µg
Salts	KH_2PO_4	850 mg	5 g
	K_2HPO_4	150 mg	0.15 g
	$MgSO_4.7H_2O$	500 mg	2.07 g
	NaCl	100 mg	4.68 g
	$CaCl_2.6H_2O$	100 mg	0.25 g

4.3.3 Experimental verification of the identified optimum

The validation was carried out under optimum conditions of the media predicted by the model. The experimental value of biomass (1.349 g/L) was closer to the predicted value (1.356 g/L) validating the model.

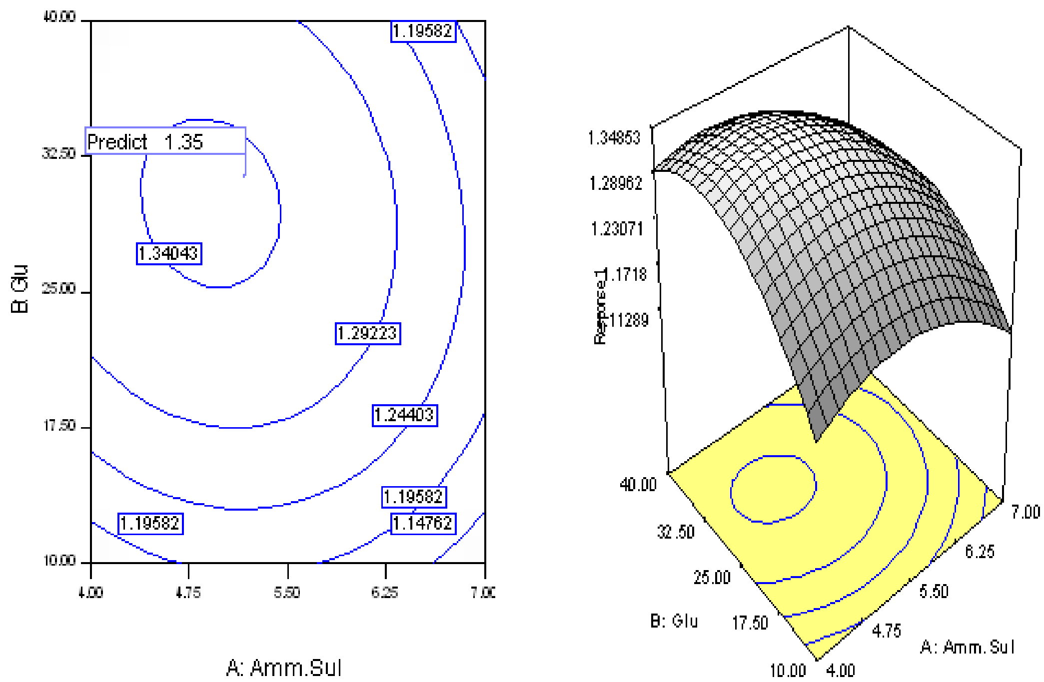


Fig 4.2 a. Contour plot and the corresponding response surface plot (3D) of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = B: Glucose

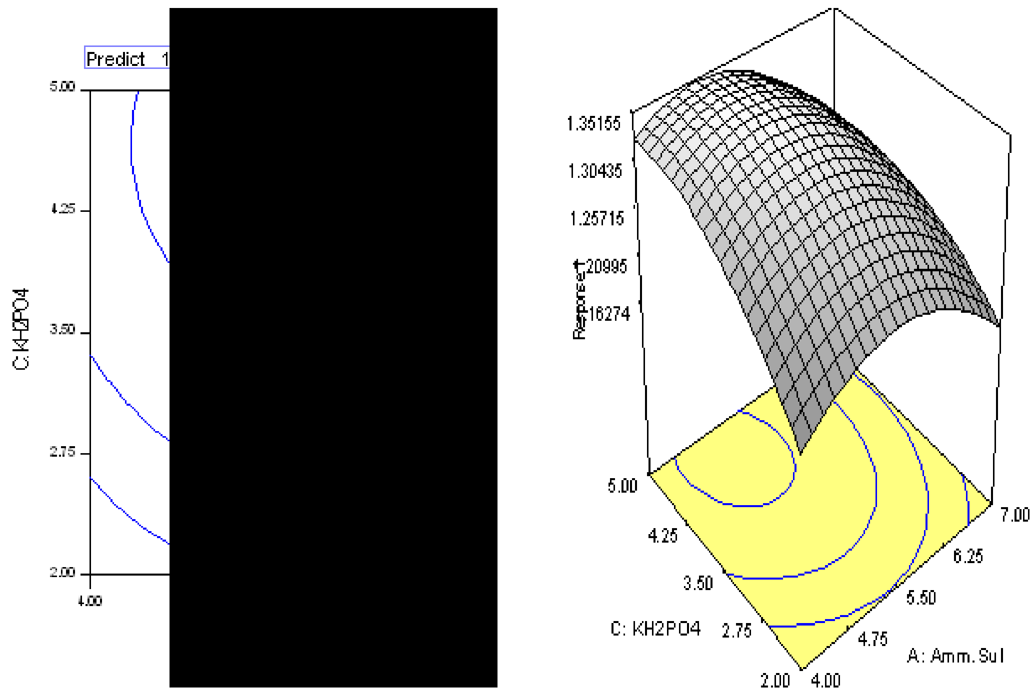


Fig 4.2 b. Contour plot and the corresponding response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = C: KH_2PO_4

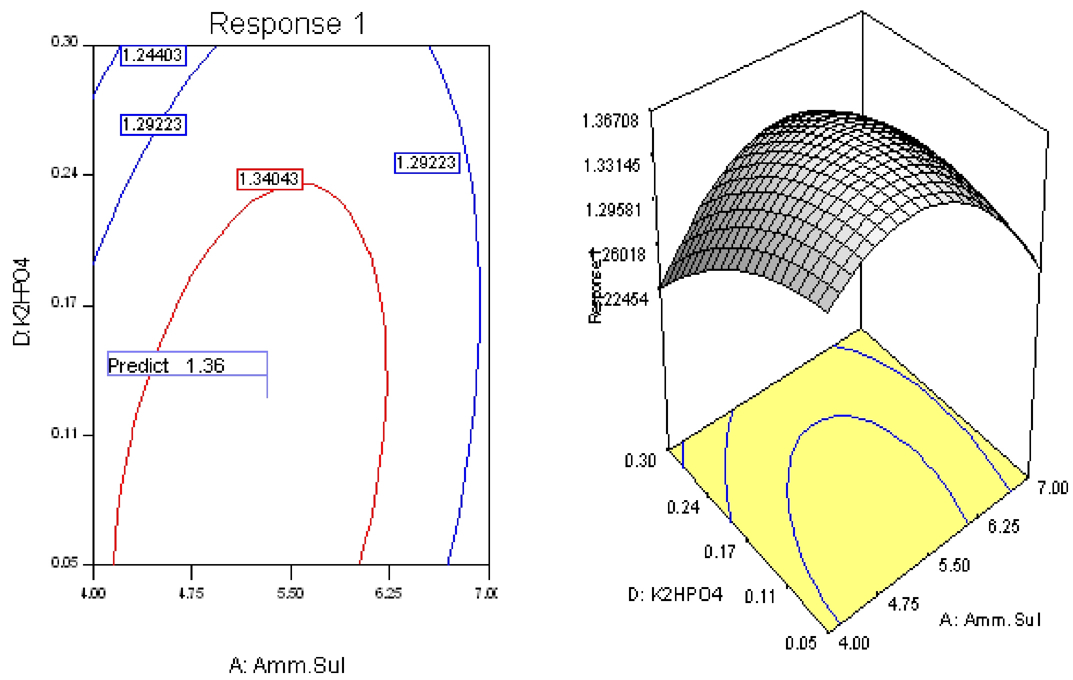


Fig 4.2 c. Contour & response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = D: K_2HPO_4

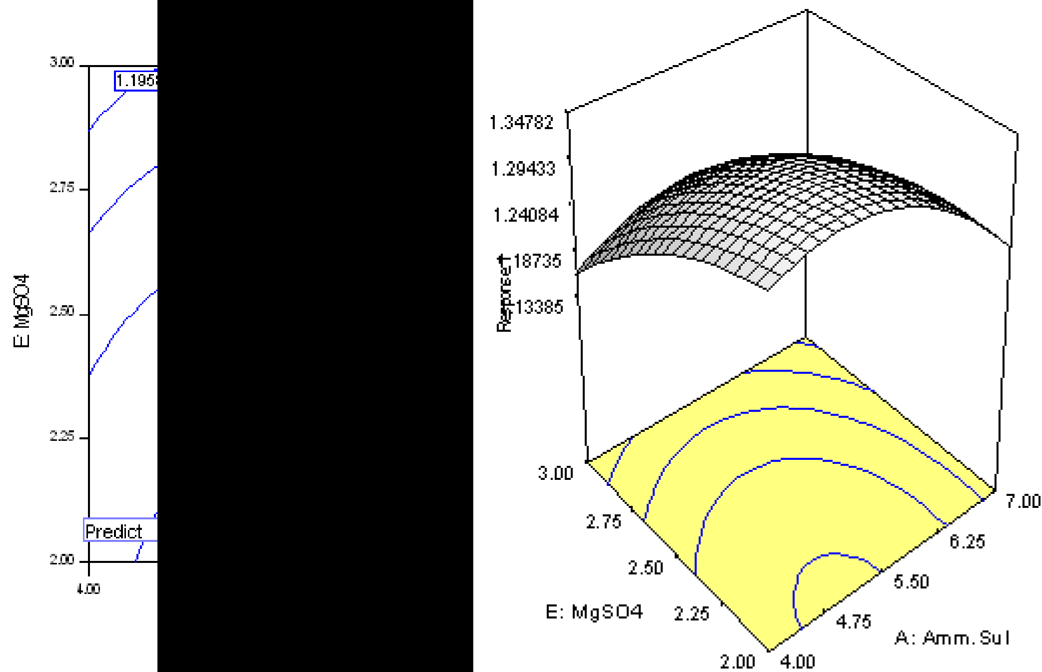


Fig 4. 2 d. Contour plot & its corresponding response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = E: $MgSO_4.\ 7H_2O$

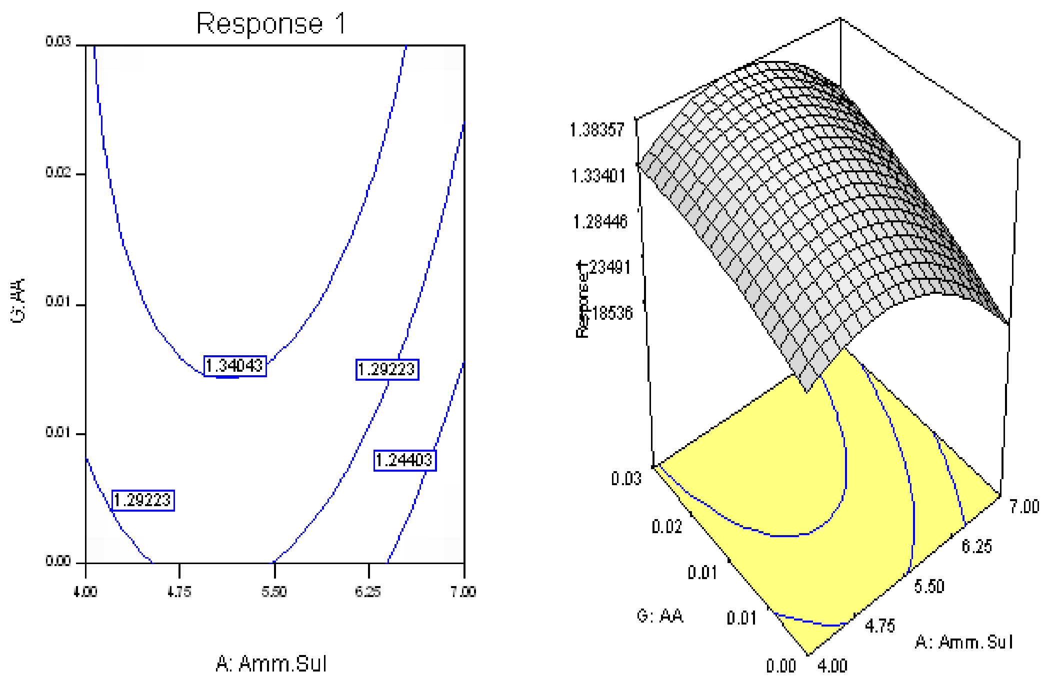


Fig 4.2.e. Contour & response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = G: Amino acid Mix

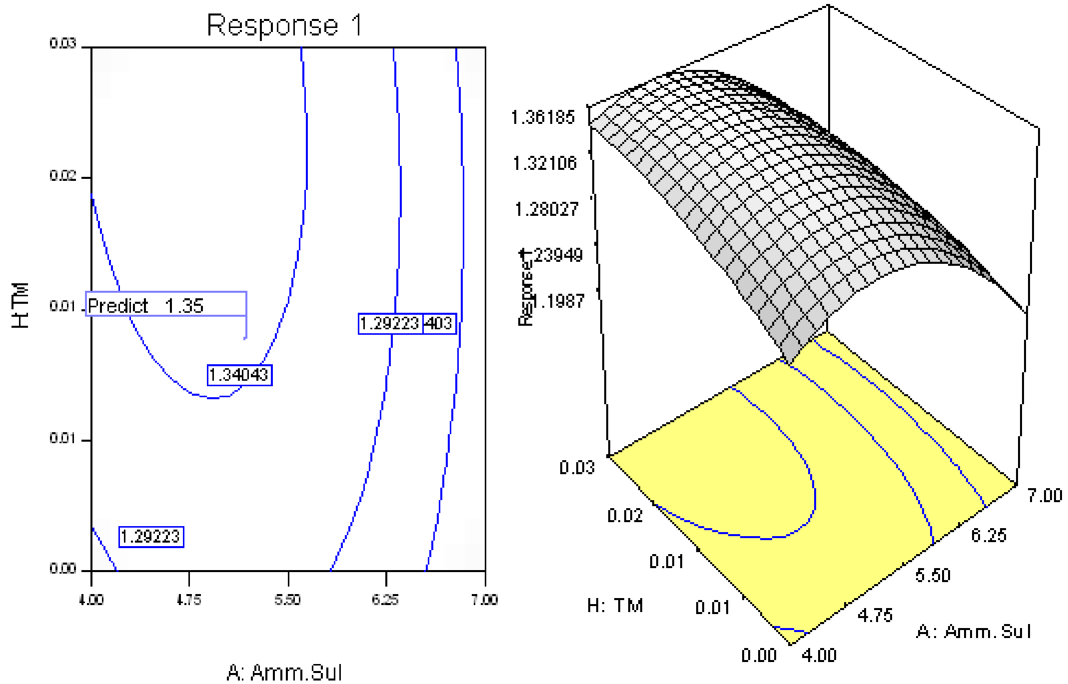


Fig 4.2 f. Contour & response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = H: Trace metal mix

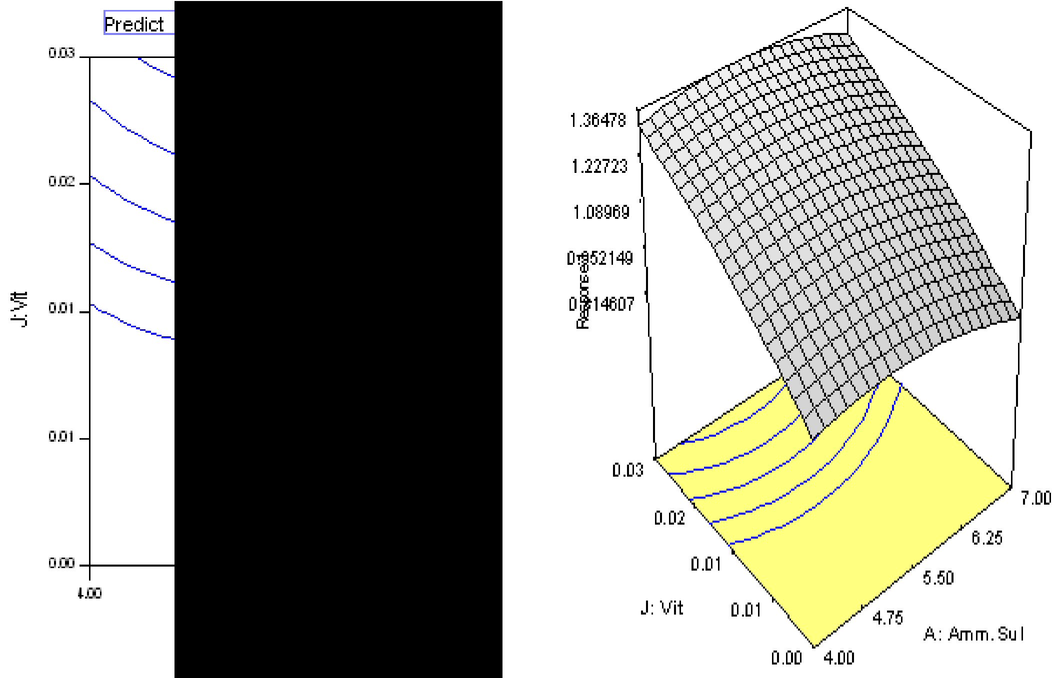


Fig 4.2 g. Contour & response surface plots of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = J: Vitamin Mix

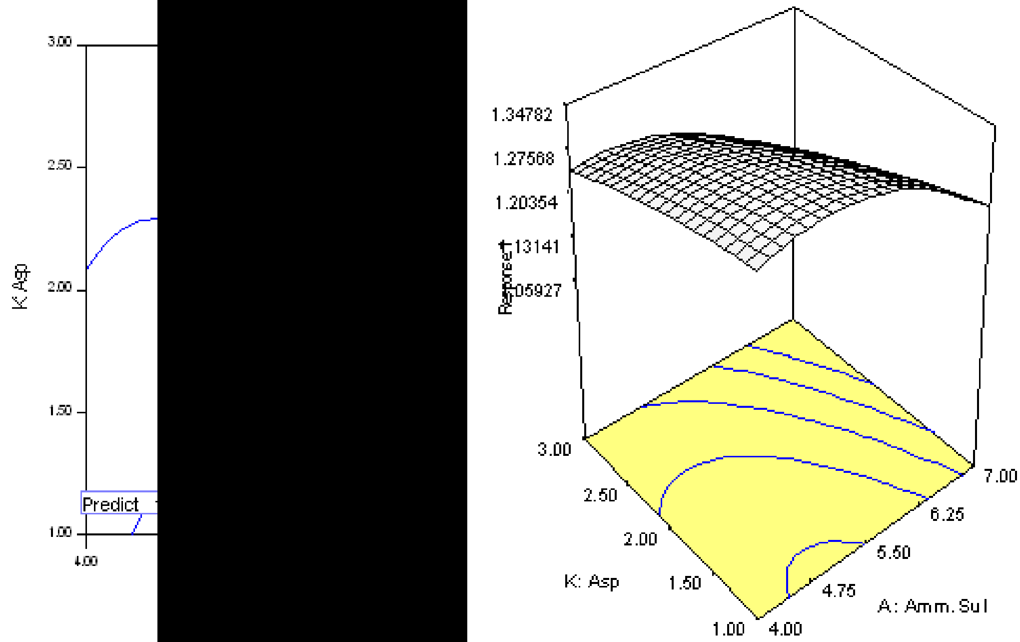


Fig 4.2 h. Contour & response surface plot of biomass vs. X = A: $(NH_4)_2SO_4$ and Y = K: Asparagine

