CRANFIELD UNIVERSITY

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Optimization of Bioprocess Design for Pharmaceutical Metabolites and Enzymes

Institute of BioScience and Technology

Applied Mycology Group

PhD THESIS

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ABSTRACT

This study examines the effect of ecophysiology on growth of cells and production of enzymes and secondary metabolites produced by the fungi *Aspergillus niger* (lysozyme) and a *Phoma* sp. (squalestatin S1). The effect of interactions of water activity (a_w) (0.99-0.90), temperature (20, 30 and 35°C) and modifying a_w solute (glycerol, NaCl) on growth and sporulation of a wild type strain of *Aspergillus niger* (W) and two genetically engineered lysozyme producing strains (L11, B1) was examined for the first time. Maximum growth rates were achieved for both strains (L11 and B1) under moderate a_w levels. Optimum conditions for growth of strain L11 were estimated by means of contour plot surfaces and found to be 0.965 a_w with glycerol as a solute at 35ºC (10.5 mm day^{-1}). A model combining the effect of a_w and temperature on growth of strains of *Aspergillus niger*, and comparison with data on food spoilage moulds in the literature was developed. The growth of two strains of *A. niger*, as a function of temperature (25-30^{o}C) and a_w (0.90-0.99) was developed. The estimation of the minimum a_w (a_{wmin}) and optimal a_w (a_{wopt}) levels were in accordance with data in the literature for a range of other *Aspergillus* and related species, regardless of the solutes used for a_w modification. A central composition design was used to describe the effects of water activity (a_w, 0.98, 0.97 and 0.96), inoculum size ($2.7x10^5$, $2.7x10^4$ and $2.7x10^3$ spores ml^{-1}), and three autoclaving procedure (A = all components autoclaved together, B = medium autoclaved + maltose filtered and, C = medium autoclaved + maltose & soya milk filtered) on the production of lysozyme by two genetically-engineered strains of *Aspergillus niger* (B1 and L11) in a liquid culture fermentation. Although both strains produced similar lysozyme concentrations (15 mg l^{-1}), different production patterns were found under the experimental conditions. However, strain B1 produced relatively higher amounts of lysozyme under water stress (0.96 a_w) with all the substrates autoclaved together. Subsequently, a central composition design was used to investigate: different immobilized polymer types (alginate and pectate), polymer concentration (2 and 4% (w/v)), inoculum support ratios (1:2 and 1:4) and gel-inducing agent concentration ($CaCl_2$, 2 and 3.5% (w/v)) on lysozyme production. Overall immobilization in Ca-pectate resulted in higher lysozyme production compared to immobilization in Ca-alginate. Similar effects were observed when the polymer concentration was reduced. A 13 fold higher

lysozyme production was achieved with Ca-pectate in comparison to Ca-alginate (20-23 and 0.5-1.7 mg l^{-1} respectively). Polymer modifications also significantly affected the final pH and a_w of the immobilized cell fermentation. The a_w factor is a very significant parameter in the immobilization design. A combined statistical methodology of orthogonal design $L_{27}(3^{13})$ and surface response methodology was applied to optimize the composition and concentration of a liquid fermentation medium for the production of squalestatin S1 by a *Phoma* species. Confirmatory experiments of the optimal medium composition produced average concentrations of 434 mg l^{-1} in five days fermentation at 25^{o}C. This represented an improvement over 60% of the maximum concentration achieved in the initial experiment and a two-fold higher productivity in comparison with reported productivities of S1 in liquid fermentations with different fungal species. Different liquid height and column diameter (H_L/H_r) ratios 3.7, 7.4 and 11.4 were studied in a bubble column (D_r=0.07 m) with a porous plate gas distributor, to find the effect on the gas hold up, power consumption (P_G/V_L) and volumetric mass transfer coefficient, k_La performance, under different superficial gas velocities calculated from the liquid properties and flow rates (2, 4, 6 and 8 l min^{-1}) and temperatures (15, 25 and 30^{o}C). Two k_La models were proposed based on the geometrical ratio (H_L/D_r) and superficial gas velocity (m s^{-1}) (R^2=0.951), and power consumption (P_G/V_L) (R^2=0.950). A free cell fermentation was performed in the bubble column, ratio (H_L/D_r)= 3.7 and superficial gas velocity U= 0.120 m s^{-1}, at 25^{o}C. The S1 production reached a level of 420 mg l^{-1}. The bioreactor scale up succeeded in maintaining the high S1 concentration obtained in our previous work 434 mg l^{-1} in Erlenmeyer flasks but in a shorter time. A Plackett-Burman design was used to improve the S1 produced by different immobilized designs. The immobilized cell fermentation design considered: polymerization with alginate and polygalacturonate and copolymerization, polymer concentration (alginate 3, 3.5 and 5 % w/v and pectate 4, 6 and 8 % w/v), 0.98, 0.96 and 0.94 a_w levels, inoculum levels of 10, 20 and 30 % wt. v/v, gel-inducer ($CaCl_2$) 3, 4 and 5 % w/v, gel-reinforce agent 0, 0.75 and 1.5 g l^{-1}, air flow 4, 6 and 8 l min^{-1}. Production of S1 reached levels of 883 mg l^{-1} which represent a 34 % improvement over the 660 mg l^{-1} produced in a stirred tank bioreactor (STR) with a free cell fermentation.

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List of contents

List of figures

List of tables

List of abbreviations

ε	Overall gas hold up	(-)
a	Correlation coefficient Eq. 5 and Eq. 6	($m^{-\beta}$ $s^{-\beta}$)
a_w	Water activity	(-)
a_{wmax}	Maximal water activity	
a_{wmin}	Minimal water activity	
a_{wopt}	Optimal water activity	
b	Exponent coefficient Eq. 5 and Eq. 6	(-)
b_0, b_1, b_2, b_3 and b_4	design parameters (Eq 3.4)	
b_w	$1-a_w$	
C^*	Steady state (or saturation) dissolved oxygen concentration	(kg m^{-3})
C_L	Oxygen concentration in the liquid at different times	(kg m^{-3})
C_{L0}	Initial oxygen concentration in the liquid	(kg m^{-3})
d_b	Effective bubble diameter	(m)
df	Degrees of freedom	
D_r	Column diameter	(m)
F	Fisher's number	
g	Acceleration due to gravity	(m s^{-2})
H_E	Aerated liquid height	(m)
H_L	Height of the liquid without aeration	(m)
H_L/D_r	Liquid height and column diameter ratio	(-)
k_La	Volumetric mass transfer coefficient	(s^{-1})
k_La_L	Volumetric mass transfer coefficient per unit volume of liquid	(s^{-1})
m	Fungal growth rate	(mm day^{-1})
MS	Mean square sum	
P	Significance descriptive level	
P	vapour pressure of water in a substrate	Pa
P_0	vapour pressure of pure water	Pa
P_G/P_V	Power consumption	(W m^{-3})
P_h	Reactor head-space pressure	(Pa)
Q_m	Air molar flow	(kmol s^{-1})
R	Gas constant	(8.134 J K^{-1} mol^{-1})
SD	Standard deviation	

SE	Standard error	
t	Time	(s)
T	Temperature	(K)
T	Temperature	(^{o}C)
T_{max}	maximum temperature for growth	(^{o}C)
T_{min}	minimal temperature for growth	(^{o}C)
U	Superficial gas velocity	(m s^{-1})
X_i	molar fraction of solutes	(-)
X_w	molar fraction of water with other solutes	(-)
ρ_L	Water density	(kg m^{-3})

CHAPTER I
LITERATURE REVIEW

1.1 INTRODUCTION

Biotechnology has been one of the scientific fields with the highest growth rates during the past 50 years which has come from the integration of biological, physical and engineering sciences in order to achieve technological application of biological systems. Most biotechnological processes make use of microorganisms such as bacteria, yeasts and filamentous fungi, but vascular plants, algae and even animal tissue can also be utilised. Biotechnology is not an industry in itself, but an important technology that is having a large impact on many different industrial sectors. This technology is not new: People have for a long time known how to crossbreed plants and animals for better concentrations for traditional products such as bread, beer, cheese, and wine. In textile processing the enzymatic removal of starch from woven fabrics has been in use for the last century and the fermentation vat is probably one of the oldest tools known for dyeing materials. These processes are characterised by the direct application of live organisms and the *in situ* production of enzymes and other products. New biotechnology has a stronger focus on the application of biological products or enzymes and these are often produced *ex situ*. One of the advantages of the new biotechnology over classical biotechnology is that the process can be improved and optimized as the mass balance is easier to control.

In recent years, the term biotechnology has also been used to refer to novel techniques such as recombinant DNA and cell fusion. Recombinant DNA allows the direct manipulation of genetic material of individual cells, which may be used to develop useful microorganisms as well as microorganisms that produce new products. The laboratory technology for genetic manipulation within living cells is also known as genetic engineering. A major objective of this technology is to splice a foreign gene for a desired product into circular forms of DNA (plasmids), and then to insert them into an organism, so that the foreign gene can be expressed to produce the product from the organism.

The applications of this new biotechnology are numerous in the production of pharmaceuticals e.g. antibiotics, antigens, antibodies and vaccines. Previously,

expensive and rare pharmaceuticals such as insulin for diabetics, human growth hormone to treat children with dwarfism, interferon to fight infections, vaccines to prevent diseases, and monoclonal antibodies for diagnostics could be produced from genetically engineered cells inexpensively and in large quantities (Lee, 1992). In the last few years there have been very rapid developments in genetic manipulation techniques (genetic engineering) which has introduced the possibility of 'tailoring' organisms in order to optimise the production of established or novel metabolites of commercial importance and of transferring of genetic material (genes) from one organism to another. Biotechnology also offers the potential for new industrial processes that require less energy and are based on renewable raw materials. It is important to note that biotechnology is not just concerned with biology, but it is a truly interdisciplinary subject involving the integration of natural and engineering sciences.

Biotechnological processes have been developed for most of the food and pharmaceutical industries and a number of them have been successfully commercialised (Leach, 1992). Some of the most important organisms used in biotechnology are fungi. Examples of the products and processes involving fungi are shown in Table 1.1.

Successful commercialization of biotechnological processes requires the development of large-scale processes that are technologically viable and economically efficient. To scale up a laboratory-scale operation into a large industrial process, it is not possible to just make the vessel bigger. For example, a small Erlenmeyer flask (100 ml) in a shaken culture can be an excellent way to cultivate fungal cells, but for a large-scale production of 2000 l, a significant number of factors need to be considered. We need to design an effective bioreactor to cultivate the cells under optimum conditions. Because of this, biochemical engineering is one of the major areas in biotechnology important for commercialization.

Table 1.1. Some examples of the use of fungi in biotechnology

Food Applications	**Useful Products**	**Other Processes**
Baking	Alkaloids	Biobleaching/biopulping
Brewing	Antibiotics	Biological control agents
Cheese-making	Ethanol	Bioremediation of soils
Mushroom cultivation	Enzymes	Coal solubilisation
Oriental food fermentations	Gibberellins	Dyes/dye intermediates
Quorn© myco-protein	Immunomodulators	Microencapsulation
	Organic acids	Mycorrhizal inoculants
	Polysaccharides	Steroid bioconversions
	Vitamins	Waste treatment

A typical fermentation process (bioprocess) involving microbial cells requires investigation of raw materials, biomass, and how they are treated and mixed with other ingredients required for cells to grow well. The medium, is sterilized to eliminate all other living microorganisms and introduced to a large cylindrical vessel, bioreactor or fermenter, typically equipped with agitators, baffles, air spargers, and various sensing devices for the control of the fermentation conditions. A pure strain of a microorganism is normally introduced into the vessel. The bioreactor supports the natural process by providing suitable conditions such as optimum temperatures, pH, sufficient substrate, nutritional salts, vitamins and oxygen (for aerobic organisms), enabling cells to grow and form metabolites and enzymes. The cells will start to multiply exponentially after a certain period of lag time and reach a maximum cell concentration as the medium is depleted. The fermentation in a batch culture will be stopped and the contents pumped out for the product recovery and purification.

Biotechnology is an interdisciplinary field with contributions from basic life science disciplines such as microbiology, molecular and cell biology, biochemistry, genetics and engineering including chemical, control and instrumentation. The entire process can be divided into three stages;

Stage I: Upstream processing which involves preparation of liquid medium, separation of particulate and inhibitory chemicals from the medium, sterilization and air purification

Stage II: Fermentation, when the conversion of substrates to the desired product with the help of biological agents such as microorganisms

Stage III: Downstream processing which involves separation of cells from the fermentation broth, purification and concentration of the desired product and waste disposal or recycling of waste

Depending on the type of product, the concentration levels it produces and the purity desired, the fermentation stage might constitute anywhere between 5-50% of the total fixed and operating costs of the process. Therefore, optimal design and operation of a bioreactor frequently dominates the overall technological and economic performance of the process. This process can be operated either in a batch mode or continuously.

To carry out a bioprocess on a large scale, it is necessary to investigate and develop three principle areas:

1. to obtain the best biocatalyst (microorganisms, animal cell, plant cell, or enzyme) and medium optimization for a desired process
2. to create the best possible environment for the catalyst to perform by designing the bioreactor and operating it in the most efficient way
3. to separate the desired products from the reaction mixture in the most economical way.

To obtain optimal biocatalyst activity (microorganisms, e.g., bacteria, yeasts or filamentous fungi) it is necessary to develop an understanding of the in behaviour under different ecological and environmental conditions for a specific microorganism. Filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* are able to secrete large concentrations of enzymes (e.g. amylases, proteases, cellulases), metabolites and organic acids into the environment. This property has been widely exploited by the food and beverage industries (Conesa *et al*., 2001). Increasingly, these species are being used to produce recombinant proteins. Filamentous fungi offer advantages for the production of recombinant proteins: they possess an efficient secretion system, are able to glycosylate proteins and have higher specific growth rates than plant, insect or mammalian cells (Lin *et al*., 1993).

1.2 WATER ACTIVITY AND FUNGAL GROWTH

The commercial use of the fungal secretory machinery and the limitations encountered in the production of heterologous proteins has stimulated research into genetics, design of fermentation media, and optimization of the environmental conditions to enhance the heterologous protein production. Growth of filamentous fungi has been shown to be dependent on thermodynamic factors such as water availability and temperature (Scott, 1957; Gervais, 1988) and chemical factors such as glucose, O_2, and CO_2, concentrations (Trinci, 1969; Trinci and Collinge, 1973). The most important environmental stress parameter that influences fungal activity and

secondary metabolite production is water availability (water activity, a_w) (Magan *et al*., 2004).

Microorganisms all require a source of water to enable cellular functioning to occur effectively. They have a semi-permeable cell membrane, which permits water molecules to enter the cell through osmosis and come to equilibrium with the outer environment. Certain groups of yeasts and filamentous fungi have over time evolved the capability to adapt to extreme environments (dry conditions) and exploit niches occupied by few other microorganisms (Magan, 1997).

The total water of any substance is the sum of the bound water (water of constitution), which is held in chemical union with other compounds by very strong forces, and free water, which is weakly bound. Free water is the most readily available for microbial growth and metabolism. Scott (1957) suggested that the water activity (a_w) would best describe the water availability for microbial activity. Thus the a_w is the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water (P_0) at the same temperature and pressure:

$$a_w = \frac{P}{P_0} \qquad \text{Eq. 1.1}$$

The a_w of pure water is given the value 1.00. As the solute concentration increases, the a_w decreases according to the second law of thermodynamics.

$P=X_w P_0$ or $P=(1-X_i) P_0$ Eq. 1.2

Then the reduction of vapour pressure is related to the molar fraction of water with other solutes X_w and ionic or no-ionic solutes X_i.

From an ecological point of view little information is available on whether, in liquid fermentation systems, genetically modified strains will behave in a similar manner to wild type strains or not. No studies have attempted to evaluate this, although it has been shown that in wild type strains of pharmaceutically useful fungi, subtle changes in water stress can result in a significant stimulation of secondary metabolite production. It has been demonstrated that significant improvements in pharmaceutical

metabolite production can occur when a_w stress is imposed during fungal growth (Baxter *et al*., 1998; Aldred *et al*., 1999).

1.3 MODELLING OF FUNGAL GROWTH AND METABOLITE / PROTEIN PRODUCTION

There has been interest in understanding and modelling the effect of and interactions between environmental conditions and effects on growth and production of useful products. In the last two decades, predictive food microbiology has included the development of models capable of describing the growth of pathogenic bacteria (Buchanan and Phillips, 1990). However, predictive modelling of filamentous fungal growth has not received the same attention (Gibson *et al*., 1994; Gibson and Hocking, 1997). Two of the most important environmental parameters that determine the ability of moulds to grow on foods are water activity (a_w) and temperature (T) (Scott, 1957). An empirical approach to modelling the effects of a_w on mould growth was used by Gibson *et al*. (1994) who found that the logarithm of the fungal growth rate (μ, measured as the increase in colony radial growth per unit of time) showed a parabolic relationship with the square root of b_w. The b_w was defined as the difference between the a_w of pure water (1) and that of a set a_w for a specific growth kinetic. They investigated the appropriateness of models that were previously used to predict bacterial growth for the interpretation of mould growth data.

Much work has been directed towards the development of models for bacterial growth as a function of temperature and a_w (McMeekin *et al*., 1987; Zwietering *et al*., 1994; Rosso *et al*., 1995). The temperature dependence of the specific growth rate of bacteria may be modelled by means of the square root model (Ratkowsky *et al*., 1983) with the cardinal parameters (e.g. the minimal temperature for growth, T_{min} and the maximum temperature for growth, T_{max}). For example, Marin *et al*. (1995) developed a predictive method modelling the effect of a_w, temperature and time on fumonisin production by *Fusarium* strains. More recently, some modelling of ochratoxin production by *Aspergillus carbonarius* has also been reported (Mitchell *et al*., 2004, Belli *et al*., 2004). This showed that optimal conditions for growth (30-35^{o}C) were

different from that for ochratoxin production (OTA). Furthermore, a_w and temperature limits for growth were different from than for secondary metabolite production. Cuppers *et al*. (1997) successfully combined the models of Rosso *et al*. (1995) and Ratkowsky *et al*. (1983) to describe the combined effects of temperature and NaCl on the growth rate of some food spoilage moulds. The four moulds used, *Penicillium chrysogenum*, *Cladosporium cladosporoides*, *Aspergillus flavus* and *Alternaria alternata*, were inoculated at the optimum temperature for growth and at pH close to optimum for growth. However, little knowledge is available on the impact of such interacting factors on pharmaceutically useful production. Much more is known of the effects on toxic secondary metabolites production (mycotoxins) (Sanchis and Magan, 2004).

1.4 IMPACT OF FERMENTATION MEDIUM ON THE PRODUCTION OF SECONDARY METABOLITES AND ENZYMES

1.4.1 Medium composition

Fungi have been widely used for the production of heterologous as well as homologous proteins. However the levels of production of heterologous proteins do not exceed a few tens of milligrams per litre (Gouka *et al*., 1997). Filamentous fungi are able to utilize a great variety of carbon and nitrogen sources by secreting a range of different enzymes into their environment.

Some key intermediates of primary metabolism serve as branching points of biosynthetic pathways leading to end products of primary and secondary metabolism. Secondary metabolism is regulated by precursors, carbon sources, nitrogen sources, phosphate, trace elements, induction of enzymes of secondary metabolism, catabolic repression and inhibition, feedback repression and inhibition, and control by auto-regulators (Betina, 1994).

The available cell precursor levels may regulate antibiotic/secondary metabolite production, especially when the specific synthase is already active in the cells (Betina, 1994). There are differences between the carbon sources for growth and

secondary metabolism. For example, glucose is usually an excellent source for growth but may interfere with secondary metabolism. Glucose has also been shown to interfere with the biosynthesis of actinomycin, benzodiazepine alkaloids, cephalosporin, chlorotetracycline, enniantin, ergot alkaloids, erythromycin, kanamycin, oleandomycin, penicillin, puromycin, tetracycline and tylosin (Demain, 1992). The synthesis of pectinolytic enzymes is considerably influenced by the glucose content of the cultivation medium containing mixed carbon sources in a predetermined optimal ratio (Panda *et al*., 2004).

The effect of nitrogen sources on secondary metabolism is conditioned by several factors including the type of metabolic pathway, the producing organism, the type and concentration of the nitrogen sources and whether cultures are stationary or submerged. Very often, secondary metabolic pathways are negatively affected by nitrogen sources favourable for growth (Betina, 1994). Negative effects of ammonium salts have been reported in the production of cephalosporine, penicillin, erythromycin, tylosin, leucomycin, chloramphenicol, macbecin, rifamycin, streptomycin, streptothricin and tetracycline (Demain, 1992).

It is known that several trace elements are essential for microbial growth because of their involvement in metalloenzymes or as enzyme activators. In secondary metabolism, zinc, iron and manganese are the most important trace elements. Several reports have been published on the importance of these three elements in secondary metabolite production (Betina, 1994).

In developing a biotechnological industrial process, designing the fermentation medium is of critical importance. The fermentation medium affects the product concentration and volumetric productivity. It is also important to reduce the cost of the medium as much as possible, as this may affect the overall process economics. Medium screening studies are very time consuming and expensive. This is because the number of possible media combinations that can be tested and the number of fermentation substrates that are available are also very large. Thus, for economy of effort and scale different approaches have been used to rapidly identify the variables which need to be controlled for optimising production of useful metabolites and heterologous proteins.

It has also been shown that the expression of heterologous proteins and production of secondary metabolites are influenced by pH (Murphy and Power, 2001). This parameter affects the morphology and the enzyme production pattern of the genus *Aspergillus* (Schügerl *et al*., 1996). Environmental pH conditions can change protease activity pattern (Schügerl *et al*., 1996; Wiebe *et al*., 2001; Bruno-Bárcena *et al*., 2002), and can enhance protein production stability (Pitson *et al*., 1996; O'Donnel *et al*.,2001; Wiebe, 2003).

1.4.2 Effect of medium sterilization on metabolite production

In industrial microbiology, most investigations into submerged aerobic cultures, especially the development of media and screening of microorganisms, are conducted using shake flask cultures. This is because the scale is convenient and replication can be easily achieved. Production variables such as autoclaving procedures, inoculum size, temperature, water activity, medium composition, pH and degree of mixing can be identified and their effect on fermentation productivity commonly determined by the conduct of sequential cultures where only one variable at a time is altered. Such investigations provide a basis for the manipulation of production variables in order to optimize product concentrations.

At this scale it is important to investigate the impact of autoclaving of the fermentation medium on production. Sterilization of fermentation media can result in vital nutrients (e.g. vitamins, amino acids, and sugars) being destroyed, while the hydrolysis of complex substances (e.g. proteins and polysaccharides) may be enhanced. The formation of insoluble compounds may eliminate the availability of a nutrient, while inhibitory compounds resulting from the interaction of medium components may result in a medium which is not conducive to growth or to product formation (Anderson *et al*., 1986). A compromise between the substrate quality and the risk of contamination of the raw materials needs to be analyzed. Sterilization conditions have been shown previously to affect fermentation performance. For example, efrotomycin production by *Nocardia lactamdurans* was greatly improved by sterilizing glucose together with the rest of the medium components (Jain and

Buckland, 1988). Zaragozic acid production by *Leptodontidium elatius* was also improved under different autoclaving conditions (Connors *et al*., 1995).

1.4.3 Effect of inoculum size

The inoculum size has an important effect on the morphology of the colony. A close relationship between a particular morphology and increased process productivity is characteristic of a number of industrially important fermentations (Calam, 1987; Papagianni, 1999). The role of fungal morphology in relation to formation and secretion of proteases has been evaluated in *Aspergillus niger* (Papagianni and Moo-Young, 2002). Morphology was manipulated by means of inoculum levels. A reduction of extracellular proteases enhanced production of heterologous proteins. Thus, morphological development in filamentous fungal fermentations can be manipulated by inoculum level (Angelova *et al*., 1998; Chen *et al*., 1999; Dominguez *et al*., 2000; Papagianni and Moo-Young, 2002).

Proteolysis has been recognised as a major problem associated with recombinant protein production, not only in filamentous fungal populations, but also in bacterial and yeast populations (Enfors, 1992; Punt and van den Hondel, 1995). Several strategies have been employed for reducing proteolysis of recombinant proteins. The primary strategy has been to use a protease deficient host strain to generate the transformant (van den Hombergh *et al*., 1995; Zheng *et al*., 1998).

In addition to the use of a protease deficient host, or when no such host is available environmental and morphological parameters can be manipulated to reduce proteolysis. The pattern of expression of proteases in *Aspergillus niger* can be modified by changes in the inoculum size (Papagianni and Moo-Young., 2002). Inoculum quality, in terms of size, type or age, is of prime importance in determining the outcome of filamentous fungal fermentations (van Suijdam *et al*., 1980; Gencheva and Dimova, 1984; Vecht-Lifshitz *et al*., 1990; Brückner and Blechschmidt, 1991).

1.5 STATISTICAL APPROACH TO INCLUDE MULTIPLE FACTORS FOR OPTIMAL CONCENTRATION OF USEFUL PRODUCTS

Companies from all over the world, including Ford, AlliedSignal, General Electric, Sony and Lockheed Martin are reporting tremendous savings and benefits from the application of statistical methods and statistically-designed experiments to their manufacturing processes. Contour surfaces, central composition, Plackett-Burman and orthogonal experimentation, which have received much attention in the scientific community, are examples of technologies that are enhancing the quality and quantity of information in biotechnological process, increasing efficiency, improving products, and decreasing costs. Traditional methods of optimization involve changing one independent variable while keeping the others fixed at a certain level. This single-dimensional approach was laborious, time consuming, expensive and incapable of reaching the optimum due to the interactions among variables (Furuhashi and Takagi, 1984). Response surface methodology is an important strategy for seeking the optimal conditions for multivariable systems. It has been successfully employed for optimizing medium ingredients and operating conditions in some bioprocesses (Kemp *et al*., 1989; Roseiro *et al*., 1992; Prapulla *et al*., 1992; Lee *et al*., 1997). In order to carry out the optimization process to screen for the optimal immobilized design, it is necessary to identify the region of optimum performance. This can be done using various mathematical tools.

Some investigators have used mapping approaches to identify the optimization space. This enables a better picture of the possible effects of each component in the medium. Orthogonal and Plackett Burman designs are important methodologies that can reduce the number of runs to an absolute minimum (Castro *et al*., 1992; Escamilla *et al*., 2000, Xu *et al*., 2003). The main disadvantage of these designs is that they consider only first order effects and ignore interactions. However, while a full factorial design (testing every combination possible) provides the most complete information, they often require such a large number of runs that they are impractical to carry out. Optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial design to obtain a model of the

production system, usually involving fitting of data to a polynomial equation, often using stepwise multiple regression. Response surface methodology has also been used to investigate the optimal regions of production of useful product (Prapulla *et al*., 1992). Detailed analyses of the optimized region using cetroidal or simple designs have also been applied for optimization processes (White *et al*., 1990). However, several interactions of the experimental design and optimization of models are required for effective application to product formation in fermentation systems. Combined statistical systems have not previously been applied to the production of pharmaceutical and heterologous proteins.