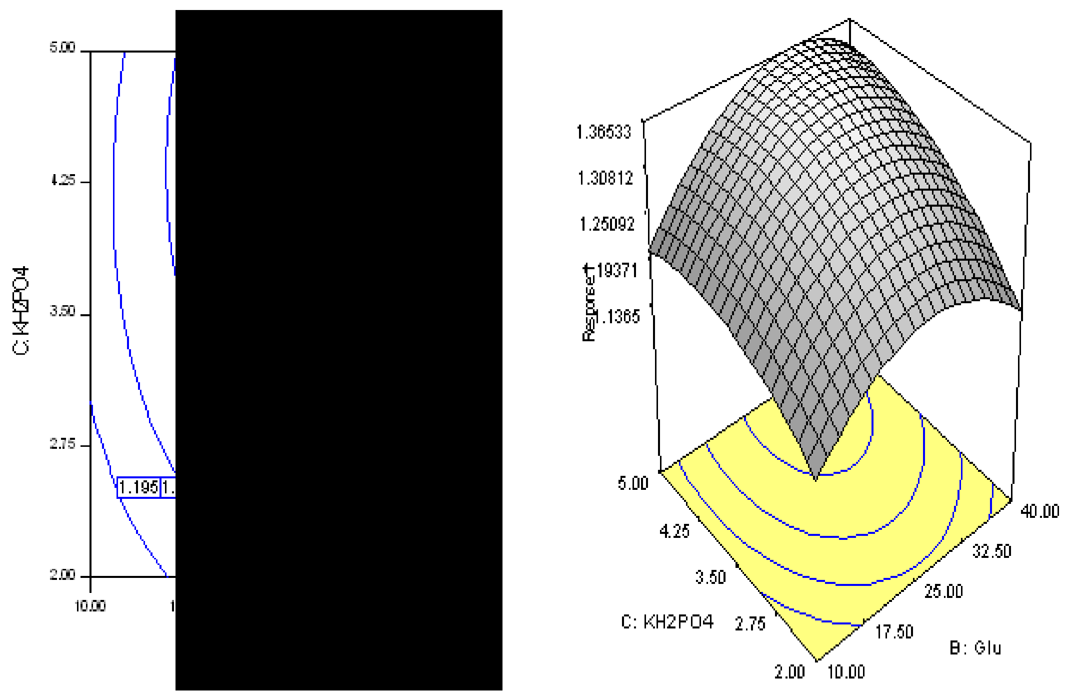


Fig 4.2 i. Contour plot and the corresponding response surface plot of biomass vs. X = B: Glucose and Y= C: KH_2PO_4

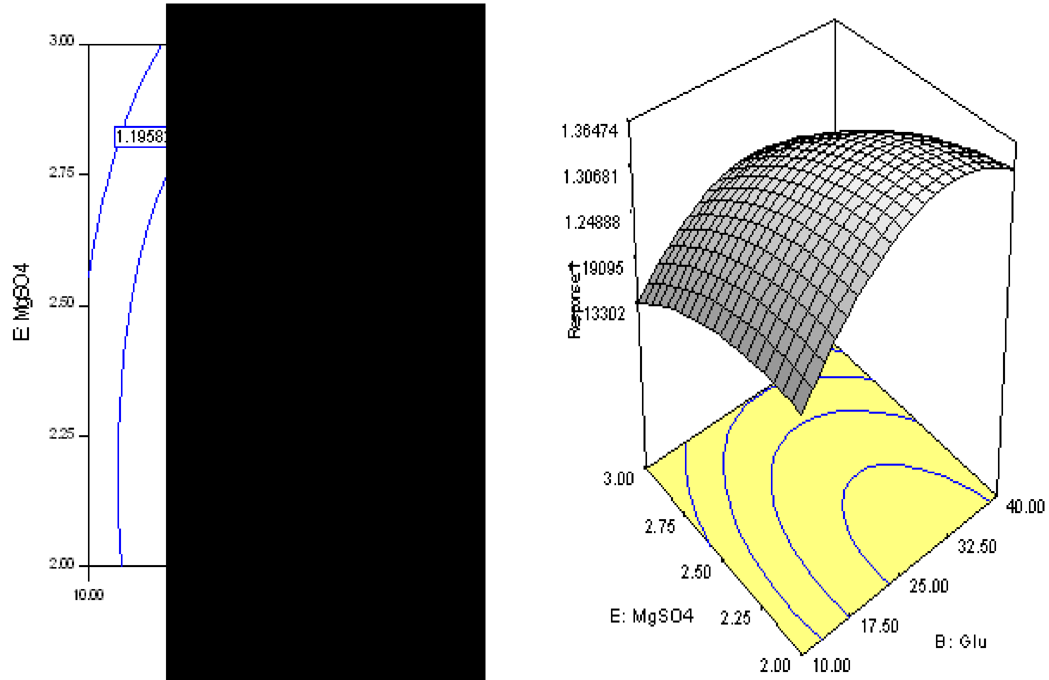


Fig 4.2 j. Contour & response surface plot of biomass vs. X = B: Glucose and Y= E: $MgSO_4 . 7H_2O$

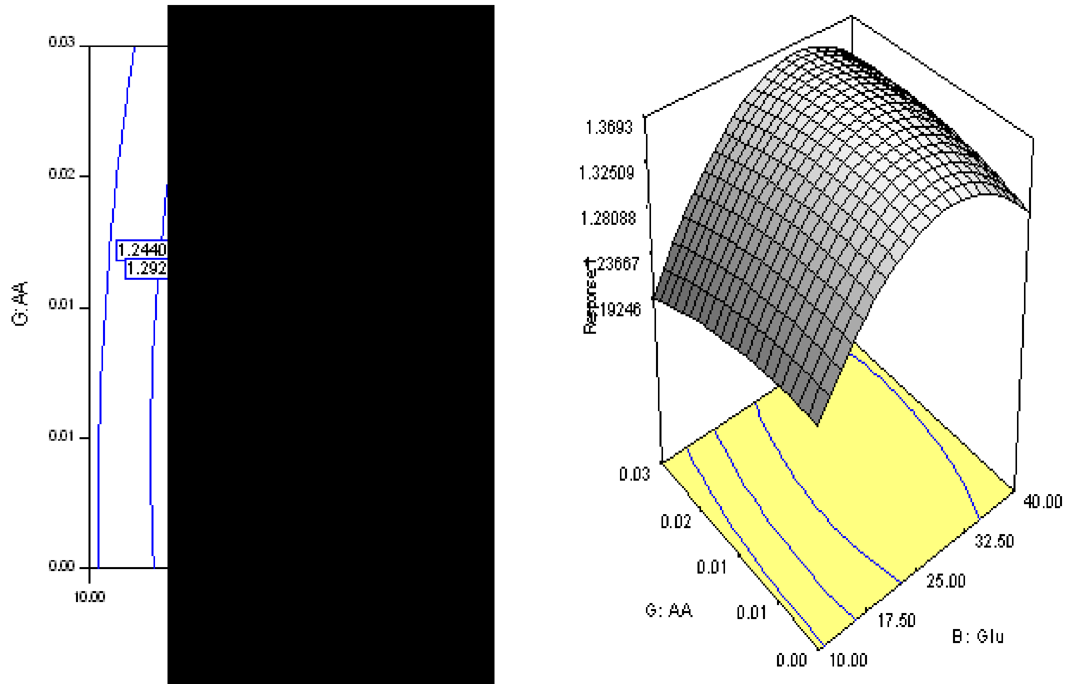


Fig 4.2 k. Contour & corresponding response surface plots of biomass vs. X = B: Glucose and Y= G: Amino acid Mix

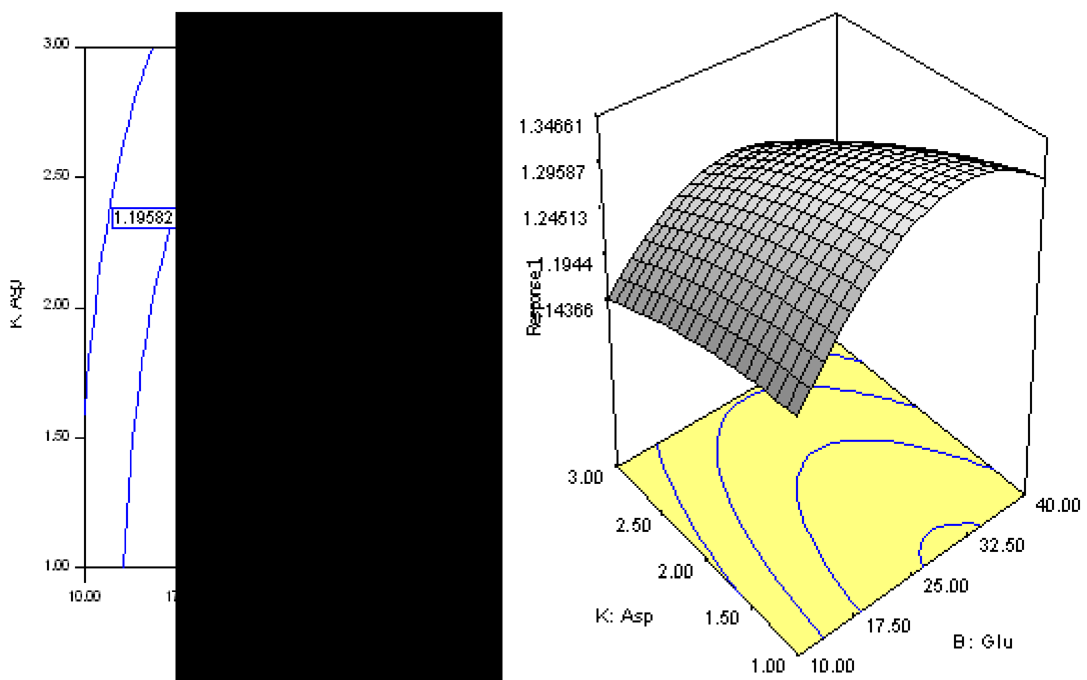


Fig 4.2 l. Contour & response surface plots of biomass vs. X = B: Glucose and Y= K: Asparagine

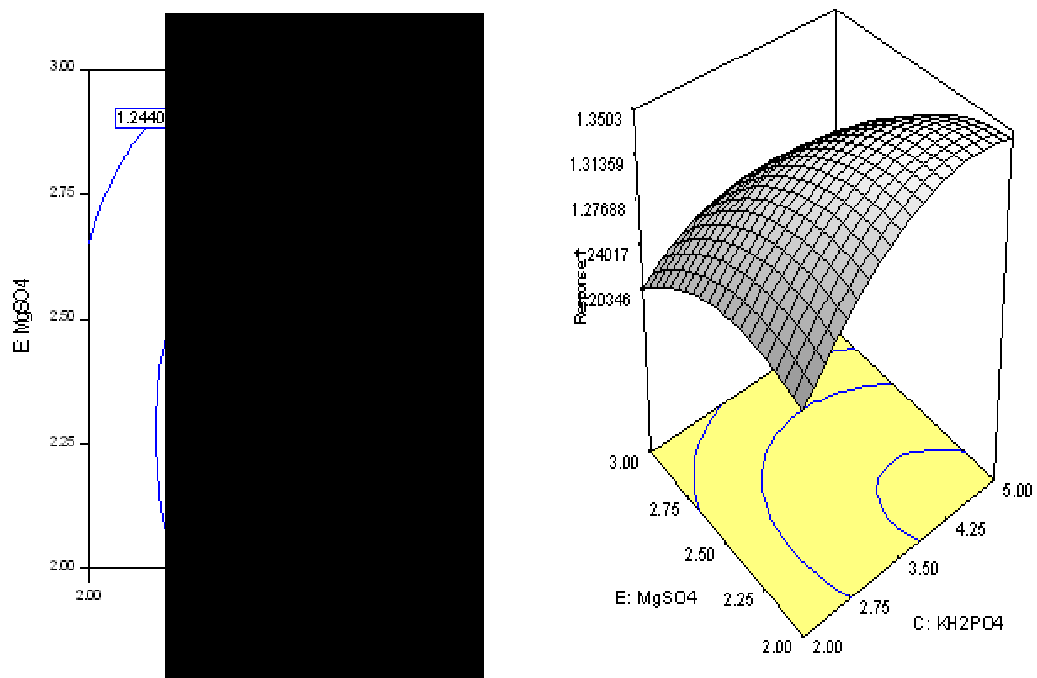


Fig 4.2 m. Contour & response surface plots of biomass vs. X = C: KH_2PO_4 and Y= E: $MgSO_4$. $7H_2O$

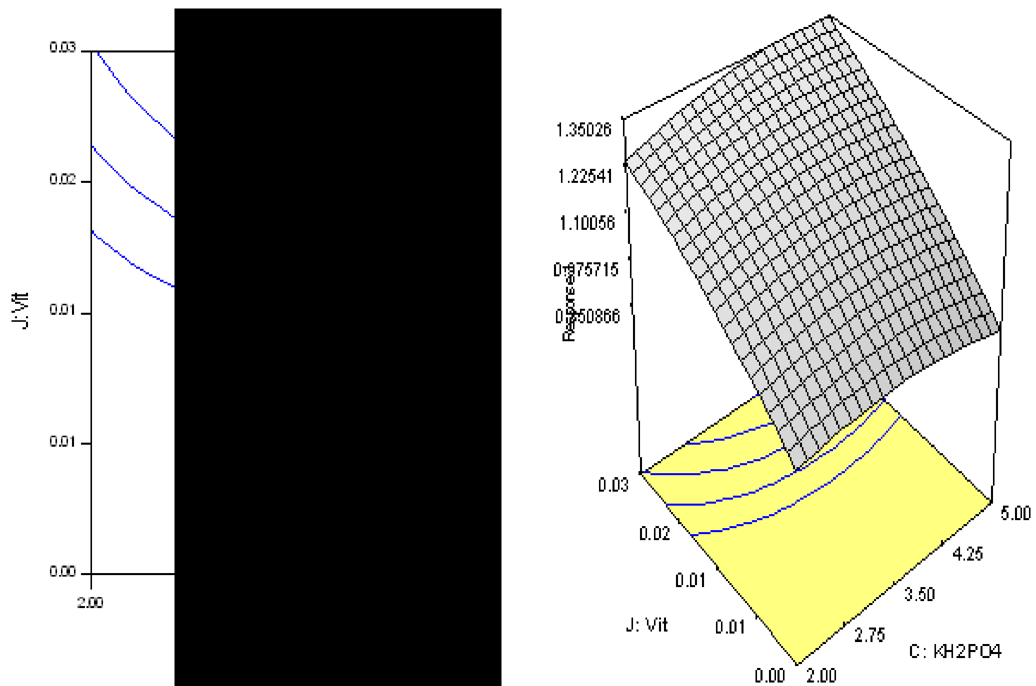


Fig 4.2 n. Contour & response surface plots of biomass vs. X = C: KH_2PO_4 and Y= J: Vitamin Mix

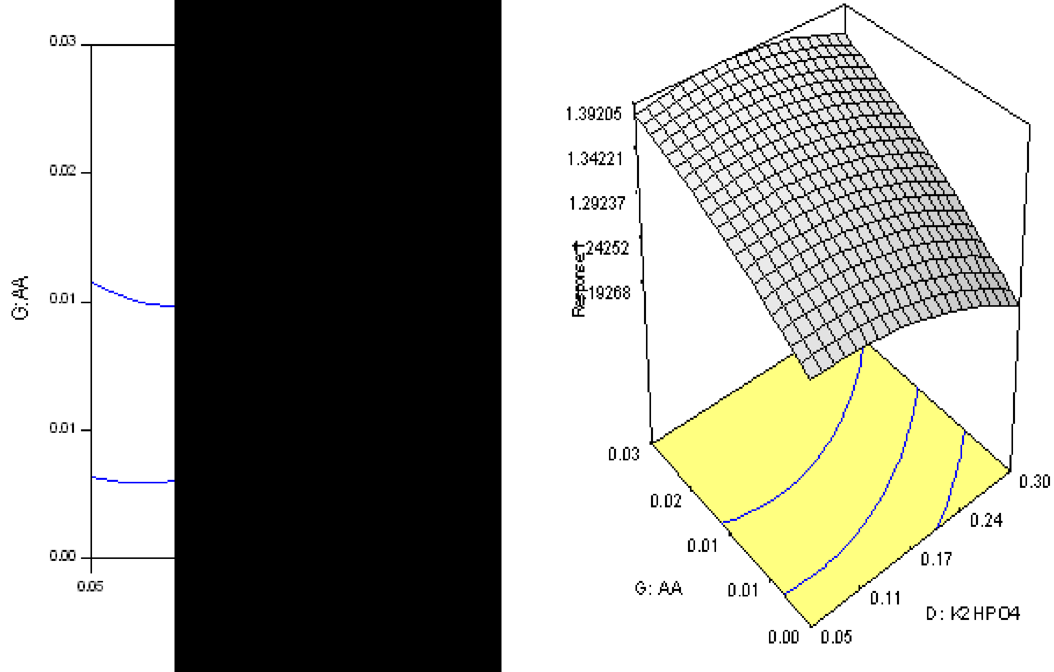


Fig 4.2 o. Contour & response surface plots of biomass vs. X = D: K_2HPO_4 and Y= G: Amino acid Mix

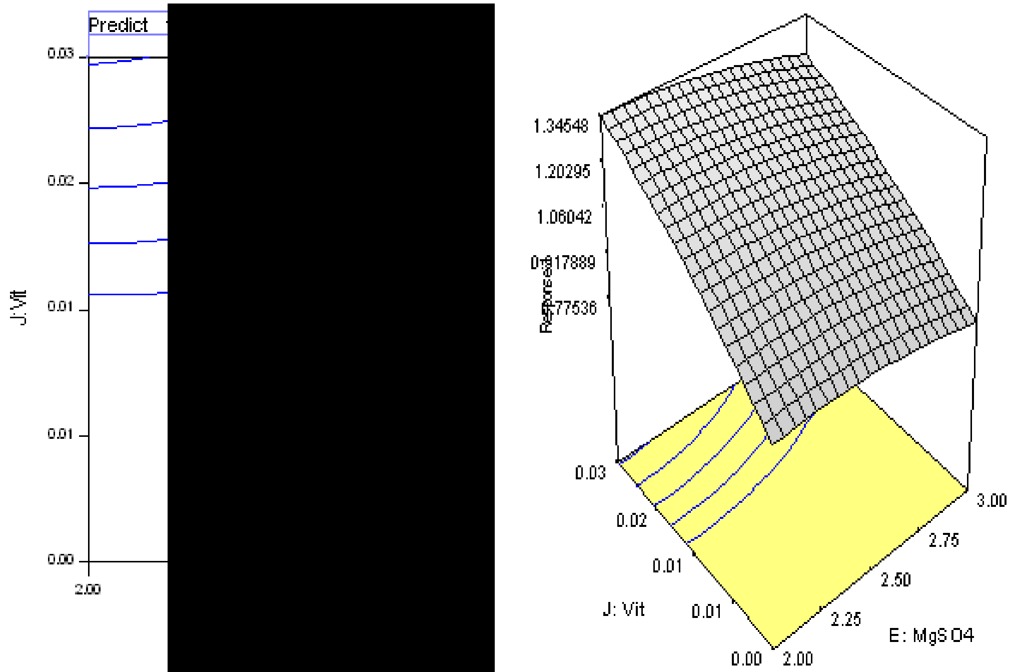


Fig 4.2 p. Contour & response surface plots of biomass vs. X = E: $MgSO_4$. $7H_2O$ and Y=J: Vitamin Mix