1.6 IMMOBILIZATION OF FUNGAL BIOMASS FOR IMPROVED PRODUCTIVITY

Fermentative production of metabolites can be obtained in both solid state and submerged cultivation. However, it has been shown that immobilization can lead to differences in physiological behaviour and to higher metabolite production which is released into the medium (Lin, 1973; Evans and Wang, 1984; Lin and Demain, 1991; Lee *et al*., 1995). Iimmobilization of microbial cells gives the advantage of using solid substrate support in a submerged culture fermentation framework. This approach has received increasing attention in recent years (Becerra *et al*., 2001).

In 1969, the first industrial application of immobilization was applied and since then numerous advances have been made in the techniques of immobilization. A large number of supports for the immobilization have been used (Ohmori and Kurokawa, 1994; Sato *et al*., 1994; Emregul *et al*., 1995; Escamilla *et al*., 2000) and the total number of their applications has also risen, particularly in the fields of environmental engineering, food processing and medicine (Gmeiner, 1992).

Gels are one of the most widely used supports; these can be either natural, derived from polysaccharides such as agar (Hayashi *et al*., 1993), alginate (Wu *et al*., 1994), k-carrageenan (Sakiyama *et al*., 1993), pectate (Escamilla *et al*., 2000) or synthetic, such as the gels derived from acrylamide (Axelsson *et al*., 1994)

The immobilization of microbial cells in different carriers leads to changes in their microenvironment (Shreve and Vogel, 1993). Because of these changes, immobilized cells show various modifications in physiology and biochemical composition when compared to suspended cells (Hilg-Rotmann and Rehm, 1990). In the past two decades advanced use of polysaccharides for gel-entrapment or encapsulation of cells and optimization of technique had become a challenging area for the biotechnologist. The immobilization procedure can be carried out in a single step process under very mild conditions and is therefore compatible with most viable cells.

The first step in the fungal production is inoculation of vegetative cultures and subsequent inoculum development. Inoculum quality and quantity strikingly affect the overall production (Gancheva and Dimova, 1984; Brückner and Blechschmidt, 1991). The optimization of the immobilization support involves several variables, and the classical strategy of changing one variable each time is very time-consuming. An optimization of the immobilization parameters would permit the development of an immobilized cell fermentation which could improve the production of metabolites such as squalestatin S1 and heterologous proteins in a bubble column bioreactor.

The immobilization of microbial cells in different carriers leads to changes in their microenvironment (Shreve and Vogel, 1993). Because of these changes, immobilized cells show various modifications in physiological and biochemical composition when compared to suspended cells (Hilg-Rotmann and Rehm, 1990; Adam *et al*., 2001). The use of polysaccharides for fungal immobilization has become one of the most important methods used in biotechnology. This technique can be carried out in a single step process under very mild conditions and is therefore compatible with most viable cells. Polysaccharide matrices generated in gel beads are strongly dependent on several variables such as polymer type, concentration and ionic strength (Skjårk-Bræk *et al*., 1989; Nava-Saucedo *et al*., 1994; Gmeiner *et al*., 1996). Such modifications may also result in changes in the water availability in the beads and may influence rates of colonisation and subsequent production of enzymes or secondary metabolites. Although this could have important implications in studies of xerotolerant or xerophilic microorganisms and impact on concentration, very few

studies have taken these factors into account. Indeed practically none have measured the a_w of such beads.

Once the ecophysiological conditions of the biocatalyst are understood, the scale up of the process can be initiated. The process consists of in developing a suitable bioreactor in order to scale up the process with the gained information of environmental conditions, and couple this knowledge with new operation conditions in order to enhance the product concentrations. Statistical and mathematical approaches are normally used to create the best possible environment for the catalyst by designing the bioreactor and operating it in the most efficient way

1. 7 BIOREACTORS

Fermentation, which involves the cultivation of bacteria, yeasts and fungi, is an ancient technology. However, the use of bioreactors to produce fermentation products is a rather modern development. This particular process involving aseptic and controlled conditions for the production of antibiotics started in the 1940's. In the scale up process it is vital to investigate the type of bioreactor suitable according to the physical and physiological requirements of the microorganisms used before starting further optimizations. In general, stirred tank reactors are the most widely used type of bioreactors in the production of pharmaceutical compounds. These type of reactors offer a few advantages, such as independent control of mixing conditions and aeration rate. The main disadvantages of these reactors are the high energy consumption and high shear stress affecting such fragile cells as fungal cells. Bubble column bioreactors (BC) are a good alternative to the stirred tank bioreactors (STR). The main advantages of bubble column reactors are, from the apparatus side, the relatively simple construction and the absence of mechanically moving parts. Thus, they are easy to maintain and have low operating costs. With regard to the internal flow and efficiency behaviour, they offer a large interfacial area and transport rates leading to excellent heat and mass transfer characteristics and more suitable shear conditions for fungal growth and production.

Scale up of newly developed bioprocesses from laboratory scale to production scale is often very complex and time consuming. In particular, the scale up from shake flask to bioreactor is a difficult step. Generally, the oxygen transfer rate (Humphrey, 1998) and the specific power input (Büchs *et al*., 2000; Büchs *et al*., 2000a) have been predominantly used for scale up from shake flasks to bioreactors.

1.8 MODEL SYSTEMS USED IN THIS WORK

1.8.1 The secondary metabolite squalestatin S1

The treatment of hypercholesterolemia with pharmaceutical agents reduces the risk of developing arteriosclerosis. Several therapies such as bile acid sequestrants or cholesterol biosynthetic inhibitors are available. In the isoprenoid biosynthetic pathway, the first step to the biosynthesis of cholesterol involves the dimerization of farnesyl pyrophosphate to squalene. This step is catalyzed by squalene synthase and is a potential drug target. Substrates analogous of the farnesyl pyrophosphate have been synthesized and found to be inhibitors of this enzyme. Squalestatins are a potent inhibitor of the squalene synthase (Blows *et al*., 1994). Among the various fungal species that produce the family of squalestatins as secondary metabolites, a *Phoma* sp. is particularly important for high concentration capacity (Baxter *et al*., 1998; Dawson *et al*., 1991).

1.8.2 Heterologous protein hen egg white lysozyme (HEWL)

Hen egg white lysozyme (HEWL) is a naturally occurring protein found in many organisms such as viruses, bacteria, plants, insects, birds, reptiles and mammals (Osman *et al*., 1995). HEWL, which attacks the cell wall of certain bacteria thus killing them, has been found effective in controlling *Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella typhimurium* (Osman *et al*., 1995). HEWL is effective against *Clostridium tyrobutyrium,* a contaminating bacteria in cheese making (Samaranayke *et al*., 1993). HEWL also shows lytic activity against fungal plant pathogens, *Phytophthora nicotinae* and *Fusarium oxysporum* (During *et al*., 1999). Immobilised in solid supports, the applications of HWEL in biomedicine are:

downstream processing, recovery of proteins (Owen and Chase 1997), cell sorting (Nandakumar *et al*., 1999), purification of nucleic acids (Ujam *et al*., 2000), and flow-ELISA analysis (Palsson *et al*., 2000).

1.9 OBJECTIVES

The overall objectives of this project were to screen and model the effect of different process parameters such as environmental factors (a_w, temperature and pH) on growth of *A. niger* strains, and the effect of free and immobilized cell fermentations on S1 and lysozyme production. To achieve these the following studies were carried out.

a) To evaluate the effects of water availability (water activity, a_w), solute used to modify a_w, and temperature on growth and spore production by a wild (W) and two genetically-engineered strains (B1, L11) of *Aspergillus niger*.

b) To develop a model for the combined effect of water activity (a_w) and temperature on growth of strains of *Aspergillus niger*, and comparison with data on food spoilage moulds in the literature.

d) To evaluate the effect of (a) water availability, (b) inoculum size and (c) sterilization procedure on the production of the heterologous protein lysozyme by two genetically engineered *Aspergillus niger* strains (L11 and B1).

e) To optimise by response surfaces the physiological and immobilization conditions to enhance the production of the heterologous protein lysozyme, by a genetically engineered *A. niger* B1 strain. The factors examined were (a) polymer type and concentration, (b) gel-inducer ($CaCl_2$) concentration and (c) inoculum levels. The importance of the fermentation pH and bead water availability on heterologous protein concentrations were also evaluated.

f) To optimize thirteen substrates, including those reported in the literature, at three different concentration levels in submerged fermentation to produce squalestatin S1 by a *Phoma* species. This approach used a combination of statistical strategies involving the use of an orthogonal design, response surfaces and polynomial

regression to find the best medium for maximising concentrations in a free fermentation system.

g) To evaluate the effect of the liquid height and the height-diameter (H_D/D_r) ratios 11.1, 7.4 and 3.7 on the total volumetric mass transfer coefficient, k_La, at different temperatures.

h) To evaluate the effect of different height-diameter (H_r/D_r) on the volumetric mass transfer coefficient.

i) Development of two models for effect of superficial gas velocity (m s^{-1}) and power consumption (W m^{-3}) combined with the geometrical parameter height of the liquid-column diameter (H_L/D_r) on the k_La with distilled water.

j) To evaluate the influence of the immobilization design parameters: polymer and copolymer (alginate and pectate) immobilization under different mixture concentrations, inoculum level, gel inducer (calcium chloride), reinforcing agent (chitosan) and air flow on the production of squalestatin S1 by a *Phoma* sp. in a bubble column bioreactor.

k) To carry out comparisons of optimal production of squalestatin between free cell fermentations in a standard stirred tank bioreactor (STR) and in a bubble column (BC) with immobilized cell fermentations.

Figure 1.1 shows in a diagrammatic form the components of the thesis. The work is presented as independent chapters (Chapter II-VIII) with integrated Materials and Methods, Result and Discussions presented in the chapters. These have either been published, are in press or are being submitted for publication.

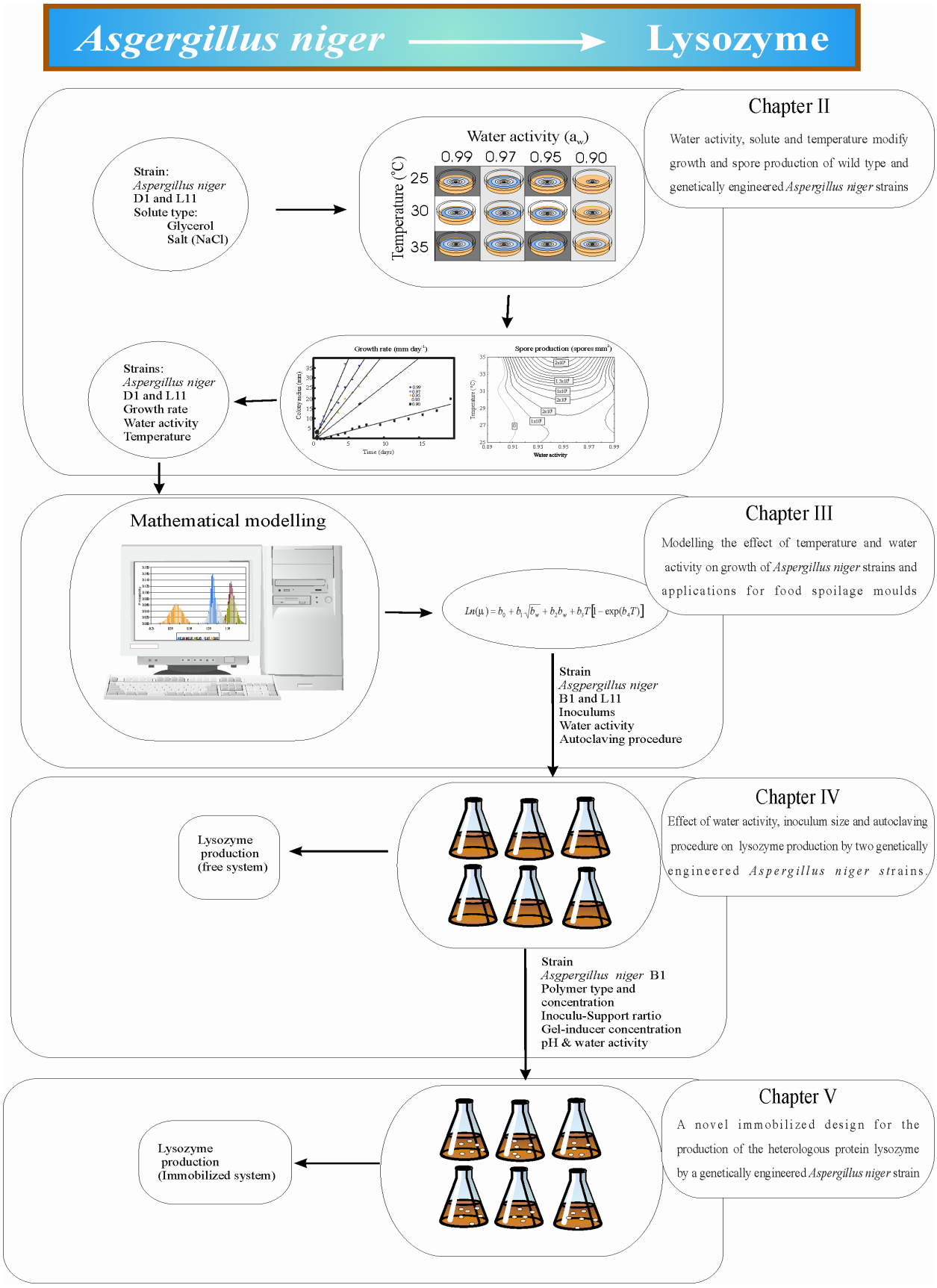

Figure 1.1. Diagram form of the components studied

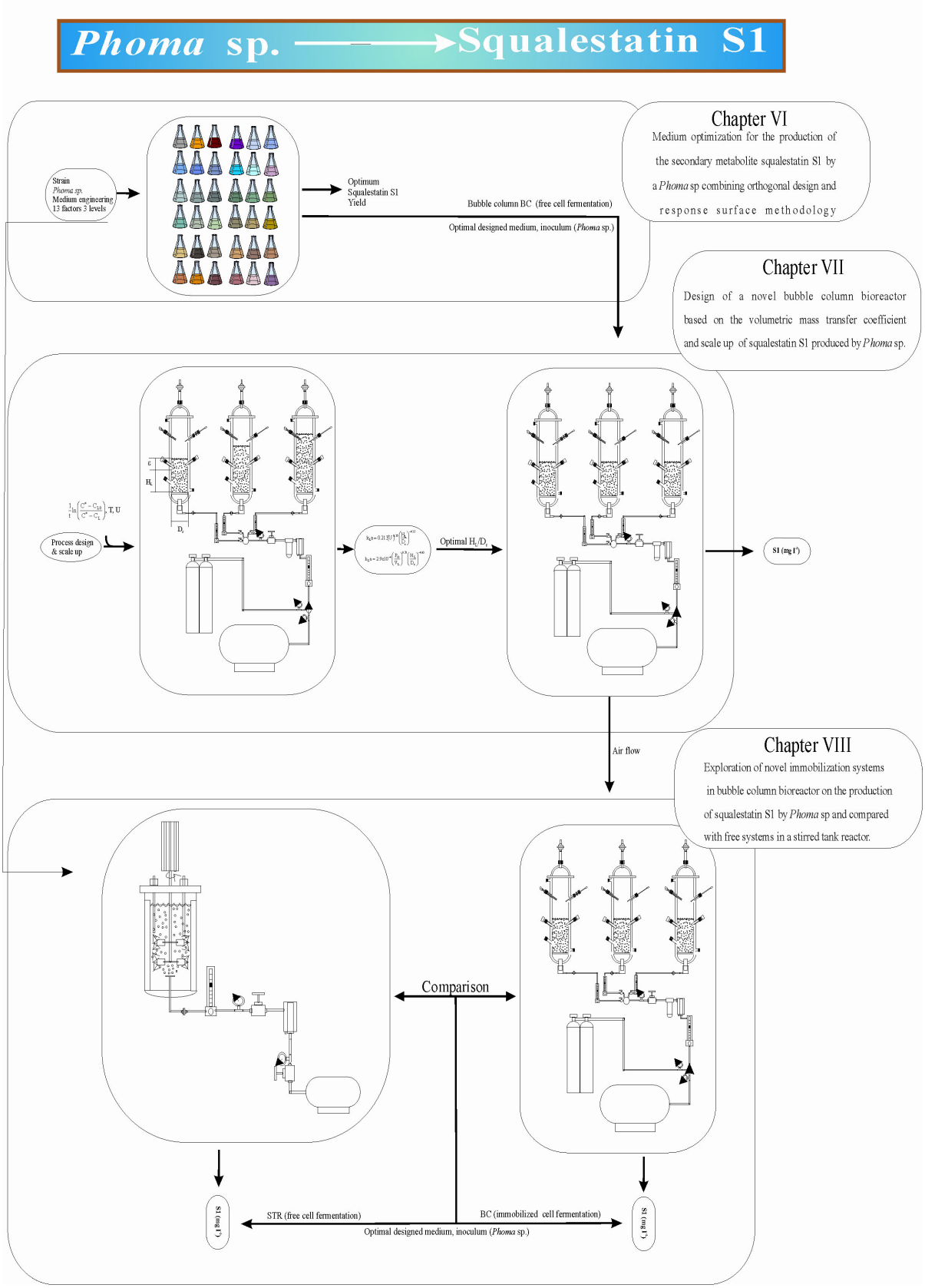

Figure 1.1. Diagram of the components studied (*cont.*.)

CHAPTER II

WATER ACTIVITY, SOLUTE AND TEMPERATURE -MODIFIED GROWTH AND SPORE PRODUCTION OF WILD TYPE AND GENETICALLY ENGINEERED *Aspergillus niger* STRAINS

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2.1 INTRODUCTION

Filamentous fungi, especially *Aspergillus* species, are attractive hosts for the production of foreign proteins because of their high secretory capability (Archer *et al*., 1990a; Jeenes *et al*., 1991; Archer, 2000; Cullen *et al*., 1987; Gwynne *et al*., 1987; Upshill *et al*., 1987; Turnbull *et al*., 1989). This has been demonstrated for a number of products from *Aspergillus nidulans* and *A.niger* (Christensen *et al*., 1988; Ward *et al*., 1990; Conesa *et al*., 2001; Punt *et al*., 2002). *A.niger* is a xerophilic species which can normally tolerate quite dry environments (Magan, 1997; Marin *et al*., 1995; Baxter *et al*., 1998). Water activity (a_w) and temperature are critical factors affecting the growth and metabolism of fungi (Scot, 1957; Gervais *et al*., 1988). From an ecological point of view little information is available on whether genetically modified strains will behave in a similar manner to wild type strains or not. No studies have attempted to evaluate this, although it has been shown that in wild type strains of pharmaceutically useful fungi, subtle changes in water stress can result in a significant stimulation of secondary metabolite production (Baxter *et al*., 1998; Gervais *et al*., 1988; Aldred *et al*., 1999). Thus, the objective of this study was to evaluate the effects of water availability (water activity, a_w), solute used to modify a_w, and temperature on growth and spore production by a wild (W) and two genetically-engineered strains (B1, L11) of *Aspergillus niger*.

2.2 MATERIALS AND METHODS

2.2.1 Fungal strains

Two genetically engineered strains *A. niger* B1 and L11 from strain AB4.1 (van Hartingsveldt *et al*., 1987) containing the full-length hen egg white lysozyme (HEWL) cDNA under the control of the *A.niger*. glucoamylase promoter were used in this study (Archer *et al*., 1990b). B1 differs from L11 in gene copy number and in having the HEWL-encoding found downstream of the region of the gala gene encoding the cathalase and in the domain of galactosidase (GAM). The gala and

HEWL-encoding genes were separated by a sequence encoding the KEXZ endoproteolytic cleavage site (Jeenes *et al*., 1994). The cultures were maintained as aliquots of spore suspensions in 5% (w/v) glycerol stored at -70^{o}C (Connors *et al*., 1995).

2.2.2 Media preparation, incubation and growth rate assessment

The water activity of 4.8 % w/v malt extract agar (MEA) (Oxoid) was modified with calculated amounts of the non-ionic solute glycerol and the ionic solute NaCl to 0.99-0.90 a_w. A Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland) was used to check the a_w levels obtained and found to be within 0.005 of the desired a_w level.

Actively growing 8-day-old colonies of W, L11, B1 on MEA were used to prepare a spore suspension (1.8 x 10^7 spores ml^{-1} ± 2%). Fungal spore suspensions were prepared in a solution of Tween 80 (100µl l^{-1}). Petri plates with glycerol or NaCl-modified MEA were inoculated with 5 µl of the spore suspension and incubated at 25, 30 and 35ºC.

The temporal mycelial extensions of treatments and replicates were measured in two directions at right angles to each other. Measurements were recorded on alternate days during the growth until the Petri plate were completely colonised (Baxter *et al*., 1998; Aldred *et al*., 1999; Baxter, 1997). A linear regression of the data was performed in order to calculate the growth rate. The calculated growth rate was used for statistical analysis. It has been previously shown by Trinci (1969) and Trinci and Collinge (1973) that the linear growth rate on a solid substrate, e.g. agar medium, is a good approximation of biomass increase in liquid culture.

Spores were recovered from Petri plates by agitating the surface with 10 ml of sterile water (+ a drop of Tween 80) twice and decanting into a Universal bottle. The number of spores was determined using a haemocytometer and microscope (Olympus ABHZ, Olympus UK) . Spore number were recorded when the treatment strain had colonised the surface of the Petri plate fully up to a maximum of 28 days.

2.2.3 Experimental design and data treatment

For growth experiments a fully randomised factorial design run in quadruplicate was used to describe the growth rate of *A. niger* in relation to the three strains, three temperatures, five a_w levels and two solute types. In all cases linear regression of increase in radial extension against time was used to obtain the growth rates under each set of treatment conditions.

A fully randomised factorial design (3^3) in triplicate was used to describe the spore production of *A. niger* in relation to the same treatments used for growth. The number of spores produced at the end of each experimental run was recorded and used in the statistical analysis. Orthogonal experiment optimization was computed in order to calculate the optimal conditions of growth rate and contour surface plots were used to find the maximum values of growth and sporulation (Wu and Hobbs, 1987).

2.3 RESULTS

2.3.1 Effect of environmental factors on growth

Figure 2.1 shows an example of the temporal radial extension of colonies of *A. niger* (L11) on media modified with the non-ionic solute glycerol or the ionic solute NaCl at three different temperatures. This shows that mycelial extension was faster at 0.95 a_w than on unmodified media with freely available water. This information was used to compare growth rates *of A. niger* in the different treatments.

Figure 2.2 compares growth of all three strains of *A.niger* at different aw levels at 35^oC, the optimum temperature for growth using both solutes. This shows that the wild-type strain grew optimally at 0.95-0.93 a_w on glycerol-modified media, but optimally at 0.99 a_w when NaCl was used. For the two genetically-modified strains growth was optimal at 0.97 a_w, regardless of solute used. Overall, strain L11 grew faster than the others examined. The overall growth pattern of the two genetically modified strains was similar, and different from the wild-type strain. In unmodified medium (0.99 a_w) growth of the wild type was faster than the two modified strains. Statistical analysis of the data showed that all factors and interactions were significant ($P<0.001$) for growth rates (Table 2.1).

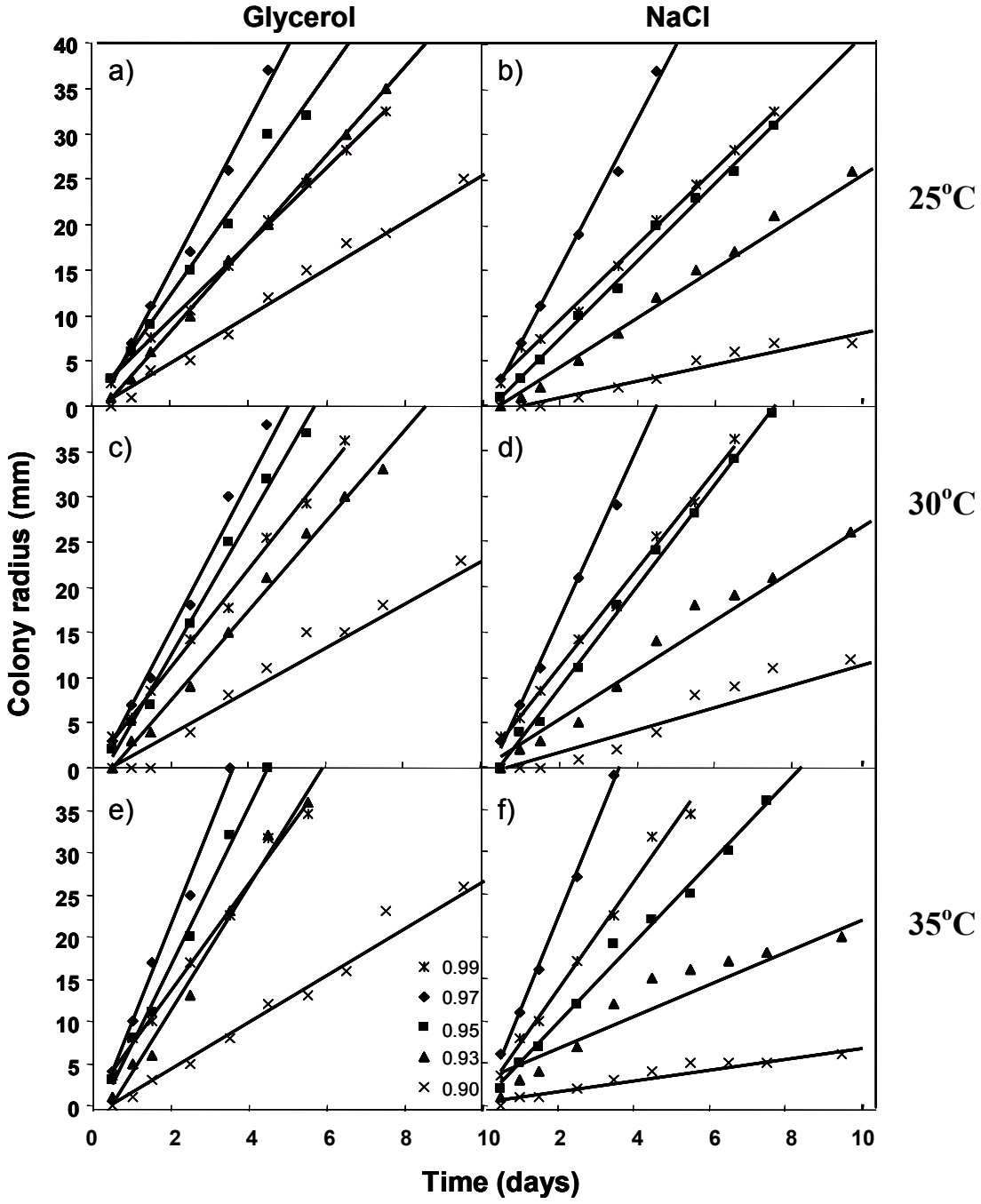

Figure 2.1. Effect of water activity modified with glycerol and NaCl at 25 ºC (a,b), 30 ºC (c, d) and 35 ºC (e, f) on colony diameter of *Aspergillus niger* L11