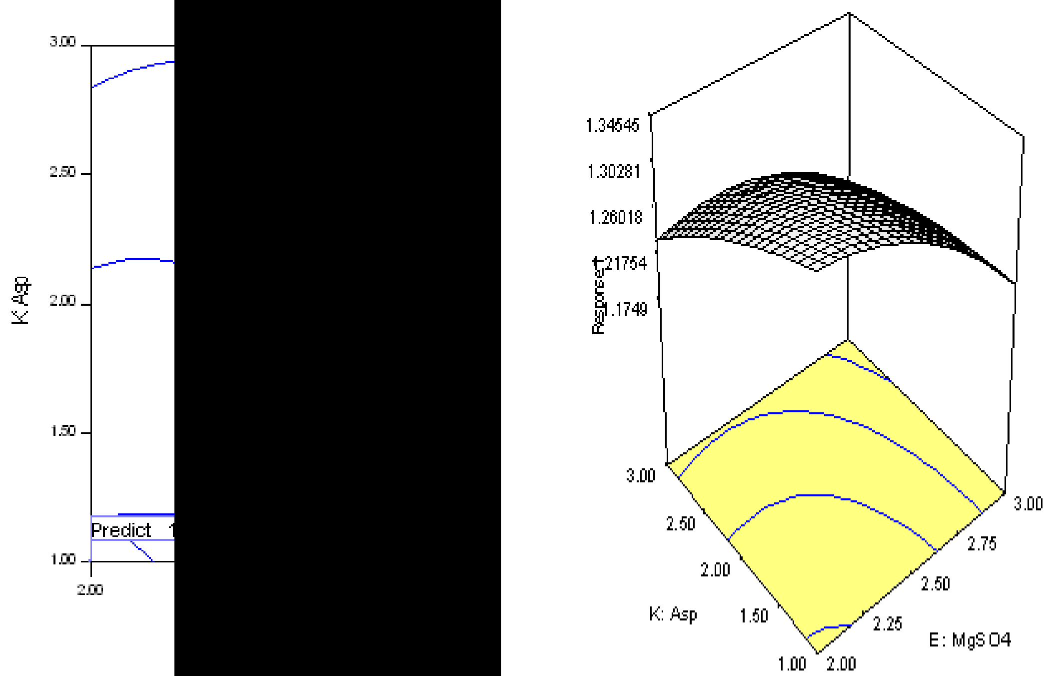


Fig 4.2 q. Contour plot and the corresponding response surface plot of biomass vs. X = E: $MgSO_4$. $7H_2O$ and Y= K: Asparagine

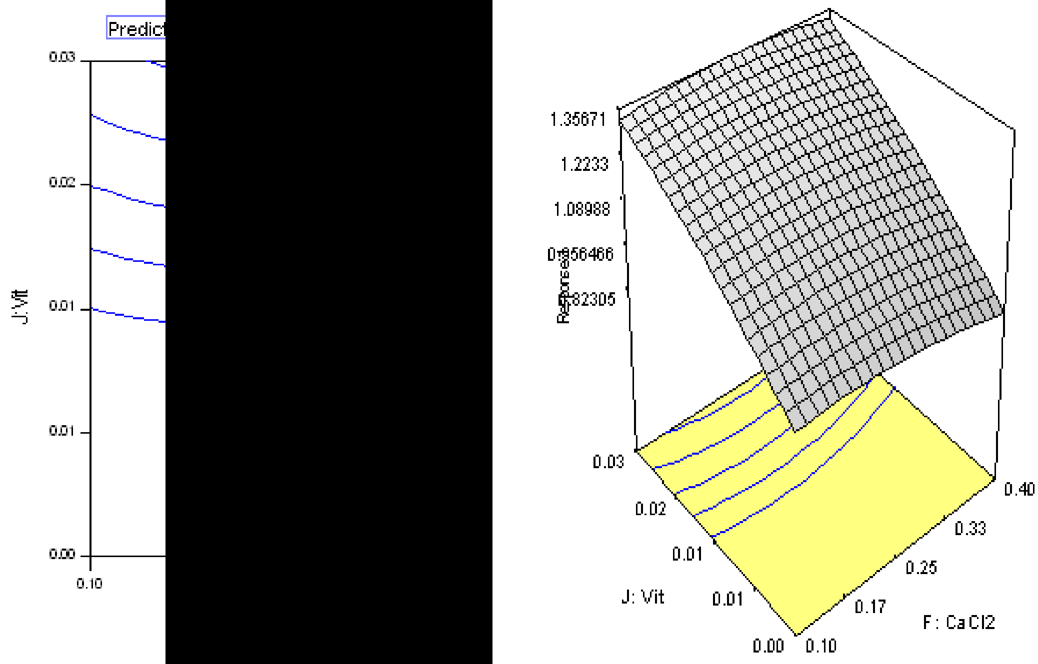


Fig 4.2.r. Contour & response surface plots of biomass vs. X = F: $CaCl_2$. $2H_2O$ and Y= J: Vitamin Mix

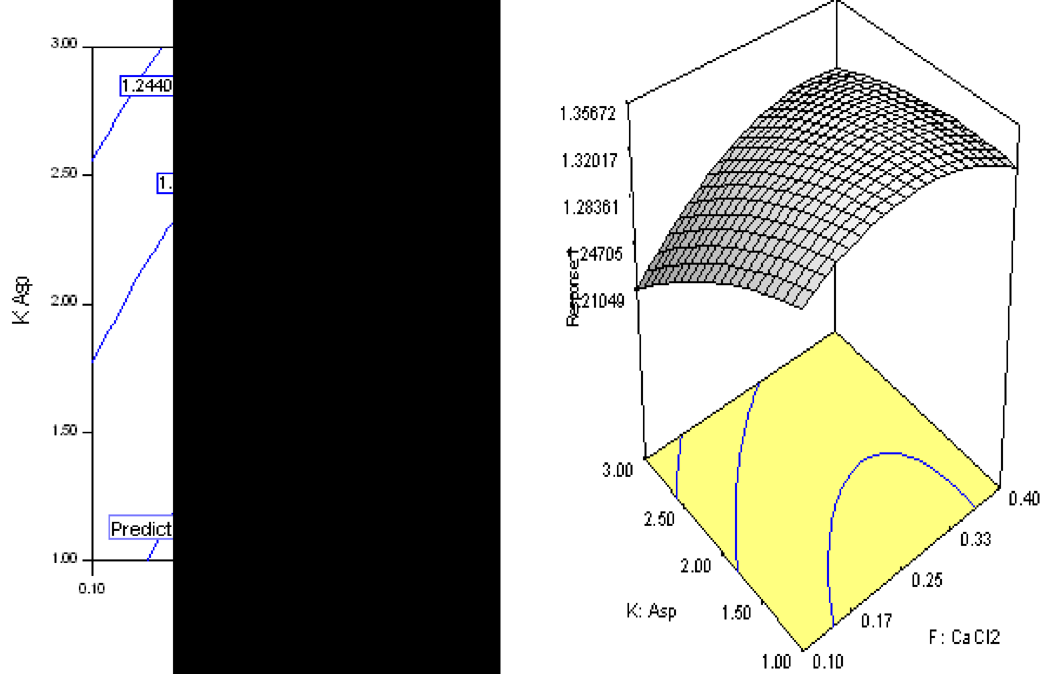


Fig 4.2.s. Contour & response surface plots of biomass vs. X = F: $CaCl_2$. $2H_2O$ and Y= K: Asparagine

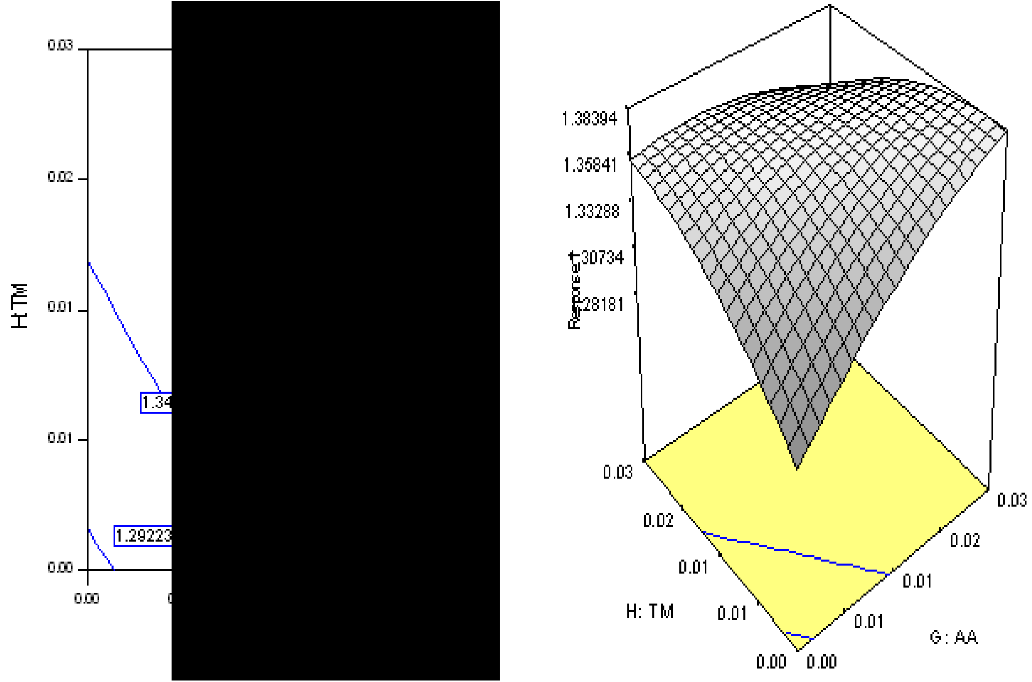


Fig 4.2.t Contour & response surface plot of biomass vs. X = G: Amino acid Mix and Y=H: Trace Metal Mix

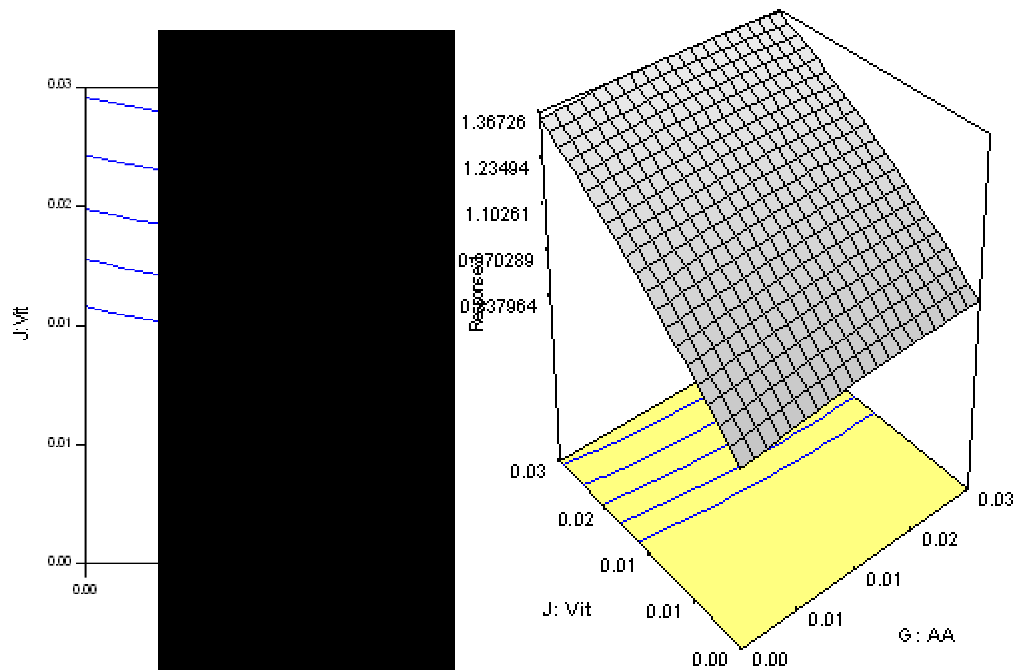


Fig 4.2 u. Contour & response surface plots of biomass vs. X = G: Amino acid Mix and Y= J: Vitamin Mix

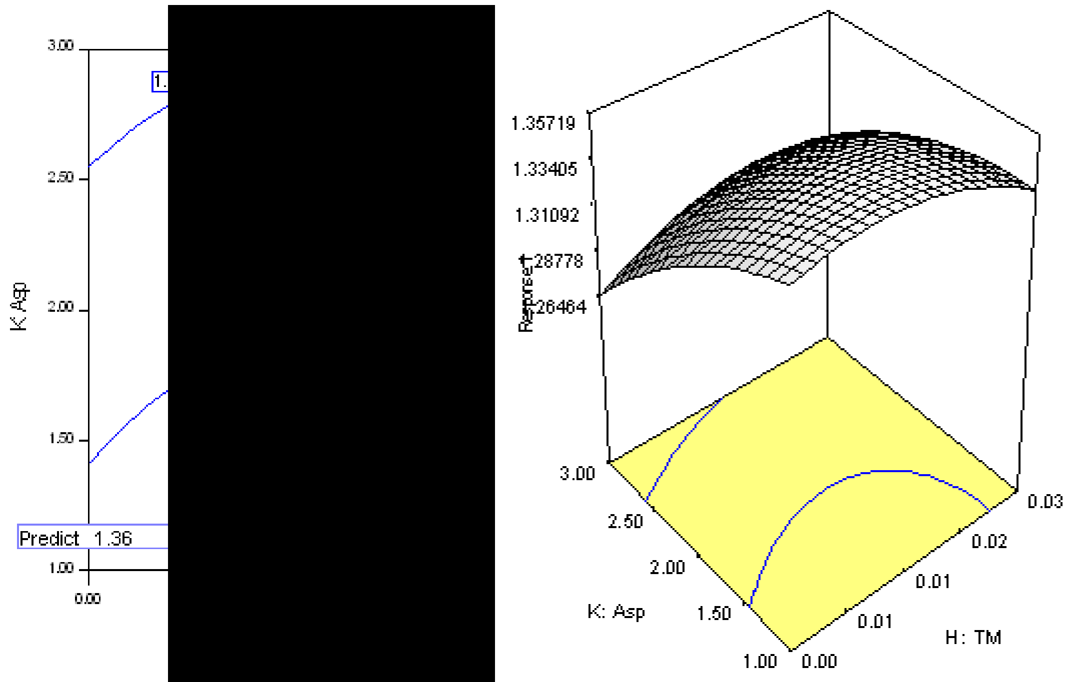


Fig 4.2 v. Contour & response surface plots of biomass vs. X = H: Trace Metal Mix and Y= K: Asparagine

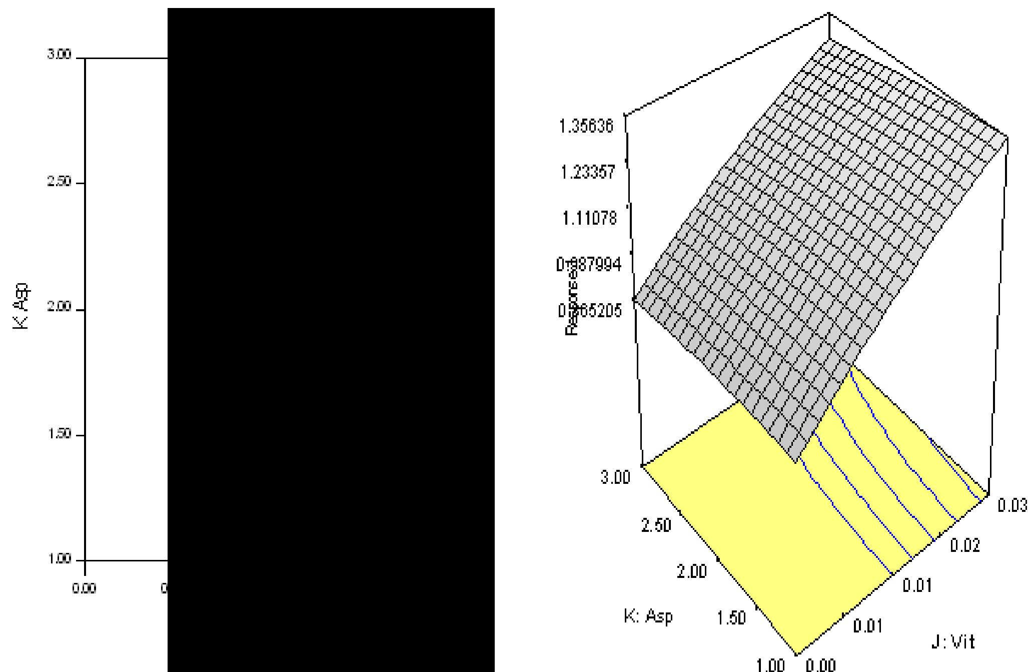


Fig 4.2.w. Contour & response surface plot of biomass vs. X = J: Vitamin Mix and Y= K: Asparagine

4.4 Discussion

Due to the lot-to-lot variation of complex nutrients it is often hard to obtain consistent results in successive experiments. Therefore, to investigate the effects of nutrients, chemically defined medium is the preferred choice. Biomass production of marine yeast *Candida* sp. S 27 was attempted in a chemically defined medium. The three-dimensional response surfaces were generated to study the interaction among the ten medium components and the optimum medium composition was found out.

The cellular functions are strongly influenced by the composition of the environment. All the required elements are taken up by the cells from their environment. In the present study, the carbon source glucose required for biomass production was 32.72g/L whereas in the basal medium suggested by Barnett and Ingram (1955) it is 10 g/L. Casas et al. (1997) stated that for a growth medium with no production purposes, moderate quantities of glucose (close to 12 kg/m^3) offered the best yield in the transformation to biomass. They found that for *Candida bombicola* growth in a synthetic medium, both nitrogen and phosphorus were

nutrients with great influence and magnesium lacked significance at the concentration interval tested.

Dalmau et al. (2000) reported that among the various carbon sources (at 2g/L) screened for the growth and lipase production by *Candida rugosa*, glucose as sole carbon source promoted good growth of the strain but no lipase activity was detected.

The nitrogen content of yeast cells is about 10 % of dry weight. Yeasts generally utilize ammonium nitrogen at different rates, depending on the anion present. Diammonium phosphate is utilized most efficiently, and ammonium chloride, least whereas some strains can utilize even urea as source of nitrogen (Spencer et al., 1997). Sreekumar et al. (1999) optimized the media components for ethanol production using *Zymomonas mobilis* and determined the composition of the medium as glucose 120.4 g/L, ammonium sulphate 0.96 g/L, potassium dihydrogen phosphate 0.02 g/L , yeast extract 6.5 g/L and magnesium sulphate 0.5 g/L. They found that excess nitrogen could lead to more biomass but less ethanol production.γ-Linolenic acid production by *Cunninghamella echinulata* growing on complex organic nitrogen sources such as corn gluten, corn steep, whey concentrate, yeast extract and tomato waste hydrolysate was studied by Fakas et al. (2008). They found that growth on tomato waste hydrolysate (TWH) yielded the maximum biomass of 17.6 g/L containing 39.6% oil and significant quantities of γ-Linolenic acid. In the present study, the concentration of $(NH_4)_2SO_4$ required was 5.42 g/L and that of asparagine was 3 g/L which in the unoptimized basal medium accounted for 3.5 g and 1.5 g/L respectively.

For pyruvic acid production by *Torulopsis glabrata* TP19 in batch fermentation, the critical components required were estimated using response surface methodology as ammonium sulfate (10.75 g/L), glucose (109.38 g/L) and nicotinic acid (7.86 mg/L) with a predicted value of maximum pyruvic acid production of 42.2 g/L. The determination coefficient (R^2) was 0.9483, which ensured adequate credibility of the model (Zhang and Gao, 2007). Singh and Satyanarayana (2008) reported that the optimum values of the critical components determined by central composite design of response surface methodology by a thermophilic mould

Sporotrichum thermophile in solid state fermentation for the maximum phytase production were glucose 3%, and ammonium sulphate 0.5%. For the production of phytase by a marine yeast *Kodamaea ohmeri* BG 3, the optimum variables determined by RSM were oats 1.0%, ammonium sulfate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3 (Li et al., 2008). For biomass production of recombinant *E.coli* cells Nikerel et al. (2006) applied RSM and found that for maximum yield, the concentrations of carbon source, inorganic nitrogen, potassium and magnesium required were glucose 15 g /L, $(NH_4)_2HPO_4$ 6.6 g/L, KH_2PO_4 20.1 g/L and $MgSO_4$. $7H_2O$1.7 g/L.

Ghosalkar et al. (2008) reported that for the maximum biomass production of recombinant *Pichia pastoris* in the fermentor, response surface methodology was effective in optimizing nutritional requirements and the optimum medium composition was glycerol 20 g/L, $(NH_4)_2SO_4$ 7.5 g/L, $MgSO_4$. $7H_2O$ 1g/L, KH_2PO_4 8.5 g/L, vitamin solution 1.5 mL/L and trace metal solution 20 mL/L.

Kilonzo (2008) commented that medium affected both the growth characteristics of recombinant *S. cerevisiae* and stability of the plasmid through different metabolic pathways and regulatory systems of the host. Superior plasmid stability of 92% was obtained in complex medium with 2% D-glucose yielding 48 units of glucoamylase/g of cells compared to 54% plasmid stability achieved with 2% soluble starch, which yielded 23 units of glucoamylase/g of cells.

Yeasts utilize inorganic phosphates for growth which is taken up as the monovalent anion, $H_2PO_4^-$ and more is taken up of the monobasic potassium salt than the dibasic form (Spencer et al., 1997).Flores-Cotera (2001) found that low concentrations of ammonium or phosphate increased the fatty acid content in *Phaffia rhodozyma* cells.

The optimum point for maximum cell mass production by *Rhodotorula glutinis* was located at a C/N ratio of 31.6 and a KH_2PO_4 concentration of 2.2 g/l (Park et al., 2005). From the response surface graph it was inferred that the carotenoid production by *R. glutinis* decreased and then increased gradually with

increasing KH_2PO_4 from 0 to 3 g/L. For biomass production of marine yeast *Candida* sp. S 27, the requisite was 5 g/L.

Kumar and Satyanarayana (2001) accounted that in submerged cultivation for glucoamylase production by *Pichia subpelliculosa* ABWF-64 the optimized medium contained 1% starch, 0.2 % yeast extract, 0.4% K_2HPO_4, 0.035 % NaCl and 0.1 % $MgCl_2$. In our study the amount of K_2HPO_4 necessary for biomass production was 0.015 %. This is in agreement with the amount in the basal medium (150 mg/L) in the present study.

Uptake of sulphate by yeasts requires energy, so the medium must contain both glucose or other metabolizable compounds and available nitrogen. Magnesium, potassium, strontium, calcium, copper, iron, manganese, chloride and zinc are essential to yeast (Spencer et al., 1997).

Yeasts require pantothenic acid, inositol, thiamine, nicotinic acid, pyridoxine, riboflavin, p-aminobenzoic acid and folic acid (Umezawa and Kishi, 1989). Supplementation with ascorbic acid, biotin, choline and pyridoxine increased xylitol production by *Candida tropicalis* in a chemically defined medium (Kim and Oh, 2003). In the present study also the growth factors were an essential ingredient for biomass production and the level required was twice the concentration in the basal medium suggested by Barnett and Ingram (1955). Song et al. (2008) noticed that the growth of *Mannheimia succiniciproducens* in a chemically defined medium could be improved by more than 30% by the addition of alanine, asparagine, aspartic acid, proline, serine, ascorbic acid, or biotin.

Among the test variables, KH_2PO_4, amino acid mix, vitamin mix and trace metal mix produced largest effect on yeast biomass production because the linear, quadratic and interaction effects of these parameters were highly significant. All the other variables had significant quadratic and interaction effects which justify their addition in the medium. Normally, a regression model having an R^2 value higher than 0.9 is considered as having a very high correlation (Guilford and Fruchter, 1973; Haaland, 1989) and a model with an R^2 value between 0.7 and 0.9 is considered as having a high correlation (Guilford and Fruchter, 1973). In the present case, an R^2

value of 0.9673 reflected a good fit between the observed and predicted responses, and it was reasonable to use the regression model to analyze the trends in the response.

The optimal design of culture media is important in the development of a bioprocess. The attainment of optimal conditions for multivariable bioprocess is often tedious. However, it is possible to undertake a rational study by using statistical designs that are adequately experimental to decrease the number of experiments. RSM, an experimental strategy for seeking the optimum conditions for a multivariable system is a much efficient technique for bioprocess development making it cost-effective and more economical.

Chapter 5

Optimization of a crude medium for biomass production of *Candida* sp. S 27

5.1 Introduction

The nutritional requirements of yeasts, like other living organisms are sources of carbon, nitrogen, phosphorus, trace elements and growth factors. Yeasts being chemoorganotrophs are unable to utilize radiant energy and thus rely on the chemical compounds provided in the culture medium. The culture medium supplies nutrients for growth, energy, building of cell substance, and biosynthesis of products. The choice of a good medium is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation. Of particular importance are the sources of carbon and nitrogen in the medium, since microbial cells and products are composed largely of these elements. In addition to carbon and nitrogen compounds, a medium contains inorganic salts, water, vitamins, and other growth factors which are all considered as nutrients. A poor choice of medium components can cause limited cellular growth and little if any yield of products. Also a poor medium can alter the types and ratios of products from among those for which a particular microorganism has biosynthetic capability. Thus the types and amounts of nutritive components of a medium are critical.

Because of the cost of the relatively pure ingredients used, the synthetic media turn out to be expensive and hence less preferred for large scale biomass production. The alternative is the non synthetic or crude medium, which usually allows much higher yields than does a synthetic medium. It contains crude or ill-defined sources of nutrients and as such they provide an excess of both nutrients and growth factors. Inorganic nutrients usually present little problem when crude media are employed, because the common anions and cations occur in sufficient quantities in the crude medium components. Crude media are more likely to provide excess of toxic ions than to be deficient in required ions. Several of the better crude nutritive sources are in themselves complex mixtures of nutrients, supplying carbon and nitrogen compounds as well as microbial growth factors (Casida, 1968). Generally, microorganisms grow more vigorously in complex media than in mineral media, because the former contain biosynthetic precursors that can be channeled directly into anabolic pathways, reducing the need to produce biosynthetic precursors and

saving metabolic energy. This has a significant effect on growth and production characteristics (Hahn-Hagerdal et al., 2005).

Substrates used

Many renewable, usually agricultural, forestry and food processing waste by-products, such as molasses, sugarcane bagasse, liquid sucrose, whey, wheat bran, confectionary effluent, wastes from paper mills and sugar syrup industries, fruit processing wastes, starch wastes, wastes from meat-processing industries, prawn shell wastes, animal manure, sulfite liquor wastes, activated sewage sludge etc were widely used for yeast based single cell protein (SCP) production.

The modern history of SCP began in late 1950 when petroleum industry began experiments on the use of microorganisms for removal of paraffin wax and sulphur containing fractions from crude oil. In 1964, the British Petroleum company announced success in cultivating a yeast *Candida lipolytica* on broad petroleum fractions such as gas oils and slack waxes with the simultaneous removal of paraffin component from crude fractions. It was found that these organisms contained over 50% high quality protein and so what started out as a by-product became the primary object of research to produce SCP. Although no signs of carcinogenicity or toxicity were observed, opposition from many countries by environmental groups prevented its marketing as a human food supplement, and it was decided to label the product as an animal feed. Petrochemical substrates such as natural gas, gas-oil, coal –gas, normal paraffins, ethanol and methanol which constitutes the unconventional substrates were also utilized for bioprotein production.

Concern on substrate safety and increase in petroleum prices shifted interest back to the utilization of renewal sources, mainly food and agriculture by-products like molasses and whey, or industrial wastes rich in starch, cellulose and hemicellulose. The major SCP projects based on petroleum derivates as substrates were abandoned in the 1980s.

Candida utilis has proved its potential to utilize sulphite waste liquor from the paper pulping industry as the sole carbon source (Wiley et al., 1950). Ornelas-Vale et al. (1977) succeeded in producing single cell protein from the nopal fruit using *C.*

utilis. The *Kluyveromyces fragilis* strains are the yeasts that have been most widely studied for the production of yeast biomass from whey (Castillo, 1990). The SCP could be produced from whey using yeasts such as *Kluyveromyces, Candida* and *Trichosporon,* as they are naturally able to metabolise lactose (Mansour et al., 1993). *C. utilis* grows well on wide variety of substrates such as sucrose, ethanol and spent-sulphite liquor. It can also grow on wood hydrolysates because of its ability to assimilate pentoses.

Single cell protein production from mandarin orange peel was studied using various yeast strains like *S. cerevisiae*, *C.utilis*, *Debaryomyces hansenii* and *Rhodotorula glutinis* by Nishio and Nagai (1981). Manilal et al. (1991) were also successful in the aerobic treatment of cassava starch factory effluent with concomitant SCP production using a mixed culture of *C.utilis* and *Endomycopsis fibuliger*. Single cell protein production from *Schwanniomyces castellii* was optimized using cassava starch as carbon source (Hongpattarakere and Kittikun, 1995). Yeasts able to assimilate hydrocarbons are *Yarrowia lipolytica, Candida tropicalis, C. rugosa* and *C. guilliermondii*, which can usually also be produced on lipids. Methanol is the preferred alcohol utilized as substrate by *Pichia spp.* (*P.pastoris,P,methanolica,* etc.), *Hansenula polymorpha, H. capsulata* and *C. boidinii*. Rishipal and Philip (1998) were successful in generating single cell protein of marine yeasts on prawn shell waste. Biomass yield and the protein ratio of the biomass of *Candida utilis* grown on ram horn hydrolysate, the waste products of the meat industry (Kurbanoglu, 2001) were found to be similar to the results of investigations in which some other yeasts *C. pseudotropicalis, C.utilis, C. krusei* and *C. tropicalis* were grown on sweet whey and vinasse medium and *C. utilis* was grown on pineapple cannery effluent (Nigam, 1998). Some yeast species of *Torulopsis* and *Candida* are also able to grow on methanol as sole energy and carbon source (Bekatorou et al., 2006).

The by-product of the sugar industry, molasses are the concentrated syrup or mother liquor recovered at any one of several steps in the sugar refining process. Beet and cane molasses have been the most widely used substrate for baker's yeast biomass production since 1939.Molasses contain 45-55% fermentable sugars such as

sucrose, glucose, fructose, raffinose, melibiose and galactose. Production of high quality edible protein from *Candida* grown on cane molasses has been reported by Lawford et al. (1979). Many workers have reported the utilization of molasses as carbon source in fermentation processes and in the production of lactic acid, citric acid etc (Żarowska et al., 2001; Young-Jung et al., 2004).

Pharmamedia (Traders Protein, USA) is an economical, finely ground, yellow flour made from the embryo of cottonseed. The principal component of Pharmamedia is nonhydrolyzed globular protein. It is used as a protein nutrient for microorganisms which produce antibiotics, enzymes, steroids & vitamins by fermentation. Commercial medium (Pharmamedia) was investigated for the production of surfactin by *Bacillus subtilis* MZ-7 by Al-Ajilani et al. (2007). As source of complex organic nitrogen source pharmamedia was utilised in thermolabile xylanase production by Antarctic yeast *Cryptococcus adeliae* (Gomes et al., 2000). The production of penicillic acid by *Aspergillus sclerotiorum* CGF for the biocontrol of *Phytophthora* disease was investigated in submerged fermentation using media composed of different nutrients (Kang et al., 2007) Several organic and inorganic nitrogen sources like bacto peptone, polypeptone, pharmamedia, soyabean meal, yeast extract, beef extract, urea, tryptone, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and KNO_3 were screened to select the suitable one.

The value of a water-extracted Pharmamedia agar medium for the culture of fungi was determined by Slifkin (2000). Chen et al. (2003) immobilized a microbial consortium having high capacity for decolorisation of azo dye by a phosphorylated polyvinyl alcohol gel. In order to enhance economical efficiency, cheaper supplementary nitrogen sources such as such as fish meal, soybean meal, pharmamedia and vita yeast powder were examined to optimize the decolorisation process. Although primarily used as a nitrogen source, this cotton seed derived nutrient contains small, but significant amounts of carbohydrate.

Corn Steep Liquor, a watery by-product resulting from the steeping of corn for corn starch production is used in the commercial manufacture of feed stuffs. Although usually included as a nitrogen source, it does contain lactic acid, small amounts of reducing sugars and complex polysaccharides. A medium containing

cornsteep liquor (CSL) as a replacement for yeast extract (YE) improved the sporulation of *Bacillus sphaericus* 2362 in fed batch culture (Sasaki et al., 1998). Alkali treated corn steep liquor containing medium enhanced protein A production by *Staphylococcus aureus* (Muley et al., 1998). Supplementation of a cheese whey medium with 5 % v/v CSL resulted in 8.7 fold increase in lactic acid production by under anaerobic condition (Agarwal et al., 2008). Sporulation performance of an aquaculture biological agent *Bacillus cereus* by supplementation with CSL has been studied by Lalloo et al. (2009). The improved sporulation performance of *B. cereu*s could be related to the increased availability of free amino acids, carbohydrates, and minerals in CSL, which had a positive effect on sporulation efficiency.

Comparatively higher biomass production by edible mushroom *Pleurotus sajor-caju* in submerged culture fermentation was reported with the supplementation of corn gluten meal (Kausar et al., 2006). Of the 30 different agricultural products and by-products tested, cottonseed meal, defatted soy flour, and corn gluten meal were the most efficient substrates for the production of spore-crystal biomass and endotoxin potency by *Bacillus thuringiensis subsp. aizawai* (HD133). Soya bean meal as a sole nitrogen source promoted gentamicin production by *Micromonospora echinospora (Me-* 22*)* (Himabindu and Jetty, 2006). Bio-ethanol production from soyabean molasses by *S. cerevisiae* at laboratory, pilot and industrial scales have been demonstrated by Siqueira et al. (2008).

Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology was done by Rao et al. (2000). A 2^{5-1} fractional factorial central composite design was chosen to explain the combined effect of the six medium constituents, viz. yeast extract, peptone, casamino acids, ammonium sulphate, potassium phosphate and galactose, and to design a minimum number of experiments. The optimized medium produced 65.3 mg/l of r-hirudin in shake flask culture, which is 35% higher than the unoptimized medium.

Studies were carried out for the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface *methodology* (RSM) experiments (Medeiros et al., 2000). Five agro-

industrial residues were evaluated as substrate for cultivating a strain of *K. marxianus*. The results proved the feasibility of using cassava bagasse and giant palm bran (*Opuntia ficus indica*) as substrates to produce fruity aroma compounds by the yeast culture. In order to test the influence of the process parameters on the culture to produce volatile compounds, two statistical experimental designs 2^5 and 2^2 factorial designs were performed. In case of cassava bagasse, the2^5 factorial design did not reveal the influence of glucose, but it was found important in the 2^2 design. The reason was attributed to the lower concentrations of glucose used in the first experimental design.

A Plackett Burman design followed by a central composite design and response surface analysis was employed to optimize the culture medium for the production of lipase with

Candida sp. 99-125 (He and Tan, 2006). The concentrations of soya bean meal, soya bean powder and K_2HPO_4 were optimized which allowed the production of lipase to be increased from 5000 to 6230 IU/ml in shake flask system.

Kluyveromyces marxianus Y-8281 yeast culture was utilized for the biological treatment of deproteinated whey wastewater in a batch system (Aktas et al., 2006). Removal of lactose was optimized by the utilization of response surface methodology. The empirical model developed through RSM in terms of effective operational factors of medium pH, temperature, lactose and ammonia concentrations was found adequate to describe the treatment of deproteinated whey. Under the optimum operating conditions determined, 95% lactose removal was achieved after an 18-h fermentation.

Xiong et al. (2007) optimized the nutrient levels for the production of inulinase by a newly strain *Kluyveromyces* S120 in solid-state fermentation (SSF) using response surface methodology (RSM) based on Plackett–Burman design and Box–Behnken design. In the first step of optimization, a Plackett–Burman design was used to evaluate the influence of related factors. Inulin, corn steep liquor and $(NH_4)_2SO_4$ were found to be more compatible supplement with the substrate of wheat bran and positively influenced on inulinase production. The concentrations of the

above three nutrients above were further optimized in the second step using a Box–Behnken design. The optimized SSF medium composition was 12.72% inulin, 10.76% corn steep liquor and 1.61% $(NH_4)_2SO_4$ by employing wheat bran as the solid substrate. By applying the RSM, the highest ever reported yield of the enzyme (409.2 U/gds) was achieved.

Li et al. (2008) applied statistical experimental designs for the optimization of phytase production by a marine yeast *Kodamaea ohmeri* BG3 in a cost-effective oats medium. Using Plackett–Burman design, oats, ammonium sulfate and initial pH were identified as significant factors and these factors were subsequently optimized using a central composite design (CCD). The optimum variables that supported maximum enzyme activity were oats 1.0%, ammonium sulfate 2.3%, glucose 2.0%, NaCl 2.0% and initial pH 6.3. The validity of the optimized variables was verified in shake-flasks level. An overall 9-fold enhancement in phytase activity (62.0 → 575.5 U/ml) was attained due to the optimization.

Response surface methodology was applied to optimize culture conditions for the growth of *Candida utilis* with bamboo wastewater (Li et al., 2009). A significant influence of initial pH, fermentation time and yeast extract on biomass of *C. utilis* was evaluated by Plackett–Burman design. These factors were further optimized using a central composite design and RSM. A combination of initial pH 6.1, fermentation time 69 h and yeast extract 1.17 g/L was optimum for maximum biomass of *C. utilis*. A 1.7-fold enhancement of biomass of *C. utilis* was gained after optimization in shake-flask cultivation.

5.2 Materials and methods

5.2.1 Organism used

Marine yeast *Candida* sp. S 27 was used for the study.

5.2.2 Process parameters

The process parameters maintained were salinity 4.68 $^0/_{00}$, pH 5.97 and temperature 32.72 ^{0}C.

5.2.3 Substrates used

The selected marine yeast *Candida* sp. S 27 was grown in different crude media to select the suitable substrate for the maximal biomass production. The substrates used were

1. Pharmamedia (Trader's Protein)
2. Corn Gluten Meal (Trader's protein)
3. Corn Steep Liquor (CSL) (Trader's protein)
4. Soyabean Meal
5. Sugarcane Molasses

5.2.4 Screening of substrates

5.2.4.1 Preparation of medium

The media in triplicates (100 ml each) were prepared in Erlenmeyer flasks with various crude substrates at 1% concentration.