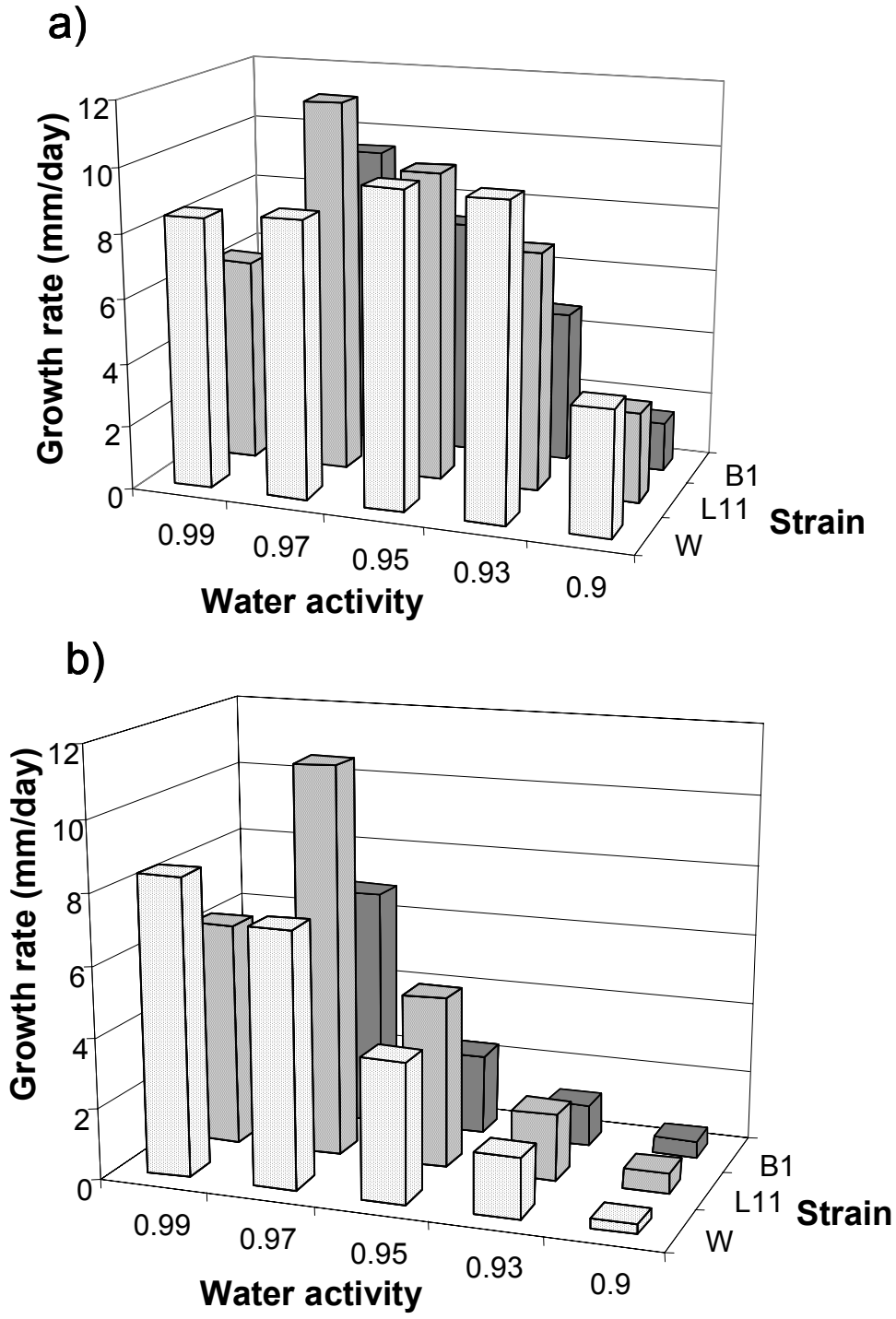

Figure 2.2. Comparison of growth rate (mm/day) of three strains of *Aspergillus niger* (L11, B1 and native strain W). Water activity modified with a) glycerol b) NaCl at 35 ºC

Table 2.1. Analysis of variance of water activity (a_w), solutes type, and temperatures in the growth rate of three strains of *Aspergillus niger* (L11, B1 and native strain W)

Factor	Df	MS	F
a_w	4	375.01	1060.29**
Strain	2	41.59	235.19**
Temperature	2	20.98	118.62**
Solute	2	209.73	1185.94**
Factor interactions			
a_w x Strain	8	12.21	17.26**
a_w x Solute	8	65.09	92.02**
Strain x Solute	4	40.94	115.76**
Temperature x Solute	4	17.85	50.48**
Residual	277	3.04	

** Significant at the level $p<0.001$

The most important factors overall were a_w, solute and their interactions. A contour surface plot summarises the optimal conditions in relation to a_w and temperature for all three strains (Figure 2.3). The data from the experiments were used to find the optimal conditions using orthogonal optimization to determine the highest growth rates (Table 2.2). To confirm the accuracy of the prediction, an experiment using these optimal conditions was carried out in triplicate. A value of 10.85 mm day^{-1} was obtained verifying a good correlation between the predicted and the measured results.

2.3.2 Effects of environmental conditions on sporulation of *A.niger* strains

Figure 2.4 shows the effect of temperature and a_w on sporulation of *A. niger* strains on glycerol-amended media. This shows that the wild type strain (W) produced most spores at 0.97 a_w, and at 35^{o}C. There were significant differences between the three strains, with L11 producing a 10-fold higher amount of spores, especially at 35^{o}C between 0.97-0.93 a_w. Thus L11 has markedly different sporulation capacity compared to the other two strains examined. Table 2.3 shows the statistically significant factors were a_w, strain, temperature, solute and a_w x strain, a_w x temperature and strain x temperature.

The contour surface plot of the impact of a_w x temperature on sporulation capacity of L11 is shown in Figure 2.5. Optimum conditions for spore production are different from that for growth. High production of spores was found with L11 at 0.95 a_w modified with glycerol at 35ºC. The range of significant spore production can be defined between 0.97-0.93 a_w and 33-35^{o}C. This suggest that higher temperatures are more suitable for sporulation and spore production.

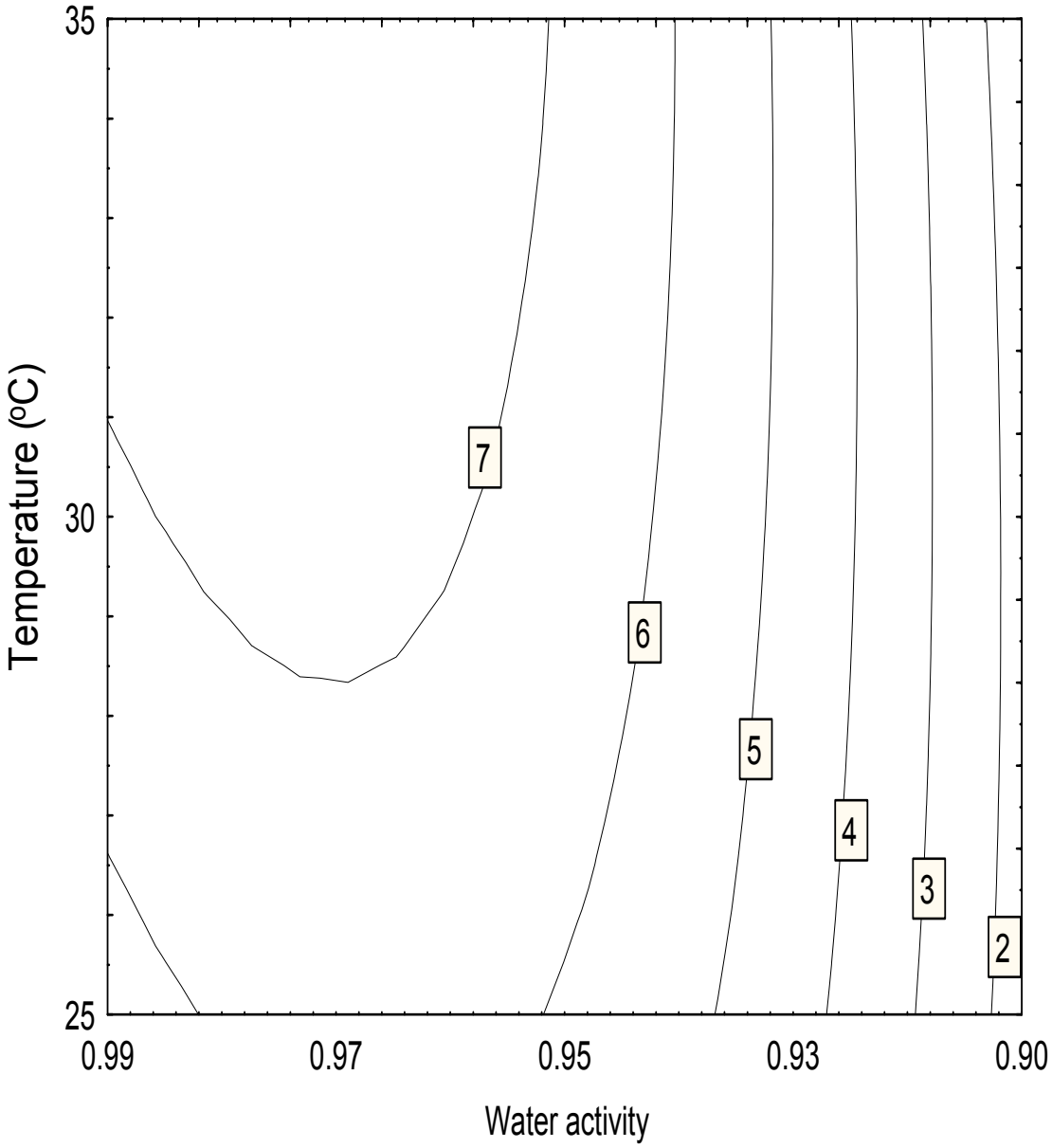

Figure 2.3. Contour surface mycelial extension of a_w against temperature effects on growth rate (mm day^{-1}). The number in the figure (□)=mm day^{-1}

Table 2.2. Optimal combination of factor and levels found with orthogonal design optimization.

Optimal Conditions	
Factors	Level
a_w	0.965
Strain	L11
Temperature (ºC)	35ºC
Solute	Glycerol
Expected	10.514 mm day^{-1}

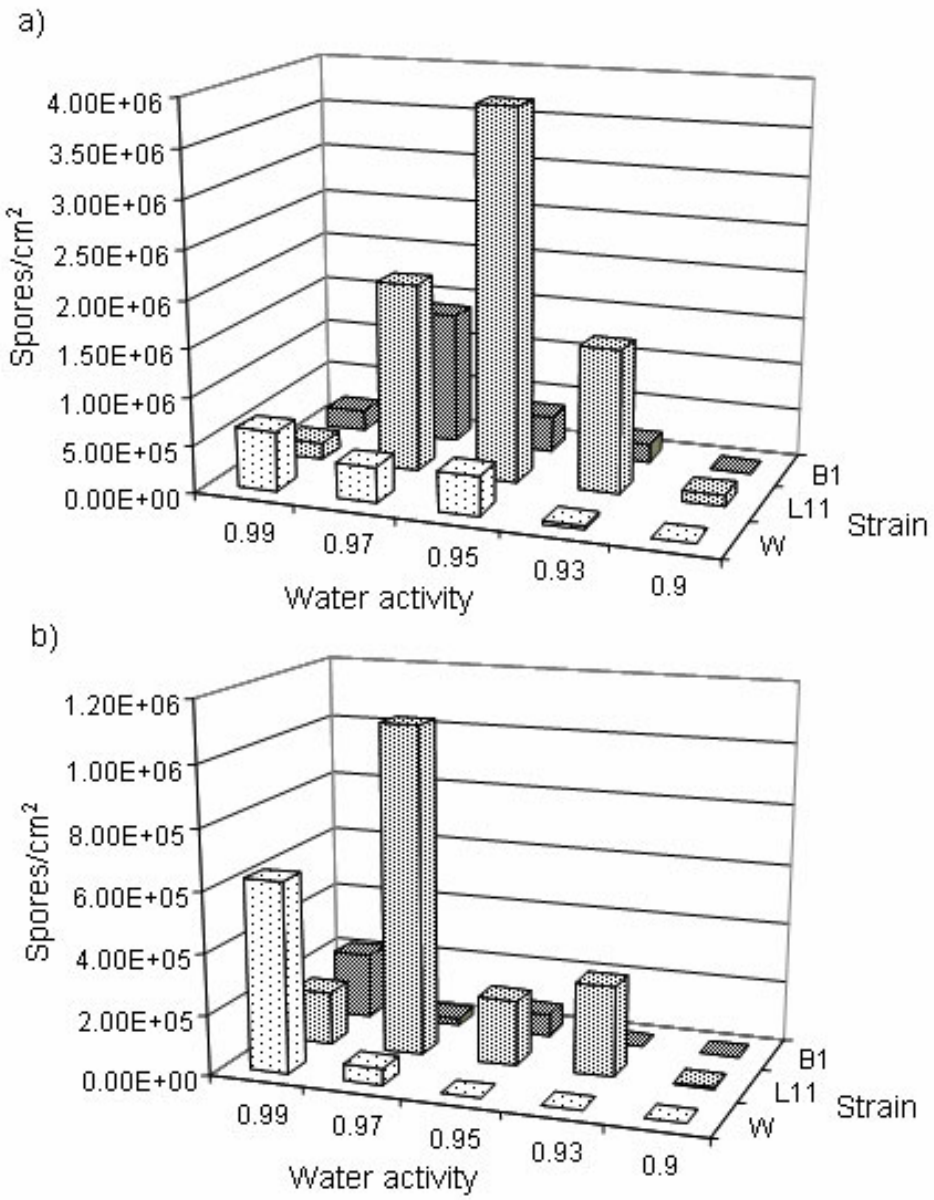

Figure 2.4. Effect of water activity modified with a) glycerol and b) NaCl on spore production (spores cm^{-2}) at 35°C in three strains of *A. niger*.

Table 2.3. Analysis of variance of water activity (a_w), solutes type, and temperatures in the spore production of three strains of *Aspergillus niger* (L11, B1 and native strain W)

Factor	df	MS	F
a_w	4	2.0E+15	2.68**
Strain	2	5.6E+15	15.22**
Temperatura	2	5.4E+15	14.69**
Solute	2	2.5E+15	6.81**
a_w x Strain	8	8.8E+14	0.60*
a_w x Temperature	8	5.9E+14	0.40*
a_w x Solute	8	7.4E+14	0.50
Strain x Temperature	4	2.1E+15	2.82*
Strain x Solute	4	7.1E+14	0.97
Temperatura x Solute	4	1.0E+15	1.40
Residual	196	3.0E+14	

*, significant at $P<0.05$

**, significant at $P<0.01$

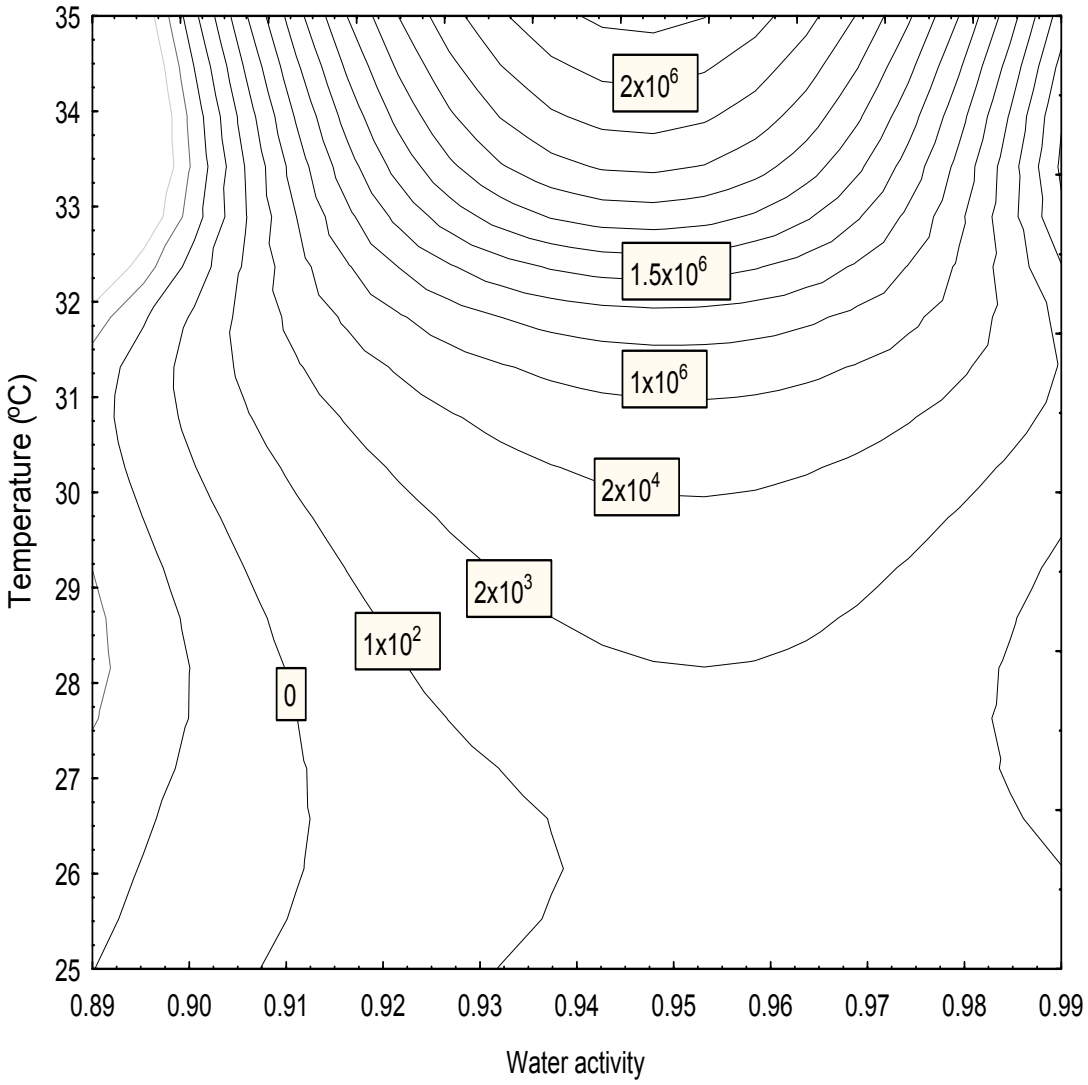

Figure 2.5. *A. niger* L11 spore production (spores cm^{-2}) contour surface of water activity and temperature.

2.4 DISCUSION

This is the first time that ecological comparisons have been made between wild-type and genetically modified strains of xerophilic fungi. We conclude that genetic modification can affect both water and temperature relations for growth, and particularly for sporulation. The optimal growth of the three strains of *A. niger* examined was found to be in the range 0.97-0.95 a_w. The growth rate of the genetically engineered strains were similar to each other, and different from the wild-type strain. Strains L11 and B1 had a higher tolerance to lower water activity than the wild type when modified with NaCl. In all cases the a_w range of 0.97-0.95 resulted in a faster growth rate when compared to the control (0.99 a_w). The use of glycerol to modify media water availability produced a higher growth rate than with NaCl, probably because it can be utilised as a carbon and energy source and can act directly as a compatible solute. In contrast, high concentrations of NaCl can be toxic and this may explain the differential growth patterns observed. The effect of the temperature and a_w were significant and both affect the growth rate. Growth patterns of both transgenic strains were similar. Growth of filamentous fungi has been shown to be dependent on thermodynamic factors such as water availability and temperature (Scott, 1957; Gervais *et al*., 1988).

Previous studies on *A.niger* suggest a range of 10-40^oC and 0.77-0.99, with the optimum at 35^oC and 0.99 a_w (Michell *et al*., 2003). However, sporulation ranges were not considered previously and this early work was carried out when no information on related *A. niger* group species (e.g. *A carbonarius*) was known. Recent studies on the *Aspergillus* section nigri group isolated from grapevine suggest optima for growth of 0.95 a_w at 30-35^oC (Ayerst, 1969).

The relative effects of the environmental factors on sporulation were more dramatic than on growth. The genetically modified strain L11 behaved in a completely different manner compared to the other two strains examined, producing substantially more conidia and over a wider a_w range, especially at the optimum temperature, 35^oC. Very few studies have examined the impact of environmental factors such as a_w or temperature on sporulation of wild type strains of fungi and none on genetically

modified ones (Magan and Lacey, 1984; Geravis *et al*., 1988; Gervais and Molin, 2003). For example, Gervais *et al*. (1988). Gervais and Molin (2003) showed that *P.roquefortii* strains from cheese grew optimally at 0.97-0.98 a_w, while maximum spore production was at 0.96 a_w. The present study suggests that genetic manipulation can alter the physiology of a fungus in an unpredictable way. The two GM-strains have multiple gene copies integrated into the genome at more than one locus, and this may have caused the changes observed.

2.5 CONCLUSIONS

From this study of the growth rate of genetically engineered *Aspergillus niger,* several parameters important for maximizing the growth rate have been identified. A combination of a_w, temperature and solute used to modify the a_w had the greatest effect on growth rate. L11 grew significantly better than the other strains examined. Thus, such screening criteria based on these environmental parameters (a_w, temperature, solute type) can facilitate the selection of appropriate transformed strains for studying secondary metabolite or heterologous protein production systems.

CHAPTER III

MODELLING THE EFFECT OF TEMPERATURE AND WATER ACTIVITY ON GROWTH OF Aspergillus niger STRAINS AND APPLICATIONS FOR FOOD SPOILAGE MOULDS

Published in *Journal of Applied Microbiology* (2004) **35**:232-237.

3.1 INTRODUCTION

In the last two decades, predictive food microbiology has included the development of models capable of describing the growth of pathogenic bacteria (Buchanan and Phillips, 1990). However, predictive modelling of filamentous fungal growth has not received the same attention (Gibson *et al*., 1994; Gibson and Hocking, 1997). Two of the most important environmental parameters that determine the ability of moulds to grow on foods are water activity (a_w) and temperature (T) (Scott, 1957). An empirical approach to modelling the effects of a_w on mould growth was used by Gibson *et al*. (1994) who found that the logarithm of the fungal growth rate (μ, measured as the increase in colony radial growth per unit of time) showed a parabolic relationship with the square root of b_w. The b_w was defined as the difference between the a_w of pure water (1) and that of a set a_w for a specific growth kinetic. They investigated the appropriateness of models that were previously used to predict bacterial growth for the interpretation of mould growth data.

Much work has been directed towards the development of models for bacterial growth as a function of temperature and a_w (McMeekin *et al*., 1987; Zwietering *et al*., 1994; Rosso *et al*., 1995). The temperature dependence of the specific growth rate of bacteria may be modelled by means of the square root model (Ratkowsky *et al*., 1983) with the cardinal parameters (e.g. the minimal temperature for growth, T_{min} and the maximum temperature for growth, T_{max}).

Cuppers *et al*. (1997) successfully combined the models of Rosso *et al*. (1995) and Ratkowsky *et al*. (1983) to describe the combined effects of temperature and NaCl on the growth rate of some food spoilage moulds. The four moulds used, *Penicillium chrysogenum*, *Cladosporium cladosporioides*, *Aspergillus flavus* and *Alternaria alternata*, were inoculated at the optimum temperature for growth and at pH close to optimum.

The main objective of this work was to develop a combined model based on the Gibson-type a_w dependence and the Ratkowasky-type temperature dependence model on growth rate that could be used to describe the effect of interacting conditions of a_w

and temperature for the first time. This was then used to estimate the cardinal a_w levels (a_{wmin}, a_{wmax}, a_{wopt}) under different temperatures for two strains of *Aspergillus niger*. Correlations with literature data were made for a wide range of *Aspergillus* and *Eurotium* species.

3.2 MATERIALS AND METHODS

3.2.1 Fungal strains

See section 2.2.1

3.2.2 Media preparation, incubation and growth rate assessment

The water activity of 4.8 % malt extract agar (MEA) (Oxoid) was modified with calculated amounts of the non-ionic solute glycerol 0.99-0.90 a_w. A Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland)was used to check the a_w levels obtained and found to be within 0.005 of the desired a_w level.

Actively growing 8-day-old colonies of *Aspergillus niger* L11 and B1 on MEA were used to prepare a spore suspension (1.8×10^7 spores ml^{-1} ± 2%). Fungal spore suspensions were prepared in a solution of Tween 80 (100µl l^{-1}). Petri plates with glycerol-modified MEA were inoculated with 5 µl of the spores suspension and incubated at 25, 30 and 35ºC.

3.2.3 Assessment of hyphal growth rate

The radial mycelial growth of each plate was measured in two directions at right angles to each other. Measurements were recorded on alternate days during the growth until the Petri plates were completely colonised. Radial mycelial growth vs. time was plotted and radial growth rates (µ, mm day^{-1}) were evaluated from the slopes by linear regression (Baxter *et al*., 1998; Aldred *et al*., 1999).

3.2.4 Experimental design and data treatment

A fully randomised factorial design run in quadruplicate was used to generate the growth rate of the *A. niger* strains L11 and B1 at three temperatures and five a_w levels modified with glycerol. In all cases a linear regression of the increase in radial

extension against time was used to obtain the growth rates under each set of treatment conditions.

3.2.5 Model development

Previously, square root-type models (Ratkowsky *et al*., 1983) to describe the effect of temperature used cardinal parameters (e.g., the minimal temperature for growth, T_{min} and the maximum temperature for growth, T_{max}) has been used :

$$\sqrt{\mu} = b(T - T_{Min})\left[1 - \exp(c(T - T_{Max}))\right] \quad \text{(Eq. 3.1)}$$

where μ= growth rate (mm day^{-1}), T= Temperature, T_{Min}= theoretical minimal temperature, T_{Min}= theoretical maximal temperature where b and c are design parameters.