5.2.4.2 Preparation of inoculum

Yeast inoculum was prepared by harvesting young culture (24 hours of growth on malt extract agar slant) of marine yeast *Candida* sp. S 27 using sterile saline of 4.68 $^{0}/_{00}$. Optical density of the culture suspension was measured at 540 nm and adjusted to 1 OD by appropriate dilution.

5.2.4.3 Inoculation and incubation

100 µl of this suspension was used as inoculum in 100 ml culture medium so that the initial O D of the culture medium is 0.001. The tubes were incubated at 32.72 ^{0}C for 48 hours.

5.2.4.4 Determination of growth

Growth was determined by measuring the optical density of the culture suspension at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601).

Among the five crude media screened, sugar cane molasses which supported maximum growth of the yeast strain was used for further study.

5.2.5 Optimization of molasses concentration

Sugar cane molasses, obtained from a private distillery in Cherthala, was diluted with distilled water (50 g in 200 ml) and used as stock solution, the total sugar of which was determined by Anthrone method (Hodge & Hofreiter, 1962). Molasses medium with varying concentrations of total sugar viz. 2, 5, 7, 10, 12, 15 and 17 mg/ml were prepared using the stock solution. After inoculation with 10µl yeast suspension (1 O D), incubation was done at 32.72 ^{0}C for 48 hours and the growth was measured at 540 nm. Molasses concentration was expressed as total sugars (mg/ml) in the medium.

5.2.6 Optimization of the nutrient supplements in molasses based medium

5.2.6.1 Experimental design

In the present study, the nutrient supplementation of a cheap complex medium was screened by the classical one at a time strategy and further optimized statistically by a full factorial central composite design (CCD) of the RSM. Initially the effect of each of these variables was independently observed keeping other variables constant in order to strike off the variables which did not have observable effect on yeast biomass production. The optimal levels of the important factors are then estimated statistically by employing RSM.

5.2.6.1.1. First step optimization

One dimensional screening was done initially to find out the range that has to be used for further optimization experiment.

5.2.6.1.1.1 Screening and optimization of nitrogen sources

The nitrogen sources screened were yeast extract, peptone, NH_4Cl and $(NH_4)_2SO_4$. Molasses based medium with five different concentrations of each of these four compounds were prepared (0, 0.25% 0.5% 0.75% and 1% for yeast extract and peptone; 0, 0.1%, 0.2%, 0.3% and 0.4% for NH_4Cl and $(NH_4)_2SO_4$) and inoculated with 10 µl of yeast suspension of 1 OD suspension such that the initial OD of the 10 ml medium is 0.001.After incubation at 32.72 ^{0}C for 48 hours, growth was measured at 540 nm.

5.2.6.1.1.2 Optimization of phosphorus

As source of phosphorus, KH_2PO_4 was incorporated at different concentrations like 0, 0.1, 0.2, 0.3, 0.4 and 0.5 % in molasses based medium and 10 µl of yeast inoculum was added to 10 ml of culture medium. Cultures were incubated at 32.72 ^{0}C for 48 hours and the growth was measured at 540 nm.

5.2.6.1.1.3 Optimization of magnesium

$MgSO_4$. $7H_2O$ was used as the source of magnesium in which different concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5%) were tested to find out the optimum concentration required for growth. 10 ml molasses based (4.68 ‰) medium were prepared and inoculation was done with 10 µl of yeast suspension. After incubation at 32.72 ^{0}C for 48 hours, the growth was measured at 540 nm.

5.2.6.1.1.4 Optimization of calcium

Different concentrations of $CaCl_2$.6 H_2O (0, 0.1, 0.2, 0.3, 0.4 and 0.5%) were tested to find out the optimal concentration required for the growth of the selected yeast strain. After inoculation with 10 µl of yeast suspension in molasses based water (4.68 ‰), incubation was done at 32.72 ^{0}C for 48 hours and the growth was measured at 540 nm.

5.2.6.1.1.5 Optimization of vitamin mix and amino acid mix

Stock solutions of vitamin mix and amino acid mix were prepared. Concentration as per Barnett (1972) was taken as single strength. 5 different levels of each of these factors (nil concentration, half strength, single strength, double strength and triple strength) were assessed to find out the optimum amounts required.

5.2.6.1.2 Second step optimization by RSM

The molasses based medium for yeast biomass production has been first optimized by 'one-variable-at-a-time' approach. The minimum and maximum ranges of the selected variables (supplements) were investigated. The medium components that resulted in maximum yeast biomass production were further optimized by RSM using CCD and a set of 13 experiments comprising of 4 factorial points, 4 axial points and 5 center points were carried out. The statistical software package Design-Expert (Version 6.0.9, Stat-Ease Inc., Minneapolis, MN, USA) was used for

regression analysis of experimental data and to plot response surface. The actual values of the variables and the combinations are presented. (Table 5.1and Table 5.2)

Table 5.1 Low and high levels of the two variables along with the coded levels

Factor		Lower limit (-1)	Higher limit (+1)
A	Molasses (total sugars)	7 mg/ml	12 mg/ml
B	Yeast extract	0.25 %	0.75 %

Table 5.2 CCD matrix of the two components of the crude medium

Run Order	Factors	
	Molasses (total sugars mg/ml) A:X_1	Yeast extract (%) B:X_2
1	+1 (12)	+1 (0.75)
2	0 (9.50)	+α (0.85)
3	-1 (7)	-1 (0.25)
4	0 (9.50)	0 (0.50)
5	0 (9.50)	0 (0.50)
6	-1 (7)	+1 (0.75)
7	+α (13.04)	0 (0.50)
8	0 (9.50)	0 (0.50)
9	0 (9.50)	-α (0.15)
10	0 (9.50)	0 (0.50)
11	0 (9.50)	0 (0.50)
12	-α (5.96)	0 (0.50)
13	+1 (12)	-1 (0.25)

5.2.6.1.3 Experimental verification

The optimum concentrations of ingredients of the media were found out from the regression equation for biomass. Experiments were conducted at the optimum levels suggested by the software. The molasses medium supplemented with 0.75 % yeast extract was prepared in 4.68 $^0/_{00}$ saline. The pH was adjusted to 5.97 and after inoculation with yeast suspension; incubation was done at 32.72 ^{0}C for 48 hours. Growth was determined by measuring the OD at 540 nm. The experimental values were subsequently compared with predicted values obtained from the model equation.

5.3 Results

5.3.1 Screening of substrates

The potential of various agro-industrial raw materials such as molasses, pharmamedia, corn steep liquor, corn gluten meal and soyabean meal were examined to select the suitable substrate for the maximal biomass production. Among the five crude media screened, sugar cane molasses supported maximum growth of the yeast strain. It was followed by corn steep liquor. There was no notable variation in the effects of crude media such as pharmamedia, corn gluten meal and soyabean meal on biomass production by *Candida* sp. S 27. (Fig 5.1)

5.3.2 First step optimization

Concentration of molasses was expressed as total sugars (mg/ml). Remarkable yeast growth could be observed with increase in sugar concentration up to 12mg/ml and thereafter the increase was only nominal (Fig 5.2).

Among the various sources screened (yeast extract, peptone, $(NH_4)_2SO_4$ and NH_4Cl), yeast extract served as the best nitrogen source followed by peptone (Fig 5.3). It was found that there was not much variation in yeast biomass production with the addition of potassium, magnesium, calcium, amino acid and vitamin supplements (Figs 5.4-5.8).

Based on the results of the regression analyses with biomass as X for molasses and yeast extract, the range was selected for second level optimization by Response Surface Methodology.

5.3.3 Second level optimization

The range of the 2 media components *viz.* molasses expressed as total sugars and yeast extract that supported significant growth was selected based on the results of first step optimization *i.e* regression analysis. RSM using CCD was applied to determine the interaction between the two parameters and their optimum levels for the maximum biomass production of marine yeast *Candida* sp.S 27.

Experiments were carried out in triplicate for all the given combinations and the mean value was taken for analysis. The observed and predicted responses of the

thirteen experiments are presented in Table 5.3.The experimental results were analyzed by standard ANOVA and the CCD was fitted with the second-order polynomial equation:

$Y = b_0+b_1X_1+ b_2X_2 +b_{11} X_1^2+ b_{22} X_2^2 + b_{12} X_1 X_2$ where Y is the estimated response, b_0 is a constant, b_i are coefficients for each term and X_i is the experimental factor and 'i' in coded units

Table 5.3 The design matrix of the CCD for two variables along with experimental and predicted values of biomass.

Run Order	Factors		Biomass (g/L)	
	Molasses (total sugars mg/ml) $A:X_1$	Yeast extract (%) $B:X_2$	Experimental value	Predicted value
1	+1 (12)	+1 (0.75)	13.139	13.14
2	0 (9.50)	+α (0.85)	12.4015	12.51
3	-1(7)	-1 (0.25)	10.5819	10.36
4	0 (9.50)	0 (0.50)	12.357	12.16
5	0 (9.50)	0 (0.50)	12.048	12.16
6	-1 (7)	+1 (0.75)	11.716	11.49
7	+α (13.04)	0 (0.50)	13.13	13.08
8	0 (9.50)	0 (0.50)	12.0305	12.16
9	0 (9.50)	-α (0.15)	10.979	11.08
10	0 (9.50)	0 (0.50)	12.145	12.16
11	0 (9.50)	0 (0.50)	12.198	12.16
12	-α (5.96)	0 (0.50)	10.308	10.58
13	+1 (12)	-1 (0.25)	12.238	12.25

The matrix was analyzed by standard analysis of variance (ANOVA) as approximate to the experimental design used (Table 5.4). The ANOVA of the quadratic regression model demonstrates that the model is highly significant for the concerned response i.e. Biomass as was evident from the Fisher's F-test (F_{model} = mean square regression/mean square residual = 45.11) with a very low probability value ($P<0.0001$). In this case, both the linear coefficients (A and B) and the quadratic coefficient (B^2) were significant model terms, where "A" is total sugars and "B" is yeast extract. The interaction coefficient was found to be not significant in

determining the response (Fig 5.9) depicted by the circular contour plot. But since it is a hierarchical model, the insignificant coefficient was not omitted from the final regression equation. The concentration of total sugars and yeast extract were exactly the same found out from ‘one- factor- at- a- time’ analysis.

Table 5.4 ANOVA for Response Surface Quadratic Model (as obtained from the software Design-Expert)

Coefficient	F value	P value
A	164.81	<0.0001
B	54.00	0.0002
A^2	4.99	0.0607
B^2	5.89	0.0456
AB	0.36	0.5683

Significant coefficients are highlighted in red

The goodness of fit of the model was checked by coefficient of determination (R^2). In this case , the value of the determination coefficient (R^2 for biomass =0.9703) indicates that sample variation of 97.03 % for biomass was attributed to the independent variables and only 2.97 % of the total variation was not explained by the model. Normally, a regression model having an R^2 value higher than 0.9 is considered as having a very high correlation (Guilford and Fruchter, 1973; Haaland, 1989) and a model with an R^2 value between 0.7 and 0.9 is considered as having a high correlation (Guilford and Fruchter, 1973). Lack of fit is a measure of how well the model fits the data. Since the model has a non significant lack of fit, it is a good predictor of the response and should be used.The value of the adjusted determination coefficient is also very high to advocate for a high significance of the model (Khuri and Cornell, 1987).A higher value of correlation coefficient (R = 0.9850) justifies an excellent correlation between independent variables. At the same time, a relatively low value of coefficient of variation (CV = 1.63 %) indicates a better precision and reliability of the experiments carried out. The adequate precision is used to measure the ratio of signal to noise ratio which is generally desirable greater than 4. As per the model, the adequate precision ratio obtained was 21.011 for biomass, indicating an adequate signal and can be used to navigate the design space (Table 5.5).

Table 5.5 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of biomass

Source	SS	DF	MS	F-value	Probability P>F
Model	8.67	5	1.73	14.10	0.0001
Residual error	0.27	7	0.038		
Lack of fit	0.20	3	0.065	3.74	0.1174
Pure error	0.070	4	0.017		
Cor total	8.94	12			

SS- sum of squares; DF- degrees of freedom; MS- mean square

The RSM gave the following regression equation for the biomass (Y_1) as a function of the 2 different media components total sugars and yeast extract designated as X_1 and X_2 (A and B) respectively.

Final equation in terms of coded factors is:

$$Y_1 = + 4.24598 + 0.90119\ X_1 + 5.77639\ X_2 - 0.026372\ X_1^2 - 2.86720\ X_2^2 - 0.093240\ X_1 X_2$$

Based on the data generated, the software suggested 3 solutions for the optimum concentration of the 2 different medium components (Table 5.6). All the three solutions had maximum desirability of 1, hence solution no: 1 was selected because there was a slight increase in predicted biomass.

Table 5.6 Optimum values of the media components recommended by the software

Solution No:	Molasses (mg/ml)	Yeast extract (%)	Biomass (g/L)	Desirability	
1	12	0.75	13.142	1.000	Selected
2	12	0.74	13.1403	1.000	
3	11.99	0.75	13.1401	1.000	

5.3.4 Experimental verification

Biomass production at optimum conditions was found to be (13.117 g/L) and there was no significant alteration from the suggested solution (13.142 g/L). The

study proves RSM as an adequate approach for modeling and optimization of media components to maximize biomass production by marine yeast *Candida* sp. S 27.

Table 5.7 Composition of optimized crude medium (Molasses-Yeast extract medium)

Ingredients	Concentration
Molasses (mg/ml)	12
Yeast extract (%)	0.75

Ph - Pharmamedia
CGM - Corn Gluten Meal
CSL -Corn Steep Liquor
S M - Soyabean Meal
Mo -Sugarcane Molasses

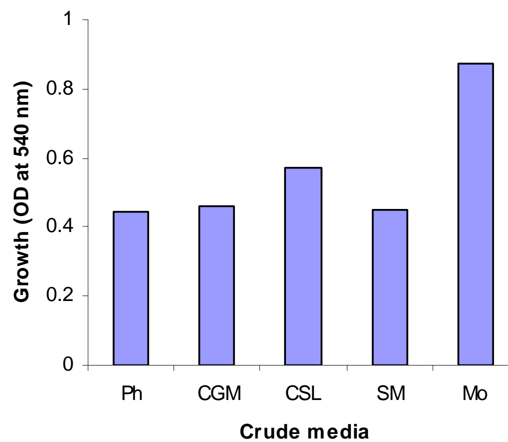


Fig 5.1 Growth of the marine yeast *Candida* sp. S 27 on different crude media

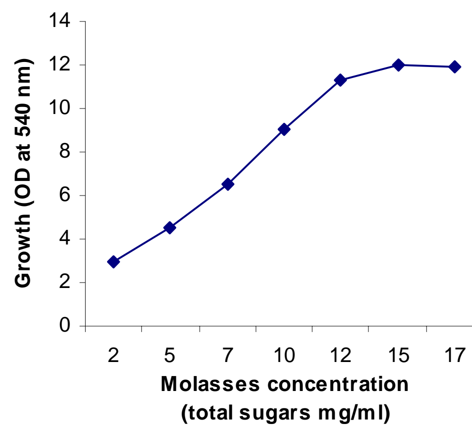
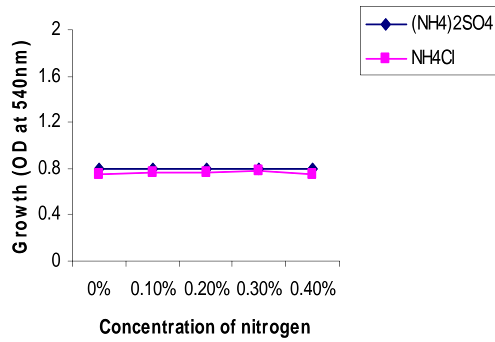


Fig 5.2 Effect of molasses concentration on growth of *Candida* sp. S 27

a) $(NH_4)_2SO_4$ and NH_4Cl **b) Yeast extract and peptone**



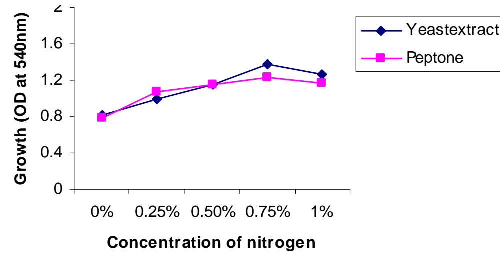


Fig 5.3 Effect of various nitrogen sources on the growth of *Candida* sp. S 27

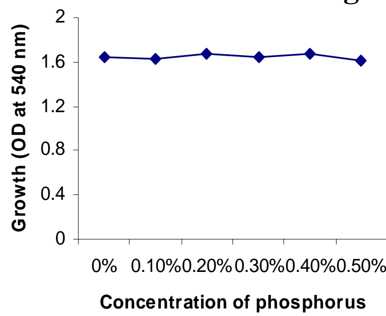


Fig 5.4 Optimization of phosphorus

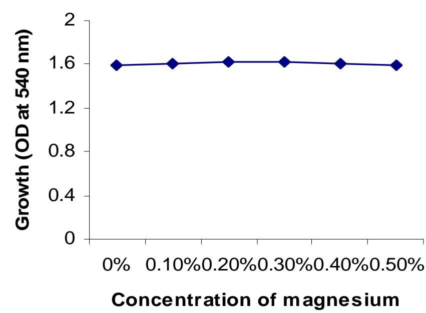


Fig 5.5 Optimization of magnesium

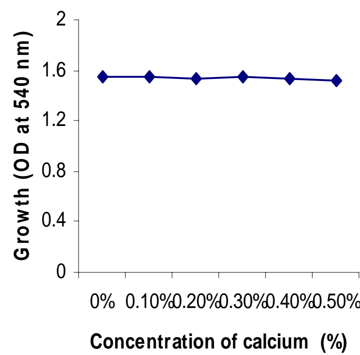


Fig 5.6 Optimization of calcium

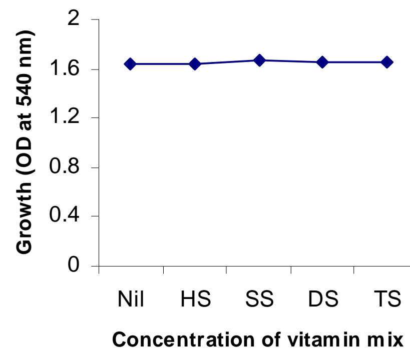


Fig 5.7 Optimization of vitamin mix

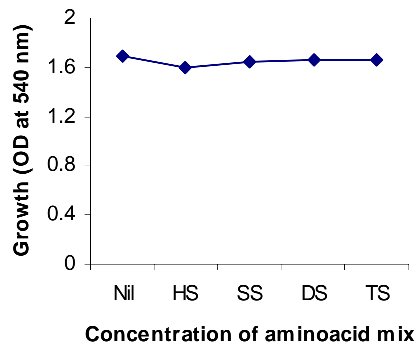


Fig 5.8 Optimization of amino acid mix

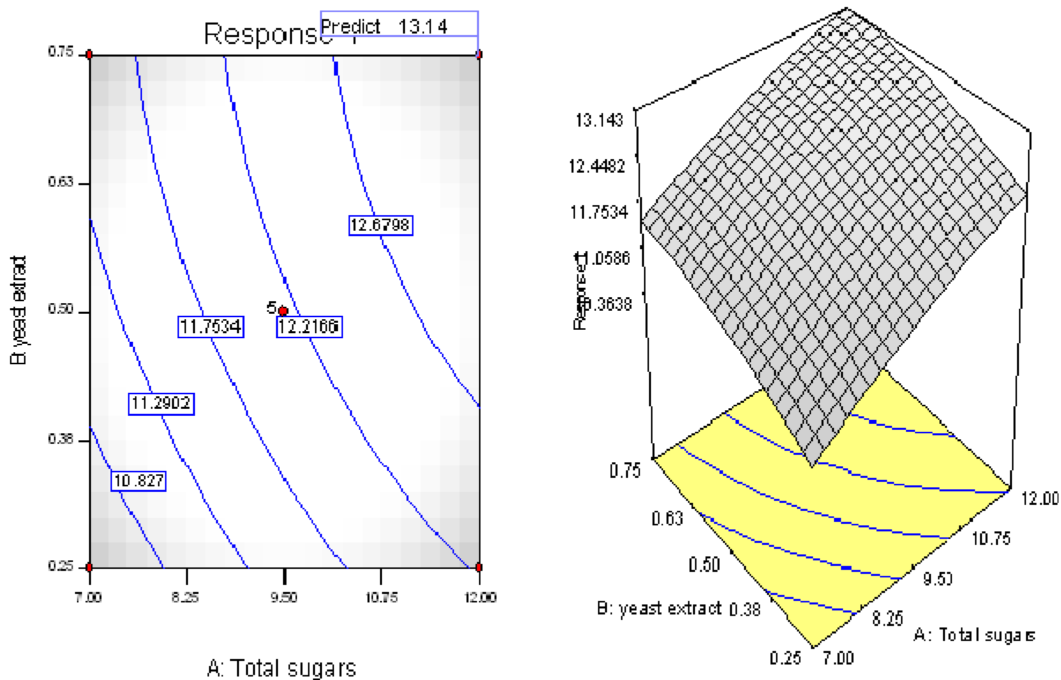


Fig 5.9 Contour and response surface plots of biomass vs. X_1 = A: Total sugars (mg/ml) and X_2= B: Yeast extract

With increase in concentration of total sugars as well as the nutrient supplement yeast extract, the biomass yield increased steadily. However no significant increase in biomass production could be noticed when the total sugar concentration and the amount of yeast extract were beyond the limit of 12mg/ml and 7.5 g/L respectively.