Gibson *et al*. (1994) found that the logarithm of fungal growth rate (μ, measured as the increase in colony radial growth per unit of time) showed a parabolic relationship with the square root of

$$b_w = 1 - a_w \quad \text{(Eq. 3.2)}$$

leading to

$$Ln(\mu) = b_0 + b_1\sqrt{b_w} + b_2 b_w \quad \text{(Eq. 3.3)}$$

The proposed model is derived from Eqs. (3.1) and (3.3) and have the general form of

$$Ln(\mu) = b_0 + b_1\sqrt{b_w} + b_2 b_w + b_3 T\left[1 - \exp(b_4 T)\right] \quad \text{(Eq. 3.4)}$$

where μ= growth rate (mm day^{-1}), b_0, b_1, b_2, b_3 and b_4 are design parameters, a_w = water activity and T= temperature (^oC). The regressed parameters were found by non-linear estimation with Hooke-Jeeves and the quasi-Newton method of Statistica v6.0 (StatSoft, Inc. 1984-2001. Tulsa USA) and simulated data and uncertainty of the

parameters were evaluated with Crystal Ball 2000 v5.2 (Decisioneering, Inc. 1988-2002, Denver, USA)

3.3 RESULTS

The growth data modelled in this work comprised the growth curves of two strains of *A. niger* L11 and B1 at five a_w and three temperatures. Mycelial extension of colonies versus time almost invariably showed a straight line, after an initial lag period. The growth rate (μ) in mm per day, was calculated as the slope of a regression line through these points. The growth rate (μ) under the conditions of a_w and temperatures recorded (data presented elsewhere) were used as inputs to calculate the design parameters of Eq.(3.4).

The mean logarithm of growth rate ($\ln(\mu)$) and standard deviation were found to be higher and lower, respectively, for L11 than for B1 (Table 3.1). A lower variability of the dispersed data of L11 could increase the robustness of the model for strain L11. Estimates of Eq. (3.4) and approximate standard errors are presented in the Table 3.2 for the strains L11 and B1 respectively. In both models there were no significant differences of the design parameters obtained in the four replicates.

Figure 3.1 and 3.2 shows the experimental data and the fitted data based on the model. The two strains of *A. niger* L11 and B1 are characterized by a sharp decrease in the radial growth rate from a_{wopt} to a_{wmax}, and from a_{wopt} to approx 0.92 a_w and from this a_w slow decrease in the growth rate until a_{wmin}. The a_w for optimal growth rate (a_{wopt}) was calculated to be 0.97 for both L11 and B1 and the minimum a_w (a_{wmin}) 0.84 and 0.82 at 25^{o}C, respectively. No differences were found in the optimal a_w (a_{wopt}) at 30 and 35^{o}C. However minimum a_w (a_{wmin}) at 35^{o}C for B1 was 0.80.

Table 3.1. Standard deviation of the model of the data for *Aspergillus niger* strains L11 and B1.

A. niger	Mean	St.dev.	Minimum	Maximum
L11	1.78	±0.445	0.880	2.447
B1	1.58	±0.530	0.418	2.247

Table 3.2. Parameter estimation for Eq. (3.4), their asymptotic standard errors and performance statistics for (a) A. niger strain L11 and (b) strain B1.

(a)

Parameter	Estimated	±SD	±SE
b0	-0.62	±0.02	±0.01
b1	27.96	±0.20	±0.05
b2	-75.92	±0.58	±0.15
b5	-1.26E-04	±8.25E-07	±2.13E-07
b4	1.33E-01	±0.01	±2.76E-03

(b)

Parameter	Estimated	±SD	±SE
b0	-1.23	±0.04	±0.01
b1	33.87	±0.39	±0.10
b2	-92.15	±1.01	±0.26
b5	-1.01	±0.13	±0.03
b4	2.47E-04	±2.02E-05	±5.21E-06

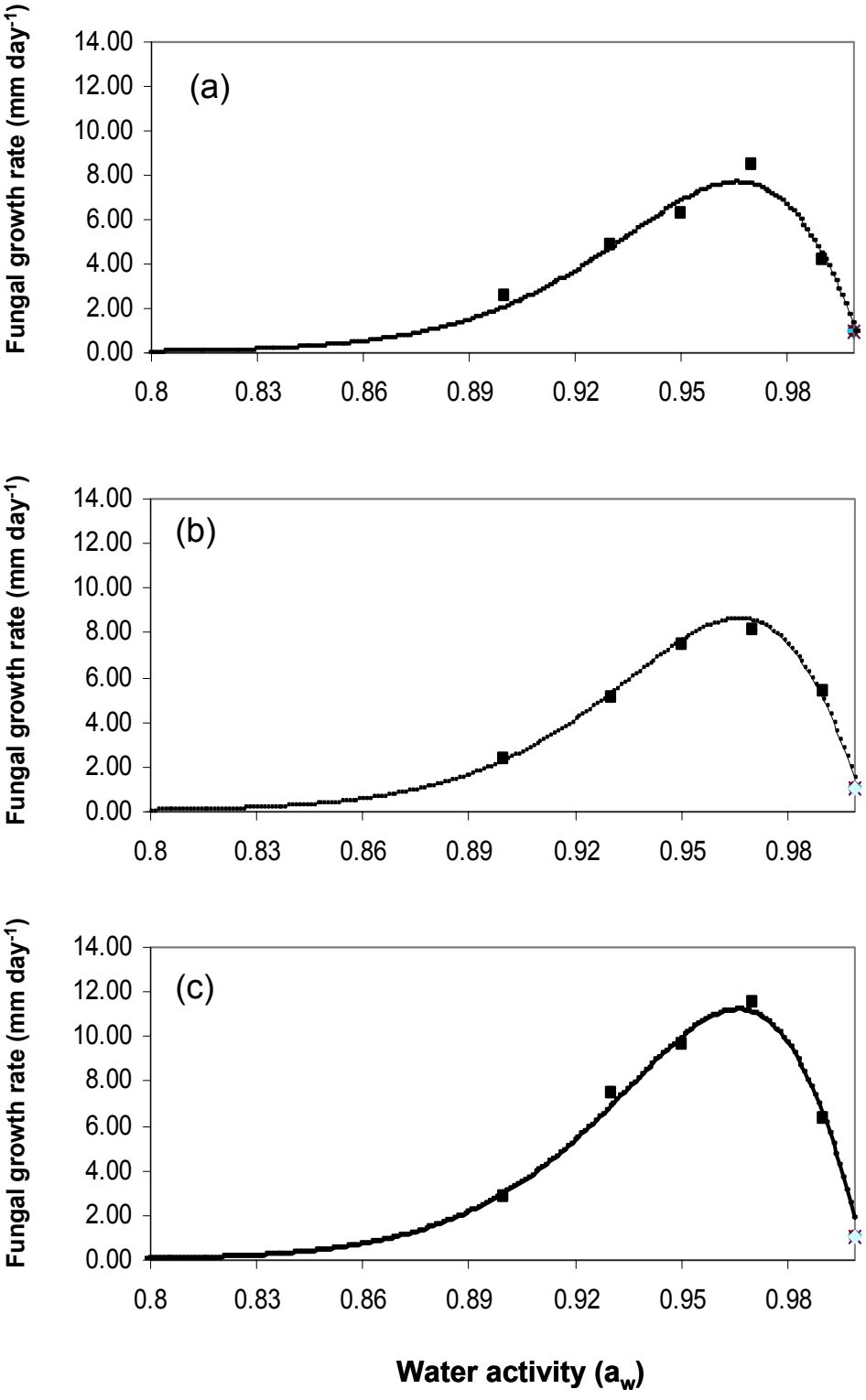

Figure 3.1. Experimental plots (■) and our model (Eq. (3.4))(—), for *A. niger* L11 at 25°C (a), 30°C (b) and 35°C (c) growing in MEA modified with glycerol as humectant (R^2=0.990).

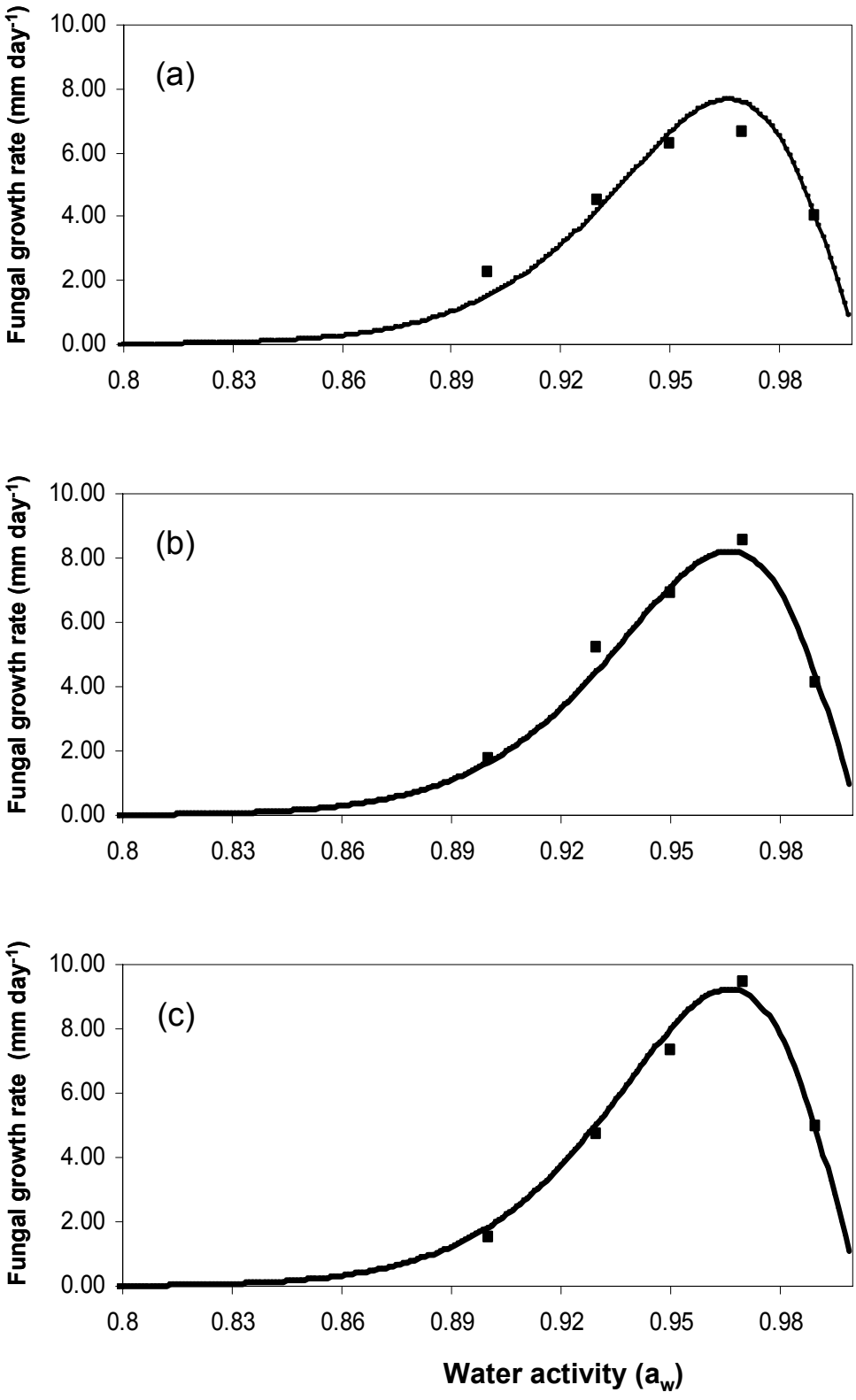

Figure 3.2. Experimental plots (■) and our model (Eq. (3.4))(—), growth rate vs a_w for *A. niger* B1 at 25°C (a), 30°C (b) and 35°C (c) growing in MEA modified with glycerol as humectant (R^2=0.990).

A computer simulated growth rate logarithm [Ln (μ)] of L11 was plotted against a_w at 25°C, 30°C and 35°C to confirm the certainty of the simulated design parameters b_0, b_1, b_2, b_3, b_4 and b_5, assuming a normal distribution under a 95% variation of the parameters (Figure 3.3). Robust model outputs growth rates (ln(μ)) were obtained for strain L11 at all temperatures at 50% variation of the design parameters. The logarithm of the growth rate at 25°C and 30°C showed very robust forecasts of the equation under all the distribution of the design parameters. The most stable design parameter was found at between 0.99 and 0.97 a_w. Moreover a reduction of aw increased the uncertainty of the model at 25 and 35°C for strain L11 and B1 (data not presented). A variation of the design parameters above 50% of the distribution in the 95% lead to a instability of the model and divergence of the results at 35°C. This result shows the high certainty of the parameters at high temperatures.

Figure 3.4 shows the generated surface of the modelled and experimental data (o) of a_w as a function of temperature on growth rate expressed as logarithm (Ln(μ, [mm day^{-1}])) for *A. niger* strain L11 and B1 (R^2=0.99). The surface generated by the model and the experimental data summarises all the interactions previously described. The surfaces show greater sensitivity of the *A. niger* L11 to the higher temperatures under optimal aw (a_{wopt}) conditions.

3.4 DISCUSSION

3.4.1 Comparisons with published data

Published reports of optimal a_w values for several species of *Aspergillus* have been obtained at temperatures of between 15-37°C, using a variety of humectants, over the range 0.85-0.995 a_w. The values predicted from the present model are that a a_w of 0.97 is optimum for growth, which is in good agreement with the published data obtained at 25, 30 and 35°C with several species of the genus *Aspergillus*, which was frequently shown to be at 0.96-995 a_w range depending on humectant used (Figure 3.5).