5.4 Discussion

Molasses, a by-product of sugar manufacturing process is rich in total sugars (50 % w/w), mainly sucrose and contains some amount of proteins and minerals and thus serve as an ideal substrate for the growth of yeast cells. Apart from sucrose (47-50 %, w/w) which is the disaccharide most easily utilized by yeast cells, molasses also contain water, 0.5-1 % of nitrogen source, proteins, vitamins, amino acids, organic acids, and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium etc. (Roukas, 1998). Hence it is a very attractive crude carbon source for yeast production from an economic point of view.

The levels of assimilable nitrogen and phosphorus are low in molasses which requires to be supplemented for proper yeast growth. The concentration of essential cations like Mg^{+} is suboptimal for yeast growth and metabolism in molasses (Walker et al., 1996). Since the common anions and cations occur in sufficient quantities in the crude medium, addition of inorganic sources of potassium, magnesium and calcium did not increase the biomass yield significantly. But the addition of yeast extract, the water soluble portion of autolysed yeast cells which is a very rich source of purine and pyrimidine bases (Amrane and Prigent, 1994), B-vitamins as well as amino acids (Miller and Chuchill, 1986) caused profound increase in yeast biomass production.

Molasses was chosen for the present study due to its high sugar and other nutrient contents, low cost, ready availability and ease of storage. A concentration of 12 mg/ml total sugars was found to be optimal for the growth of the marine yeast *Candida* sp. S 27. Sarlin (2005) found that for a few strains of the genus *Debaryomyces* (S8, S100, and S186) maximum growth was observed at 9mg/ml total sugars based on 'one factor at a time' analysis. But Sajeevan (2006) was successful in producing maximum growth of marine yeast *Candida sake* S 165 at a total sugar concentration of 2 mg/ml by factor at a time' analysis.. He found that ammonium sulphate at 0.3 % concentration was the most preferred nitrogen supplement. KH_2PO_4 at 0.3 % and $CaCO_3$ at 0.2 % also significantly increased the growth of *C.sake*. Cane molasses (3.5–17.5% w/v total sugar) and yeast powder (1.5–5% w/v) were used in the formulation of media during the cultivation of *Aspergillus japonicus*-FCL 119T and *Aspergillus niger* ATCC 20611 (Dorta et al., 2006). They found out that lower sugar concentration produced lower cellular growth.

Many workers have reported the utilization of molasses as carbon source in fermentation processes and in the production of lactic acid, citric acid etc (Żarowska et al., 2001;Young-Jung et al., 2004) In a recent study by Dhillon et al. (2007), cane molasses was supplemented with cauliflower waste and yeast extract for ethanol production by *Saccharomyces cerevisiae* MTCC 178. They have reported that the addition of 0.2% yeast extract to molasses increased the ethanol production by about 49 % as compared to control containing molasses alone.

For the mass production of *Rhizobium*, yeast extract solution (200ml/l) and cheap carbon source molasses (15g/l) was found to be suitable (Nandi and Sinha, 1970). The addition of yeast extract in the medium improved its efficiency. Greater production of xylanase in the presence of yeast extract could be noted by Gomes et al. (2000) which may be due to better amino acid composition, mineral and vitamin content. In the present study also yeast extract enhanced the growth of yeast. Although, molasses alone contains some amount of vitamin B6, there is no report suggesting the presence of other B vitamins in it. Nancib et al. (2005) reported an increase in lactic acid production from date juice by the use of yeast extract in the medium. Stehlik-Tomas et al. (2004) have reported that molasses alone does not contain elements like manganese, zinc and copper which are required for adequate growth of yeast cells. Supplementation of molasses with yeast extract and cauliflower waste could have resulted in higher concentration of minerals, essential vitamins and amino acids which in turn might have stimulated the yeast cell growth and subsequent ethanol production capability. Rapid growth of nine epiphytic isolates of the genus *Bacillus* was observed in a medium containing molasses and yeast extract as C-source and N-source respectively (Luna et al., 2002).

Cottonseed-derived medium proved to be a suitable substrate for the production of bioactive substances including surfactin, a useful compound in both medical and biotechnological fields. The medium provided not only higher product accumulations but at considerably lower cost with potential for large scale industrial applications (Al-Ajilani et al., 2007). The growth of the fungi on Pharmamedia medium was compared to that on Sabouraud brain heart infusion agar which is a general purpose medium for the isolation and cultivation of all fungi (Slifkin, 2000). The Pharmamedia extract agar also permitted the conversion of the mold-to-yeast phase with *Blastomyces dermatitides* and *Penicillium marneffei.*This investigation supports the use of Pharmamedia as a culture medium for the clinical microbiology laboratory. Its relative simplicity of preparation and effective use as a culture medium, as well as a conversion medium, emphasizes its useful application for clinical mycology.

Though all the nitrogen supplements such as fish meal, soya bean meal, pharmamedia and vita yeast powder supported the decolorization process, soya bean meal was the most effective one for economical consideration (Chen et al., 2003). Organic nitrogen sources pharmamedia, yeast extract, and polypeptone-S were found to be suitable for the production of penicillic acid by *Aspergillus sclerotiorum* CGF (Kang et al., 2007). The highest concentration of penicillic acid obtained was 2.71mg/ml when pharmamedia was used as a substrate.

There is a growing acceptance for the use of statistical experimental designs in biotechnology. The application of statistical design for screening and optimization of culture conditions allow quick identification of the important factors and the interaction between them. The CCD plan allowed studying and exploring culture conditions for yeast biomass production in a nutrient supplemented crude medium in just 13 experimental runs. The relatively low growth temperature and minimal supplementation requirements make this strain of *Candida* sp. a good candidate for yeast biomass production. This biomass can be used in the protein enrichment of aquaculture feed supplements. Pillai (2007) found that this strain *Candida* sp. S 27 acts as a good immunostimulant and antioxidant when applied as a dietary supplement in *Penaeus monodon*. Shameeda (2007) observed that Candida sp. S 27 promote growth in *Penaeus monodon* while supplied in diet at 5 % level. This study shows that molasses supplemented with yeast extract can be used as a good production medium for large scale production of yeast biomass which has got wide application in food and feed industry.

Chapter 6

Laboratory scale production of *Candida* sp. S 27 biomass using optimized media

6. 1 Introduction

Shaking bioreactors are the most frequently used reaction vessels in academia and in bioindustry for screening and bioprocess development. Shaking flasks or tanks with a typical nominal volume of 25 ml to 6 L (or even up to 60 L) and shaking test tubes of about 10–25 ml volume belong to this group of bioreactors (Liu and Hong, 2001).Insufficient oxygen supply is one of the most frequent problems associated with the microbial culture experiments. This may result in 1. slowing down of the metabolism of microorganisms 2. mode of metabolism switching on to a partially anaerobic phase resulting in unwanted by-products and 3. microorganisms become unable to synthesize enough energy leading to quick death. Agitation facilitates the even distribution of nutrients, O_2 and microbes. It ensures the homogeneity of the vessel contents to promote mass and heat transfer (especially O_2 transfer in aerobic fermentation) and to suspend particles in a fluid. Increased agitation will improve the performance of the system in these respects. Another possible reason is the fact that shaking bioreactors are mainly used in the first step of bioprocess development. Kennedy et al. (1994) argued that “there is no other way to do medium optimization apart from shake flasks simply because the number of experiments to be conducted is very large. The effect of the different medium components is relative, and therefore the best medium in shake flask culture will also be the best medium in a stirred tank”.

Archambault et al. (1996) alleged that “shake flask cultures are primitive systems in terms of gas transfer, mixing efficiency and continuous monitoring”. “Although shake-flask fermentations are relatively crude and physically unrelated to stirred vessels, they have proven to be quite valuable. Shaking bioreactors are well established and have proven to be a very valuable and useful tool for initial culture experiments, screening purposes and bioprocess development. They have to be regarded as the simplest and cheapest bioreactor technology imaginable. Many reactors can be used in parallel which make them extremely feasible for mass screening. Especially for such cases when very large numbers of experiments have to be conducted, there seems to be no other choice than to apply shaking bioreactors. They are most flexible and different vessel sizes may be densely packed on the

shaker tables. They possess certain unique and favourable properties and reveal some characteristic hydrodynamic phenomena which are specific for them. The very well-defined gas/liquid mass transfer area, which is almost independent of media changes, is an extremely important advantage of unbaffled shaking bioreactors in contrast to all other types of bioreactors. Furthermore, in this type of bioreactors no foaming problems and no cell flotation can be expected.

Quite often the wetting of the sterile closure (e.g., a cotton plug) due to splashing may result in a significant reduction of the gas permeability of the plug. A wet plug may also cause sterile problems. “Wetting of closures due to splashing is a limitation of using flasks with deeper indentations”, McDaniel et al. (1965) Accordingly, a convincing general directive was provided by Henzler and Schedel (1991) “the speed of rotation must be limited because of the risk of contamination from the plug which arises when the plug is moistened.

Freedman (1969) deduced from his investigations: “where uniform results among large numbers of flasks are necessary, standard unbaffled flasks should be employed”. Delgado et al. (1989) reported that their “data from baffled flasks were less reproducible”. Baffles that are too large may completely disturb the circulating movement of the liquid. Excessive wall growth may also be a larger problem in case of baffled flasks. Another disadvantage of baffled flasks is the highly complicated and partially chaotic flow regimes which are generated. Freedman (1969) concluded that “compared with the reciprocating shaker, the swirl pattern set up in the rotary shaker is not nearly as affected by media, viscosity, machine start-up or flask volume. This is undoubtedly the reason that the rotary shaker is in such common use in the culture of microorganisms”.

Screening of wild type strains with specific activities, conventional strain development using mutation and selection, strain development with recombinant techniques, elucidation of metabolic pathways, medium development, establishment of analytical protocols, investigations of basic process conditions like strain stability, inoculum ratio, optimal pH and temperature, total culturing time and the evaluation

of fundamental kinetic data are all performed employing extended parallel experiments in shaking bioreactors (Buchs, 2001).

Fermenter

Continuous stirred tank reactor (CSTR) is commonly used in many of the bioprocess as it allows efficient contact of three phases, i.e. gas, liquid medium and solid cells. Growth of organism like yeast, in a medium rich in nutrients requires aeration for effective utilization of nutrients for building of cell mass. Nutrient rich medium can be fully utilized by yeast cells to give cell mass if dissolved oxygen concentration can be maintained at a desired level in the direct vicinity of the cells. Operating conditions (aeration, agitation, pH and temperature) as well as medium constituents may affect the quality and quantity of the tested microorganisms (Churchill, 1982).

Parameters such as pH and aeration can be controlled to achieve high cell densities only in fermenters and it is ensured that all parts of the system are subjected to the same conditions. The purpose of aeration is to provide micro-organisms in submerged culture with sufficient oxygen for metabolic requirements, while agitation should ensure that a uniform suspension of microbial cells is achieved in a homogeneous nutrient medium (Stanbury et al., 1995). At the same time shear due to aeration and agitation should be kept at a reasonable level as the higher shear rates may physically damage the cells, thereby affecting the microorganisms. The shear can be reduced by operating the impellers at lower speed.

In case of shake flask, the growth rate is less as the gas induction is due to only surface aeration. Initially sufficient dissolved oxygen is available for growth of organisms as the medium is saturated with air. The drop in dissolved oxygen is very rapid in the initial phase and remains more or less same with time. This oxygen tension which decides the mass transfer coefficient is responsible for the growth in the shake flask. But there is no depletion of oxygen in a reactor. The mixing in the bioreactor is more compared to shake flask which maintains the sufficient oxygen levels. This could be contributing for the formation of more cell mass in a bioreactor. Aeration and agitation provides a well mixed system which may be equivalent to the

optimum respiration rate required for the organism for a better metabolic activity (Shukla et al., 2001).

6.2 Materials and methods

6.2.1 Microorganism used

Marine yeast *Candida* sp. S 27 was used for the study.

6.2.2 Media used

The media used for the study were

1. Chemically defined medium modified by RSM designated as Barnett and Ingram's medium Modified i.e. BIM medium (Table 6.1) pH 5.97
2. Molasses medium designed by RSM designated as MY medium (Molasses Yeast extract medium) (Table 6.2) pH 5.97
3. Malt extract medium, specified as ME medium (Malt extract 17 g/L; Peptone 3g/L; Distilled water 1000 ml, pH 5.97) was used as a control for comparison

Table 6.1 Composition of modified chemically defined medium (BIM) (L^{-1})

Nitrogen sources	$(NH_4)_2 SO_4$	5.42 g
	L- Asparagine	1 g
Carbon source	D-Glucose	32.17 g
Aminoacids (DS)	L-Histidine	20 mg
	DL- Methionine	40 mg
	DL-Tryptophan	40 mg
Growth factors (Vitamin mix) (DS)	p-Aminobenzoic acid	400 µg
	Biotin	40 µg
	Folic acid	4 µg
	Myo-inositol	20 mg
	Nicotinic acid	800 µg
	Pantothenate (Ca)	4 mg
	Pyridoxine HCl	800 µg

	Riboflavin	400 µg
	Thiamin HCl	800 µg
Trace element sources (HS)	H_3BO_3	250 µg
	$CuSO_4.5\ H_2O$	20 µg
	KI	50 µg
	$FeCl_3.\ 6H_2O$	100 µg
	$MnSO_4.4H_2O$	200 µg
	$Na_2MoO_4.2H_2O$	100 µg
	$ZnSO_4.7H_2O$	200 µg
Salts	KH_2PO_4	5 g
	K_2HPO_4	0.15 g
	$MgSO_4.7H_2O$	2.07 g
	NaCl	4.68 g
	$CaCl_2.6\ H_2O$	0.25 g
pH	5.97	

Table 6.2 Composition of Molasses Yeast extract Medium (g/L)

Ingredients	Amount
Molasses	Total sugars 12 g/L *
Yeast extract	7.5 g/L
NaCl	4.68 g/L
Distilled Water	1000 ml

* *Molasses quantity equivalent to Total sugars 12g/L in the final volume*

6.2.3 Shake flask experiment

Shake flask experiment was carried out at optimized conditions using the chemically defined and crude medium. 100 ml each of the three media were prepared in triplicate in 250 ml Erlenmeyer flasks. The effect of agitation or shaker speed on

yeast growth was checked at 100, 150 and 200 RPM (Revolutions per minute) to find out the optimum for biomass production.

Inoculation and incubation

Yeast inoculum was prepared by harvesting young culture (24 hours of growth on malt extract agar slant) of marine yeast *Candida* sp. S 27 using sterile saline of 4.68 $^{0}/_{00}$. Optical density of the culture suspension was measured at 540 nm and adjusted to 1 OD by appropriate dilution. 100 µl of this suspension was used as inoculum in 100 ml culture medium so that the initial O D of the culture medium is 0.001. The flasks were incubated on a rotary shaker (Scigenics, India) for a period of one week. Growth (OD at 540 nm) was measured every 24 hours till it reached the stationary phase.

Significant difference ($P<0.05$) between different groups were analyzed by One-way ANOVA and Duncan's multiple range test using SPSS 10.0 for Windows. All the data are presented as mean ± Standard deviation (SD).

6.2.4 Comparison of Biomass production in three types of Barnett and Ingram's media

1) Barnett and Ingram's Original (BIO) (Refer Table 4.1)
2) Barnett and Ingram's One Factor (BIOF) - Based on one factor at a time optimization- (Table 6.3)
3) Barnett and Ingram's modified – Developed through RSM – (BIM) (Table 6.1)

Barnett and Ingram's medium – Original (BIO), Barnett and Ingram's Medium – One factor (BIOF) and Barnett and Ingram's Medium – Modified by RSM (BIM) were prepared.

100 ml each of the three media were prepared in triplicate in 250 ml Erlenmeyer flasks. The media components (glucose, $(NH_4)_2SO_4$, L- Asparagine, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.6\ H_2O$) were weighed and transferred to distilled water containing sufficient quantity of NaCl (30 $^{0}/_{00}$ for BIO and BIOF 4.68 $^{0}/_{00}$ for BIM media respectively) and sterilized at 10 lbs for 10 minutes. Stock solutions of growth factors, amino acids and trace element sources were prepared,

filter sterilized and the required quantities were added to the cooled sterile medium and pH was adjusted. All the flasks were inoculated with 100 µl of 1 OD yeast culture suspension. The flasks were incubated at respective incubation temperatures ($28 \pm 2\ ^0$ C, 40 ^{0}C and 32.72 ^{0}C for BIO, BIOF and BIM media respectively).

Table 6.3 Optimum level of ingredients in Barnett and Ingram's medium based on 'One factor at a time' optimization. (per Litre)

Nitrogen sources	$(NH_4)_2\ SO_4$	6 g
	L- Asparagine	2 g
Carbon source	D-Glucose	30 g
Aminoacids (SS)	L-Histidine	10 mg
	DL- Methionine	20 mg
	DL-Tryptophan	20 mg
Growth factors (Vitamin mix) (SS)	p-Aminobenzoic acid	200 µg
	Biotin	20 µg
	Folic acid	2 µg
	Myo-inositol	10 mg
	Nicotinic acid	400 µg
	Pantothenate (Ca)	2 mg
	Pyridoxine HCl	400 µg
	Riboflavin	200 µg
	Thiamin HCl	400 µg
Trace element sources (SS)	H_3BO_3	500 µg
	$CuSO_4.5\ H_2O$	40 µg
	KI	100 µg
	$FeCl_3.\ 6H_2O$	200 µg
	$MnSO_4.4H_2O$	400 µg
	$Na_2MoO_4.2H_2O$	200 µg
	$ZnSO_4.7H_2O$	400 µg
Salts	KH_2PO_4	3 g
	K_2HPO_4	0.2 g
	$MgSO_4.7H_2O$	2.5 g
	NaCl	30 g
	$CaCl_2.6\ H_2O$	0.2 g
pH	5.97	

6.2.5 Biomass production in bench top fermenter

Fermenter

A continuous stirred tank bioreactor with a total volume of 5 L (B-Lite, Laboratory fermenter system, Sartorius, Germany) was used. It is a glass-jacketed system with a typical round bottom. The top plate is made from stainless steel and is compressed onto the vessel flange by an easily released clamping system. The top plate has port fittings of various sizes provided for addition of the inoculum, acid and alkali, insertion of probes, inlet pipes, exit gas cooler etc. These work by compressing the sides of the probe/ pipe against an O-ring seal. A narrow platinum resistance (Pt-100) temperature sensor is also fitted on the top plate. Culture can be withdrawn into a sampling device or a reservoir bottle *via* a sample pipe situated in the bulk of the fermenter fluid. A gas sparger is also fixed into the top plate and this terminates in a special assembly which ensures that incoming air is dispersed efficiently within the culture by the flat-bladed 'Rushton-type' impellers fixed to the drive shaft. A variable area flow meter or rotameter is used to control the air flow rate into the fermenter vessel. A drive motor provides stirring power to the drive shaft and is usually fitted directly to the drive hub on the vessel top plate. An exit gas cooler works like a simple Liebig condenser to remove as much moisture as possible from the gas leaving the fermenter to prevent excessive liquid loss during the fermentation and wetting of the exit air filter.

Preparation of medium

6.2.5.1 Barnett and Ingram's Medium Modified (BIM)

Three Litre (3 L) medium was prepared. All the ingredients (except carbon source, amino acid mix, asparagine and vitamin mix) in required amounts were dissolved in 2.5 L of distilled water and transferred into the fermenter. The vessel was assembled as directed in the operation manual. The vessel and the reagent/ sampling bottles already connected by silicone tubing were sterilized at 121 ^{0}C for 60 minutes. After cooling, D-glucose, amino acid mix and asparagine dissolved in 500

ml of distilled water (sterilized at 10 lbs pressure for 10 minutes) and vitamin mix (filter sterilized) were added (Fig 6.1)

Fig 6.1 Barnett and Ingram's Modified medium in Bench-Top Fermenter

6.2.5.2. Molasses- Yeast extract medium

3 L of the crude medium (Molasses- Yeast extract medium) was prepared with total sugar concentration of 12 mg/ml (36g) and of yeast extract, 22.5 g. 2.5 L of distilled water was autoclaved in a fermenter for 60 minutes at 121 ^{0}C. After assembling the vessel and the necessary reagent bottles, molasses and yeast extract

dissolved in 500 ml of distilled water (sterilized at 10 lbs pressure for 10 minutes) was added (Fig 6.2).

Fig 6.2 Molasses-Yeast extract medium in Bench- Top Fermenter

Antifoam

Silicone antifoaming agent (HiMedia Laboratories Limited, Mumbai) at 10 % concentration was prepared, autoclaved and used for foam control.

pH

For automated pH control, 500 ml each of 1 N NaOH and 1 N HCl were provided.

Gas supply

Air was provided from an oil-free pump (Model OF01080, Elgi Equipments Limited, Coimbatore) with a working pressure of 8 kgf/cm^3). Rotameter controlled the air flow rate into the fermenter vessel. A sterile filter (0.22 µm) was used as a bridge between the tubing from the rotameter and that connected to the air sparger of the fermenter. A second filter on the exit gas cooler stopped the microbes from releasing into the laboratory air as the gas left the fermenter under a slight positive pressure.

Preparation of Inoculum

Yeast inoculum was prepared by harvesting young culture (24 hours of growth on malt extract agar slants) of marine yeast *Candida* sp. S 27 using sterile saline of 4.68 $^0/_{00}$. Optical density of the culture suspension was measured at 540 nm and adjusted to 0.1 OD (1.829 x 10^6cells/ml) by appropriate dilution.

Inoculation

30 ml of this 0.1 O D suspension was used as inoculum in 3 L culture medium (1 % v/v) so that the initial O D of the culture medium is 0.001. The inoculum was aseptically transferred into the fermenter through the inoculum port.

Fermentation conditions

Unless otherwise specified, fermentation was performed under the following conditions: Temperature - 32.72°C and pH - 5.97 as derived from response surface methodology; Aeration rate- 0.5v/v/m and agitation speed 300 rpm. For the control of pH, 1 N NaOH and 1 N HCl were provided and pH was maintained at 5.97 ± 0.1 by the automatic addition of the acid or base. Silicone at 10 % constituted the antifoam.

O D of the culture suspension was taken at every 24 hours interval. Fermentation was carried out (for 72 hrs) until no appreciable change could be detected in the cell concentration. Observations were periodically made under

the microscope to check contamination, assuring sterility for biomass production.

Harvesting

Cell biomass was harvested at the late exponential phase/ beginning of stationary phase. Cells were separated by centrifugation at 3500 rpm in a refrigerated centrifuge at 4 ^{0}C (Remi K-70; Remi Instruments Ltd. Mumbai). The biomass was repeatedly washed with sterile 4.68 $^{0}/_{00}$ saline to remove the medium components. Wet weight and dry weight were determined.

6.2.6 Determination of proximate composition

CHN Analysis

Carbon, Hydrogen and Nitrogen content of yeast biomass prepared in both Barnett and Ingram's modified medium and Molasses- Yeast extract medium were determined. Protein content of yeast biomass was also estimated by the method of Lowry et al. (1951). The lipid content of the yeast biomass was analysed by Phosphovanillin method following chloroform-methanol extraction of the sample (Barnes and Blackstock, 1973).Total carbohydrates in the yeast biomass was determined spectrophotometrically by Anthrone method (Hodge and Hofreiter, 1962). Ash content was determined by incineration of yeast biomass in a silica crucible at 550^{0}C in a muffle furnace for 5 hours. (Refer Section 2.2.3.1)

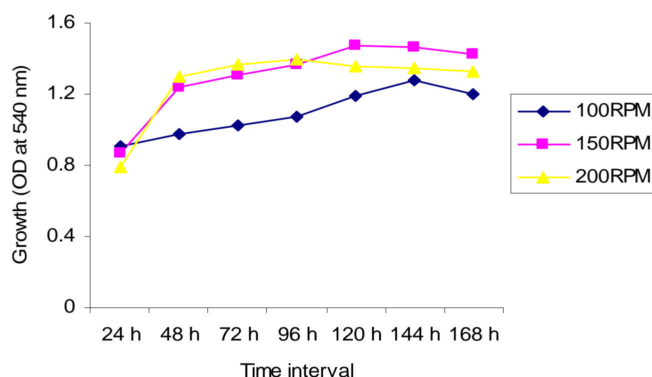
6.3 Results

6.3.1. Shake flask experiment

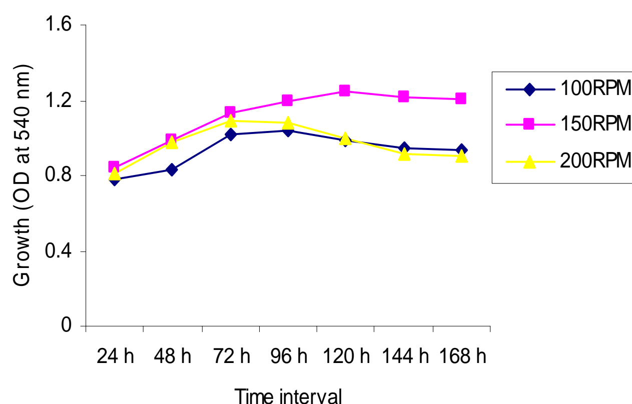
Post hoc tests have shown that there was significant variation ($P<0.05$) in biomass production in three different media. Maximum production was noticed in Barnett's Modified medium (chemically defined) (11.85 g/L) followed by Molasses- Yeast extract medium (crude) modified by RSM (11.11 g/L) (Fig.6.4). When compared to malt extract medium, there was 25.39 % and 17.56 % of increase in biomass production in the Barnett and Ingram's Modified

medium and Molasses- Yeast extract medium respectively. Shaking speed of 150 rpm was found to be optimal for growth. (Fig.6.3 a-c))

a. Barnett and Ingram's medium Modified



b. Molasses- Yeast extract medium



c. Malt extract medium

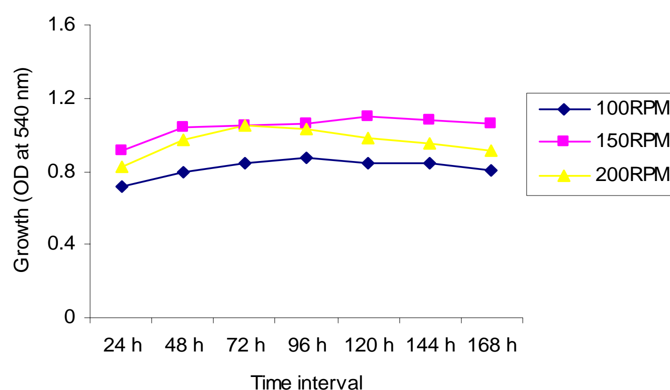


Fig. 6.3 a-c Growth of *Candida* S27 in various media under different agitation rate

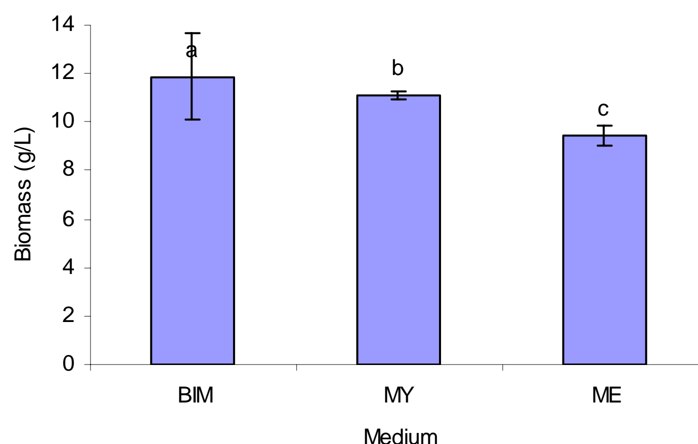


Fig 6.4 Yeast Biomass (*Candida* S 27) production (g/L) in various media - Shake flask experiment

S.No	Medium	Biomass (Mean ± SD)
1	Barnett and Ingram's medium modified (BIM)	11.85 ±1.78[a]
2	Molasses-Yeast extract medium (MY)	11.11 ±0.1909[a]
3	Malt extract (ME)	9.45 ±0.448[c]

Values with different superscripts vary significantly (P<0.05)

6.3.2 Comparison of Biomass production in three types of Barnett and Ingram's media

Biomass production was found to be highest in Barnett and Ingram's Modified (BIM) medium followed by Barnett and Ingram's One Factor (BIOF) medium and Barnett and Ingram's Original (BIO) medium (Fig.6.5).By one factor optimization an increase of 34.2 % could be obtained whereas through RSM an increase of 79.27 % yeast biomass could be observed in Barnett and Ingram's medium. When one factor optimized and RSM optimized media alone were compared. 33.6% increase could be obtained through RSM optimization over the one factor method.

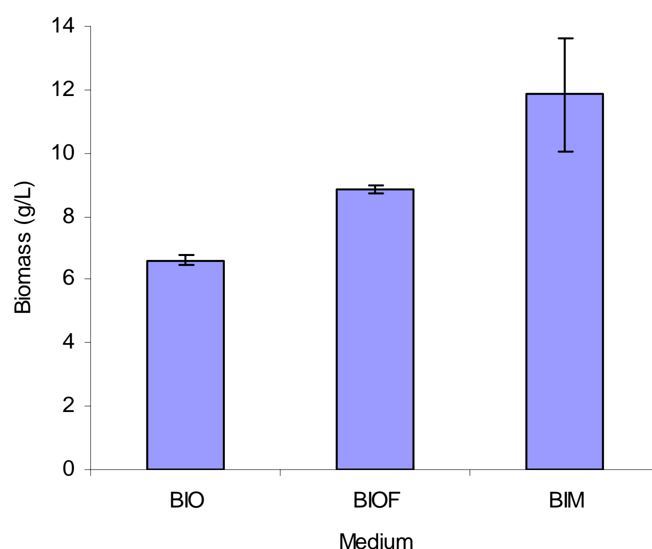


Fig. 6.5 Biomass production (g/L) in various types of Barnett and Ingram's media - Shake flask experiment

S. No	Medium	Biomass g/L
1	Barnett and Ingram's Original (BIO)	6.61 ± 0.158[a]
2	Barnett and Ingram's One Factor (BIOF)	8.87 ± 0.12[b]
3	Barnett and Ingram's Modified (BIM)	11.85 ± 1.78[c]

6.3.3 Fermenter

6.3.3.1 Barnett and Ingram's medium Modified

When the culture reached stationary phase (after 72 hours), biomass was collected through the harvest line. Wet weight of freshly harvested yeast biomass was determined. The wet weight corresponded to 50.66g/L and the dry weight 14.18g/L (Fig.6.6, 6.7 & 6.11) A 327.5 % increase in biomass production could be noticed compared to shake flask.

Fig 6.6 Biomass production of *Candida* S27 in a fermenter using Barnett and Ingram's Modified medium

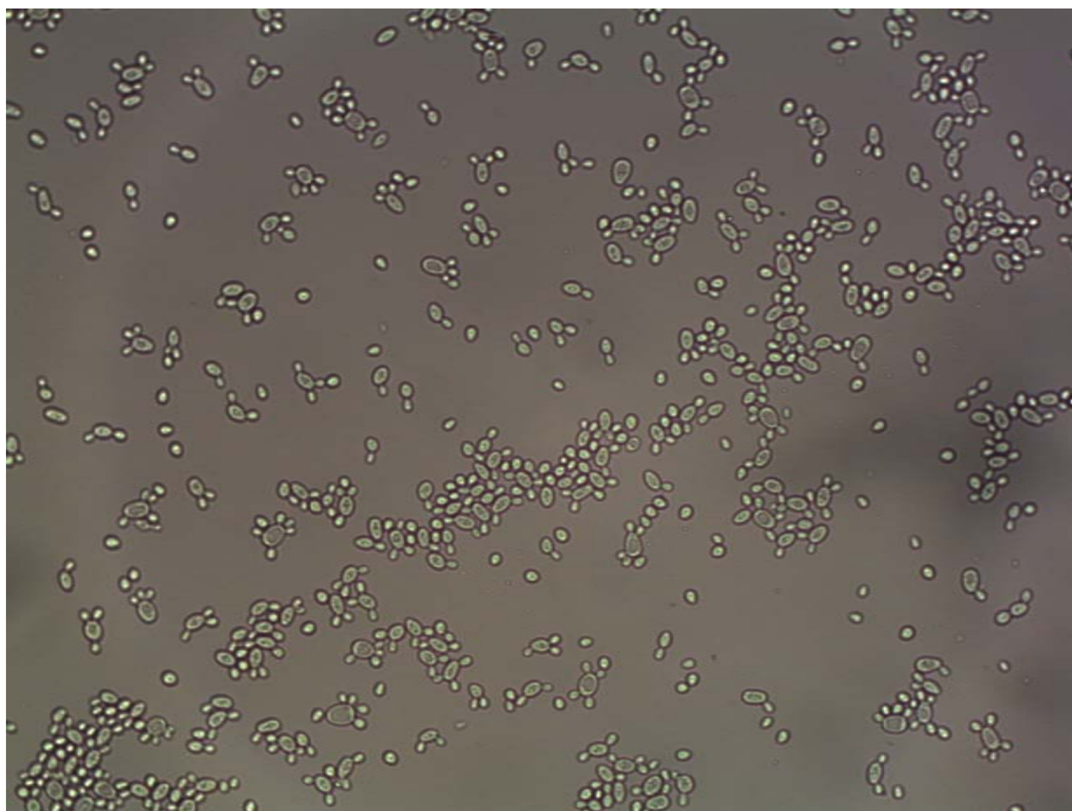


Fig 6.7 Marine Yeast *Candida* sp. S 27 in Barnett and Ingram's modified medium (100X)

6.3.3.2. Molasses-Yeast extract medium

The biomass produced in crude medium was harvested at 72 hours of growth in fermenter Wet weight of the freshly harvested biomass accounted to about 42.66 g/L and the dry weight 11.95 g/L (Fig 6.8,6.9 & 6.12). A 283.97 % increase in biomass production could be observed on scaling up.

Fig 6.8 Biomass production of *Candida* sp.S27 in a fermenter using Molasses-Yeast extract medium

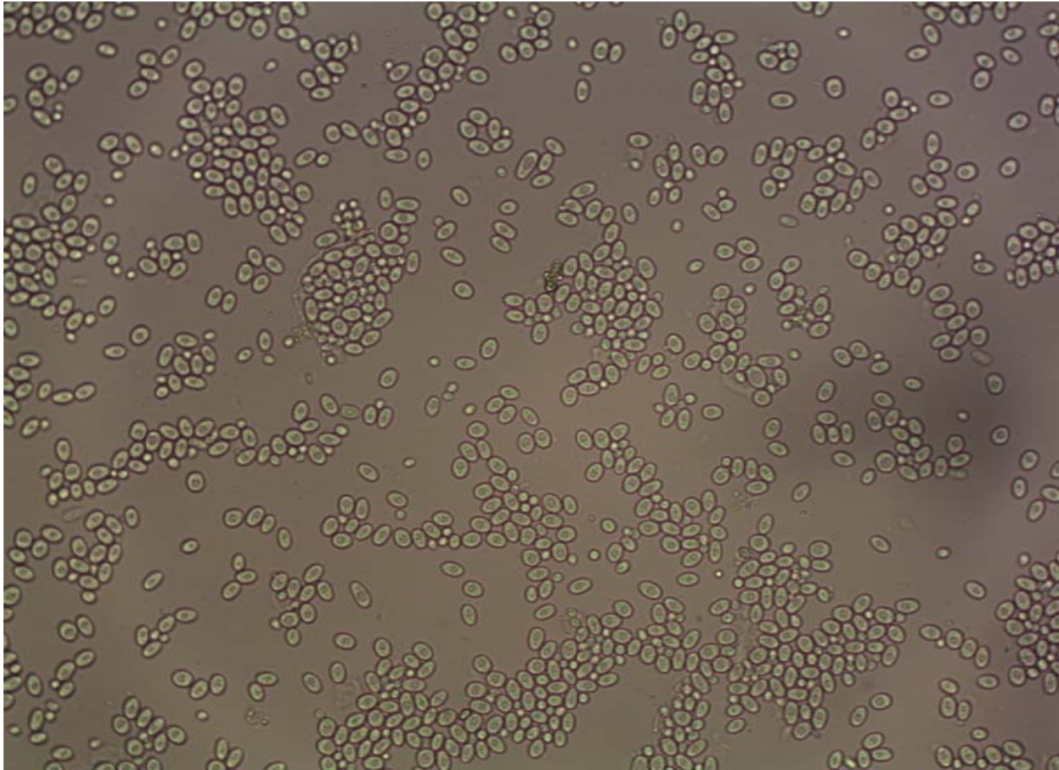


Fig 6.9 *Candida* sp. S 27 in Molasses-Yeast extract medium (100 X)

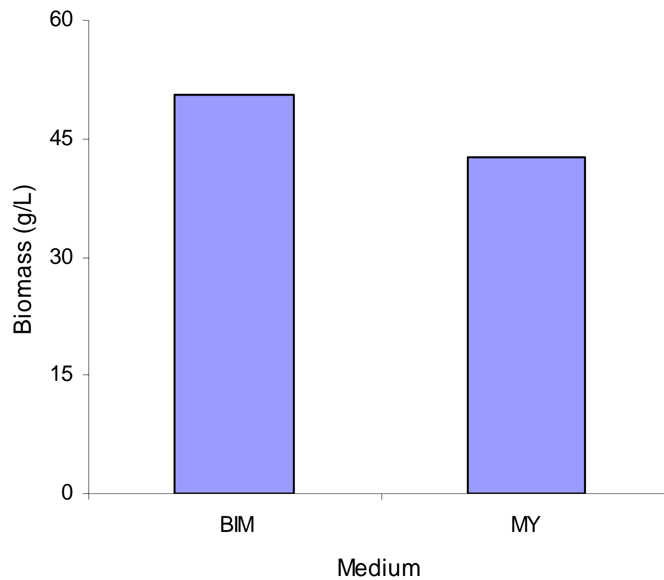


Fig. 6.10 Biomass Production in Bench Top Fermenter using BIM and MY medium

S. No	Medium	Biomass g/L
1	Barnett and Ingram's Modified (BIM)	50.66g/L
2	Molasses- Yeast extract Medium (MY)	42.66 g/L

The biomass production by marine yeast *Candida* sp. S 27 in Barnett and Ingram's Modified (BIM) medium has an increase of 18.75 % when compared to Molasses-Yeast extract medium (Fig.6.10). Proximate composition showed that there is not much variation between the nutritive value of biomass produced using the two different optimized media (BIM & MY) (Table 6.4). CHN (Carbon, Hydrogen and Nitrogen) analysis also revealed that irrespective of the media, the Carbon, Hydrogen and Nitrogen content remained more or less same in both the biomass (Table 6.5).

Table 6.4 Comparison of proximate composition of yeast biomass prepared in chemically defined (BIM) and crude media (MY).

S No:	Medium	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)
1.	Barnett and Ingram's Modified (BIM)	33.3	29.6	2.23	6.2
2	Molasses-Yeast extract medium (MY)	34.23	30.1	2.58	7

Table 6.5 CHN content of yeast biomass (*Candida* sp. S27)

S. No:	Medium	Carbon %	Hydrogen %	Nitrogen %
1	Barnett and Ingram's Modified (BIM) medium	42.46	7.80	6.07
2	Molasses-Yeast extract (MY) medium	43.01	7.79	7.52

Fig 6.11 *Candida* sp. S 27 Biomass prepared in modified chemically defined (BIM) medium

Fig 6.12 *Candida* sp. S 27 Biomass prepared in Molasses-Yeast extract medium

6.4. Discussion

Yeasts are more convenient than algae or moulds for large-scale production in fermenters, due to their unicellular nature and high growth rate. Shaking facilitates even distribution of nutrients, oxygen and microbes thereby ensuring homogeneity of the vessel to promote mass and heat transfer (especially O_2 transfer in aerobic fermentation) and to suspend particles in a fluid.

The oxygen demand of an industrial fermentation process is normally satisfied by aerating and agitating the fermentation broth. We have noticed that agitation or shaker speed has significant effect in yeast biomass production and an average speed of 150 rpm was found to be most suitable. Increase in agitation speed can lead to cell damage. This might have been the reason for decreased biomass production at high shaker speed.

Biomass production in two different media has been compared with a control. Maximum biomass production was noticed in chemically defined medium compared to the complex media. When compared to control medium, there was 25.39 % and 17.56 % of increase in biomass production in the chemically defined and crude medium respectively.

An effort was made to produce marine yeast biomass in various versions of chemically defined media. Least biomass production was noticed in the Barnett and Ingram's original medium (6.61 g/L). In the medium developed through 'one factor at a time' optimization, 8.87 g/L biomass was produced. However the chemically defined medium developed through response surface methodology was found to be the best with a biomass production of 11.85 g/L. A79. 82 % of increase in marine yeast biomass production could be noticed when compared to the Barnett and Ingram's original medium.

The main objective of scaling-up a process in a fermenter is to identify problems that were not significant at shake flask levels, and also to check whether the yield is maintained. Increased biomass production in the fermenter compared to shake flasks, is perhaps due to higher oxygen transfer in the fermenter compared to shake flasks. By scaling up the process, from flask to bench scale fermenter, we

obtained promising results. The amount of biomass obtained on scale up in chemically defined medium was 50.66 g/L and that of crude medium was 42.66 g/L. An increase in production by 327.5 % and 283.97 % could be obtained in the chemically defined and crude medium respectively when compared to shake flask.

Several wild strains and mutants of *Rhodotorula* spp. were screened for growth, carotenoid production and the proportion of β- carotene produced in sugarcane molasses by Bhosale and Gadre (2001). A better producer, *Rhodotorula glutinis* mutant 32, was optimized for carotenoid production with respect to Total Reducing Sugars (TRS) concentration and pH. In shake flasks, when molasses was used as the sole nutrient medium with 40 g /L TRS, at pH 6, the carotenoid yield was 14 mg /L and β- carotene accounted for 70% of the total carotenoids. In a 14 L stirred tank fermenter, a 20% increase in torulene content, (its characteristic carotenoid) was observed in plain molasses medium. However, by addition of yeast extract, this effect was reversed and a 31% increase in β- carotene content was observed.

A chemically defined medium was optimized for the maximum biomass production of recombinant *Pichia pastoris* in the fermentor cultures using glycerol as the sole carbon source and $(NH_4)_2SO_4$ as the nitrogen source (Ghosalkar et al., 2008). Optimization was done using the statistical methods for getting the optimal level of salts, trace metals and vitamins for the growth of recombinant *P. pastoris*. The response surface methodology was effective in optimizing nutritional requirements using the limited number of experiments. The optimum medium composition was found to be 20 g/L glycerol, 7.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4 . 7H_2O$, 8.5 g/L KH_2PO_4, 1.5 mL/L vitamin solution and 20 mL/L trace metal solution. Using the optimized medium the biomass produced was 11.25 g dry cell weight/L giving an yield coefficient of 0.55 g biomass/g of glycerol in a batch culture.

Recently a comparison between shaker and bioreactor performance based on the kinetic parameters of xanthan gum production was made by Faria et al. (2009). They obtained values of μmax as 0.119/h and 0.411/h and of $Y_{P/S}$ as 0.34 g /g and 0.63g/g in shaker and bioreactor respectively.

Abelovska et al. (2007) opined that the concentrations of some elements substantially differ between minimal and complex media and these differences may be responsible for phenotypic variations observed for microbial strains grown on synthetic and complex media.

Composition of growth medium governs the protein and lipid contents of microorganisms. Yeasts, moulds and higher fungi have higher cellular lipid content and lower nitrogen and protein contents, when grown in media having high amount of available carbon as energy source and low nitrogen (Litchfield, 1979).The proximate composition of the biomass generated through chemically defined medium and crude medium was found to be almost similar which shows that the medium ingredients does not affect the overall composition of yeast biomass. However only through detailed analysis of the biochemical composition of the biomass generated using various media we can come to a conclusion in this aspect.

Being expensive, the use of yeast extract is only justified if an economical gain in the product recovery is obtained, or it preserves some fundamental characteristic of high value to the product. So the addition of yeast extract to molasses can be justified from our study where the biomass production enhanced with the addition of yeast extract.

The bioprocess technology developed would be highly useful in the mass production of yeast biomass with better yield. Biomass thus produced can be pressed into 'Cake Yeast 'or 'Yeast Granules 'for marketing purpose. Yeast biomass being highly nutritious has tremendous applications in food and feed industry, however, yet to be exploited to its full potential. Among the various microorganisms, yeast has a long history of its use in food industry for fermentation purpose and often been a part of the human food as a constituent of fermented products. This marine yeast biomass would definitely be a valuable addition to the feed industry especially in aquaculture as a feed supplement where it has already proved its growth promoting and immunostimulant property.

Summary and conclusion

The main source of protein for human and animal consumption is from the agricultural sector, where the production is vulnerable to diseases, fluctuations in climatic conditions and deteriorating hydrological conditions due to water pollution. Therefore Single Cell Protein (SCP) production has evolved as an excellent alternative. Among all sources of microbial protein, yeast has attained global acceptability and has been preferred for SCP production. The screening and evaluation of nutritional and other culture variables of microorganisms are very important in the development of a bioprocess for SCP production. The application of statistical experimental design in bioprocess development can result in improved product yields, reduced process variability, closer confirmation of the output response to target requirements and reduced development time and overall cost.

The present work was undertaken to develop a bioprocess technology for the mass production of a marine yeast, *Candida* sp.S27. Yeasts isolated from the offshore waters of the South west coast of India and maintained in the Microbiology Laboratory were subjected to various tests for the selection of a potent strain for biomass production. The selected marine yeast was identified based on ITS sequencing. Biochemical/nutritional characterization of *Candida* sp.S27 was carried out. Using Response Surface Methodology (RSM) the process parameters (pH, temperature and salinity) were optimized. For mass production of yeast biomass, a chemically defined medium (Barnett and Ingram, 1955) and a crude medium (Molasses-Yeast extract) were optimized using RSM. Scale up of biomass production was done in a Bench top Fermenter using these two optimized media. Comparative efficacy of the defined and crude media were estimated besides nutritional evaluation of the biomass developed using these two optimized media.

The results of the present study are summarized as follows:

- Based on Internal Transcribed Spacer (ITS) sequencing, the selected marine yeast was identified as *Candida* sp. The nucleotide sequence has been deposited in GenBank and assigned the Accession No: FJ652102.

- Nutritional quality of the marine yeast *Candida* sp. S 27 was determined. It had a protein content of 32 %, carbohydrate content of 30.35 % of which the crude fibre portion was 11 %, lipid 2.224 %, nucleic acid 11.94 % and ash content , 6.8 % Wet yeast biomass contained approximately 72 % moisture.

- By FAME analysis, fatty acid profiling was done. Dominant fatty acids in marine yeast *Candida* sp. S 27 were oleic acid (35.91 %) and linoleic acid (25.53 %). Saturated fatty acids like palmitic acid, lauric acid, myristic acid and stearic acid were present in appreciable amounts. Highly unsaturated fatty acids were not present in *Candida* sp. S 27.

- Nineteen (19) aminoacids in yeast biomass were analysed by HPLC and Tryptophan by spectrophotometry. All the 20 aminoacids were present in the marine yeast *Candida* sp. S 27.

- Glucan content in *Candida* sp. S 27 accounted to 17.5 %. Structural elucidation by NMR spectroscopy revealed that it has a linear (1-3)-linked backbone with poly-(1-6)-linked side chains spaced every 15.3 repeat units on average. The (1-6) linked side chain has an average length of 5.4 repeat units.

- The process parameters salinity and pH of the culture medium and incubation temperature were screened by conventional one-factor at a time analysis and further optimized statistically by Response Surface Methodology using the software Design-Expert (version 6.0.9, Stat-Ease, Minneapolis, MN, USA)

- Central composite design of RSM was employed. A total of 20 different experiments (2^3 full factorial points, 6 axial points and 6 center points) were suggested by the software for optimization of culture conditions.

- Statistical analysis (ANOVA) showed that the model was significant (P = 0.0001) with an F value 14.10. The determination coefficient (R^2) 0.9270 for biomass indicated that sample variation of 92.7 % for biomass was attributed to the independent variables and only 7.3 % of the total variation was not explained by the model.

- A higher value of correlation coefficient (R = 0.9628) indicated an excellent correlation between independent variables but a relatively low value of coefficient of variation (CV = 10.83 %) showed the reliability and improved precision of experiments.
- The circular contour plots indicated that the interactions between the process parameters salinity, temperature and pH were not significant (P>0.05). but the linear coefficient of salinity and the quadratic coefficients of salinity, temperature and pH were significant.
- The optimum conditions for yeast biomass production were Salinity 4.68 $^0/_{00}$, pH 5.97 and temperature 32.72 ^{0}C.
- Optimization of a chemically defined medium for yeast biomass production was done. The basal medium selected was the one suggested by Barnett and Ingram (1955). The initial range of the 10 media components were screened by OVAT method. Following this, RSM was employed for optimization of the media components.
- Central composite design of RSM (chemically defined medium) proposed 158 different combinations ($^1/_8$ fractional factorial design i.e. Here $2^n = 2^{10} =$ 1024; 1/8 of 1024= 128; 20 axial points for the 10 different factors i.e. $-\alpha$ and $+ \alpha$ for each factor and 10 center points) for the different ranges of the 10 different media components. ANOVA of the statistical model showed that it was highly significant (<0.001). The model had CV 7.28 %; R^2 0.9673 and R 0.9835.
- The location of optimum obtained by the differentiation of the quadratic model for achieving maximum biomass production was: $(NH_4)_2SO_4$ = 5.42 g/l; Glucose= 32.17 g/l; KH_2PO_4 = 5g/l; K_2HPO_4 = 0.15g/l; $MgSO_4$. $7H_2O$ = 2.07 g/l; $CaCl_2$. $2H_2O$ = 0.25 g/l; Amino acid mix = DS; Trace metal mix = HS; Vitamin mix = DS; Asparagine = 1g/l.
- Among the test variables, KH_2PO_4, amino acid mix, vitamin mix and trace metal mix produced largest effect on yeast biomass production because the linear, quadratic and interaction effects of these parameters were highly

significant. All the other variables had significant quadratic and interaction effects which justify their addition in the medium.

- Because of the cost of the relatively pure ingredients used, the synthetic media turn out to be expensive and hence less preferred for large scale biomass production. Hence marine yeast biomass production was attempted in a complex medium. The various complex media screened were molasses, pharmamedia, corn steep liquor, corn gluten meal and soyabean meal. Molasses supported maximum growth of *Candida* sp. S 27 followed by corn steep liquor.
- Total sugar concentration of molasses was determined and it was found that a concentration of 12 mg/ml (total sugars) in the medium was found to be optimum for growth of *Candida* sp. S 27.
- Supplementation of molasses based medium with sources of nitrogen, phosphorus, magnesium, calcium, vitamin and amino acids was done to check the effect of these additional nutrients in increasing yeast biomass production.
- Among the various nitrogen sources screened (yeast extract, peptone, $(NH_4)_2SO_4$ and NH_4Cl), yeast extract served as the best nitrogen source followed by peptone. It was found that there was not much variation in yeast biomass production with the addition of potassium, magnesium, calcium, amino acid and vitamin supplements.
- The experimental design consisted of two steps with screening using OVAT method and optimization employing RSM. A set of 13 experiments comprising of 4 factorial points, 4 axial points and 5 center points were carried out.
- Analysis of variance showed that the model was significant (P =0.0001). The value of R^2 0.9703 indicated that sample variation of 97.03 % for biomass was attributed to the independent variables and only 2.97 % of the total variation was not explained by the model. The values of CV and R were 1.63 % and 0.9850 respectively.

- Only the linear coefficients of total sugars and yeast extract and the quadratic coefficient of the former were significant terms. The interaction coefficient was found to be not significant in determining the response depicted by the circular contour plot. But since it is a hierarchical model, the insignificant coefficient was not omitted from the final regression equation. The concentration of total sugars and yeast extract were exactly the same found out from 'one- factor- at- a- time' analysis.
- The composition of the optimized complex medium (Molasses Yeast extract) was molasses (in terms of total sugars) 12 g/L and yeast extract 7.5g/L.
- Shake flask experiment for biomass production with the chemically defined medium (BIM),crude medium (YM) and Malt extract medium (ME) showed that maximum production was in Barnett and Ingram's Modified medium (11.85 g/L) followed by Molasses -Yeast extract medium (11.11 g/L) and the lowest in Malt Extract Medium (9.45 g/L)
- A comparison of the Barnett and Ingram's Original medium (BIO), Barnett and Ingram's One Factor medium (BIOF) and Barnett and Ingram's Modified (BIM) medium showed that there was remarkable increase in biomass production in BIOF (8.87 g/L) and BIM (11.85g/L) compared to BIO (6.61g/L).
- Scale up of yeast biomass production was carried out in 5 L Bench Top Fermenter and the comparative efficacy of chemically defined (BIM) and complex media (MY) was tested. BIM supported higher biomass production (50.66g/L) followed by MY (42.66 g/L). Biomass produced through Barnett and Ingram's Modified (BIM) medium showed an increase of 18.75 % when compared to Molasses-Yeast extract medium.
- Proximate composition showed that there is not much variation between the nutritive value of biomass produced using the two different optimized media (BIM & MY) (Table 6.4). CHN (Carbon, Hydrogen and Nitrogen) analysis also revealed that irrespective of the media, the Carbon, Hydrogen and Nitrogen content remained more or less same in both the biomass.

Significant increase in biomass production could be achieved through the optimization of media and conditions applying Response Surface Methodology. This study proved that the interactions between various parameters are very important and therefore 'one factor at a time' approach is not effective enough in optimization. Significant biomass production in the crude medium deserve much attention ,since the raw material is a byproduct from sugar industry and the production cost will be considerably low. Biomass thus generated would be a valuable addition to the food and feed industry .Yeast biomass being highly nutritious can be used as a good substitute for protein, vitamins and other nutrients. Scale up from shake flask to Bench Top Fermenter displayed tremendous increase in biomass buildup. Further scale up and downstream processing for marketing are the future line of work.

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** Not referred in original*

Appendix

Candida sp. S27 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       FJ652102          512 bp    DNA     linear   PLN 23-FEB-2009
DEFINITION  Candida sp. S27 18S ribosomal RNA gene, partial sequence; internal
            transcribed spacer 1, 5.8S ribosomal RNA gene, and internal
            transcribed spacer 2, complete sequence; and 28S ribosomal RNA
            gene, partial sequence.
ACCESSION   FJ652102
VERSION     FJ652102.1  GI:223712816
KEYWORDS
SOURCE      Candida sp. S27
  ORGANISM  Candida sp. S27
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            Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales;
            Candida.
REFERENCE   1  (bases 1 to 512)
  AUTHORS   Joseph,S.P., Sarlin,P.J., Bright Singh,I.S. and Philip,R.
  TITLE     Development of a bioprocess technology for the mass production of
            marine yeast
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 512)
  AUTHORS   Joseph,S.P., Sarlin,P.J., Bright Singh,I.S. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JAN-2009) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            FineArts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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                     /strain="S27"
                     /db_xref="taxon:600621"
     misc_RNA        <1..>512
                     /note="contains 18S ribosomal RNA, internal transcribed
                       spacer 1, 5.8S ribosomal RNA, internal transcribed
                       spacer 2, and 28S ribosomal RNA"
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