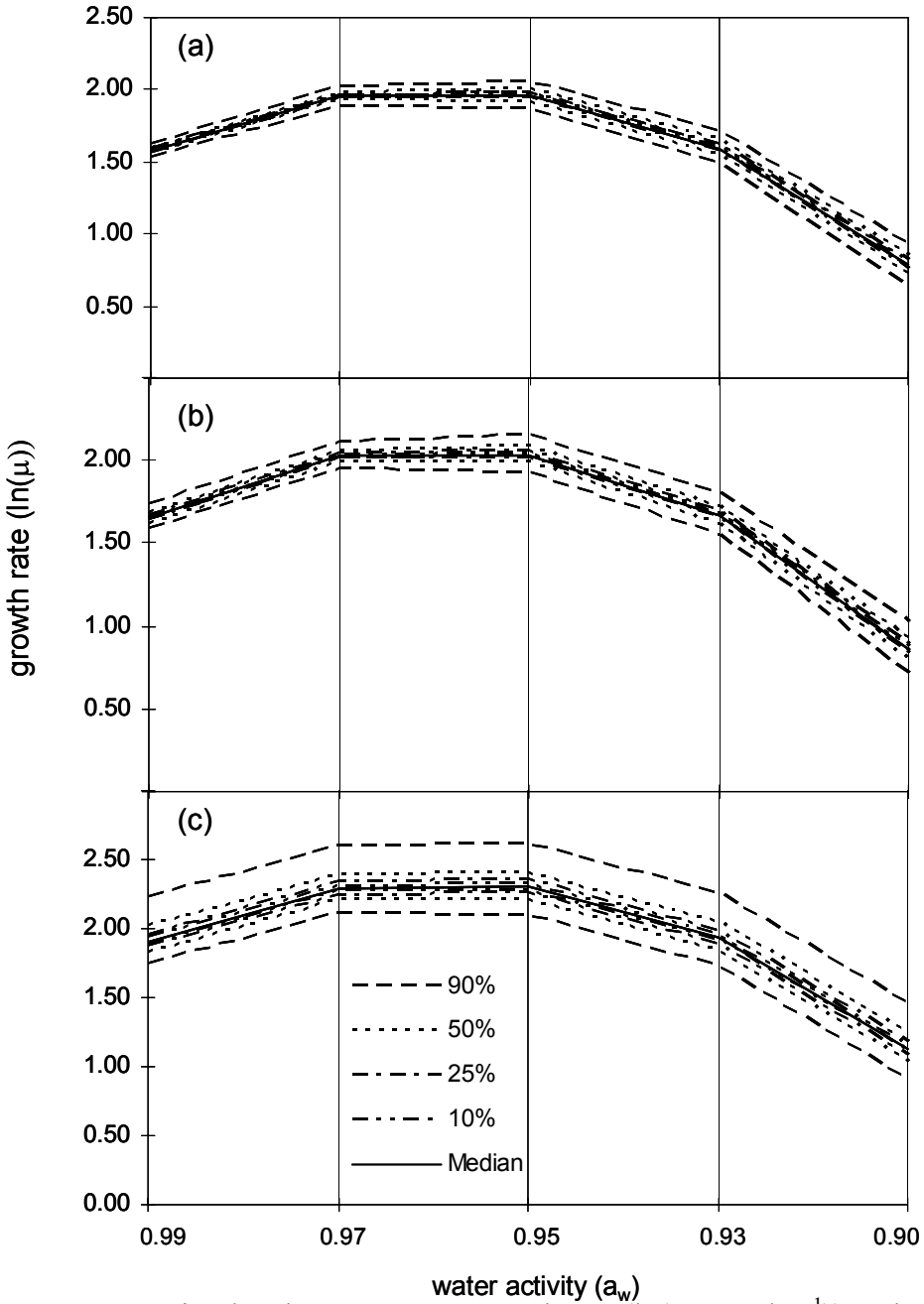

Figure 3.3. Simulated *A. niger* L11 growth rate (ln(μ, mm day^{-1})) under a generated set of the normal distributed design parameter within the a_w range at (a) 25°C, (b) 30°C and (c) 35°C.

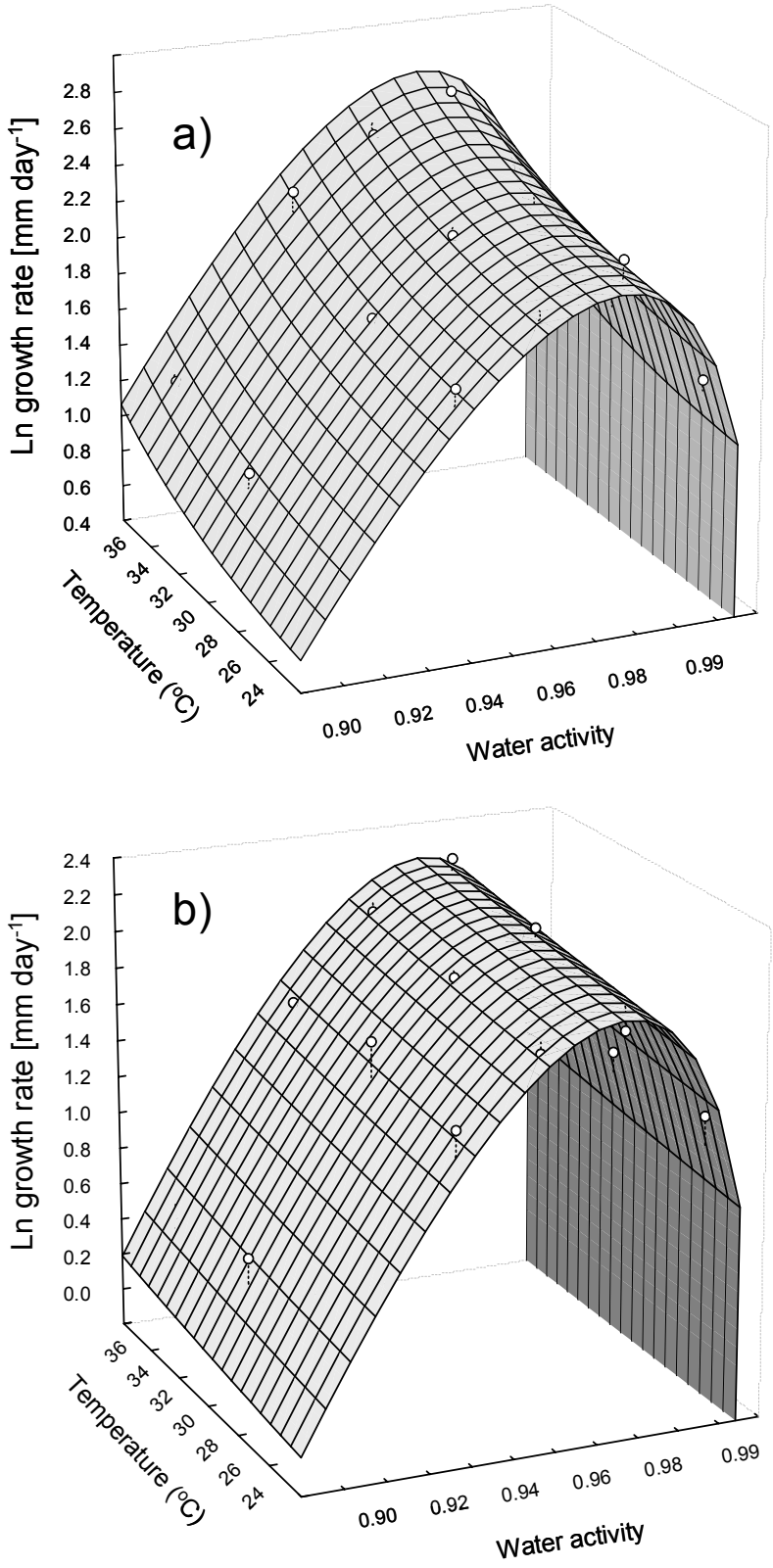

Figure 3.4. Generated surface of the modelled and experimental data (o) of a_w and temperature on growth rate expressed as logarithm (Ln(μ, [mm day^{-1}])) for *A. niger* L11 (a) and B1 (b) [R^2=0.99].

The predicted optimum a_w of different species of *Aspergillus* using our model shows good agreement with the values published as optimal (Ayerst, 1969; Horner and Anagnostopoulos, 1973; Holmquist *et al*., 1983; Magan and Lacey, 1984; Ramos *et al*., 1998; Marin *et al*., 1998; Mitchell *et al*., 2003) when using glycerol as a humectant within the confidence intervals.

The predicted optimum a_w of different species of *Aspergillus* at different temperatures (15-37^{o}C) shows good agreement with the values reported at 25^{o}C (Cuero *et al*., 1987; Horner and Anagnostopoulos, 1973; Magan and Lacey, 1984; Marin *et al*., 1998; Ramos *et al*., 1998; Sautour *et al*.,2001), at 30^{o}C (Magan and Lacey, 1984; Gibson *et al*., 1994; Marin *et al*., 1998; Ramos *et al*., 1998) and at 35^{o}C (Magan and Lacey, 1984; Marin *et al*., 1998; Mitchell *et al*.,2003) as shown in Figure 3.6.

A forecasted value of 33^{o}C as an optimum for growth was found at the 95% confidence level. This would also be partially influenced by the range of humectants and species used in the literature. At >35^{o}C and <25^{o}C, which are the limits of the model, good agreement was again seen with published work. Discrepancies were found of the forecasted and reported values at 20^{o}C (Wheeler *et al*., 1988). However, they used glucose/fructose as the humectant. More information on growth rates at this temperature is needed to obtain more accurate optimal a_w ranges at this temperature.

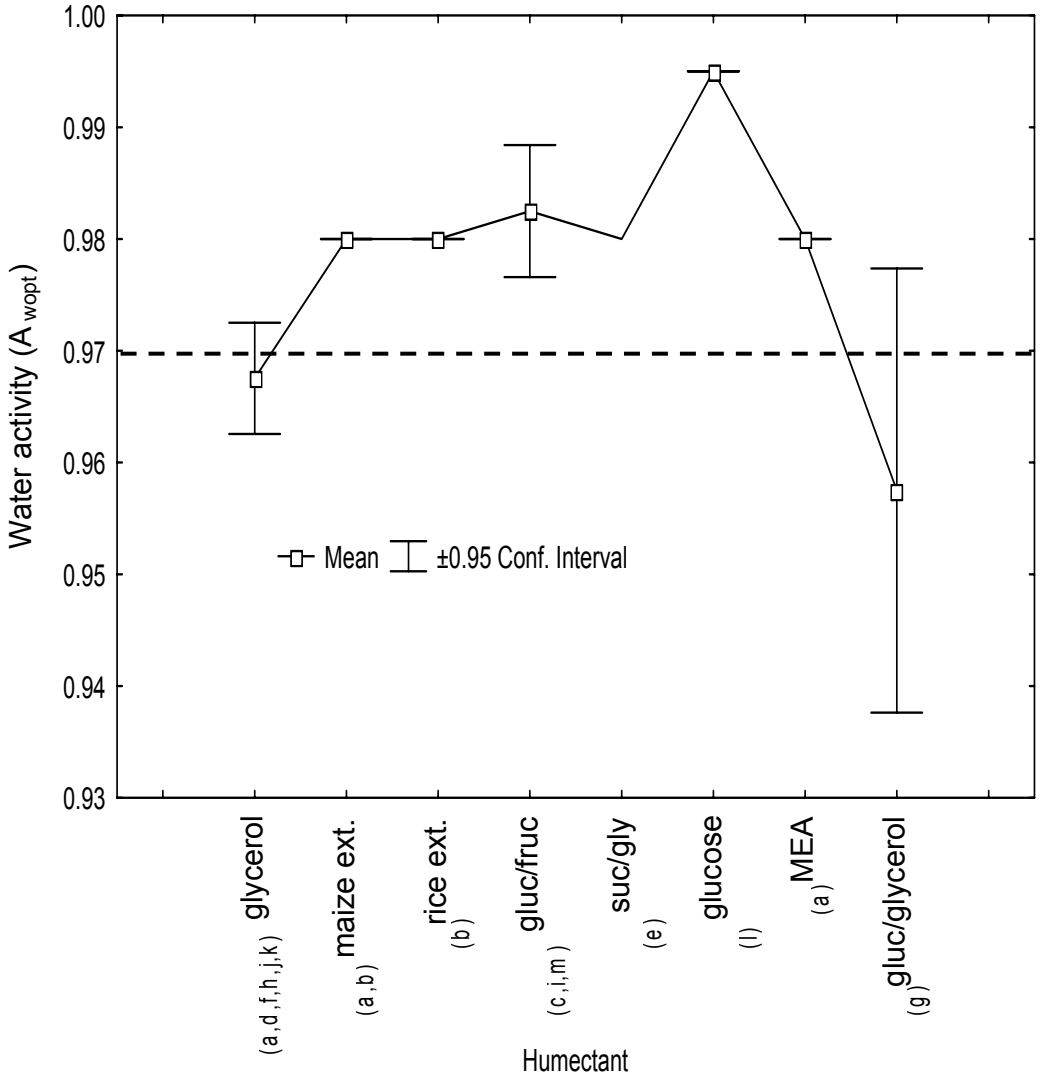

Figure 3.5. Optimal water activities (a_{wopt}) confidence intervals vs. reported humectants and forecasted a_{wopt} (—)(Eq. 3.4) for *A. niger* L11 and B1.

Key to references: (a) Ayerst, 1969; (b) Cuero *et al*., 1987; (c) Gibson *et al*., 1994; (d) Holmquist *et al*., 1983; (e) Horner and Anagnostopoulos, 1973; (f) Marin *et al*., 1998; (g) Mitchell *et al*.,2003; (h) Magan and Lacey, 1984; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari 1983.

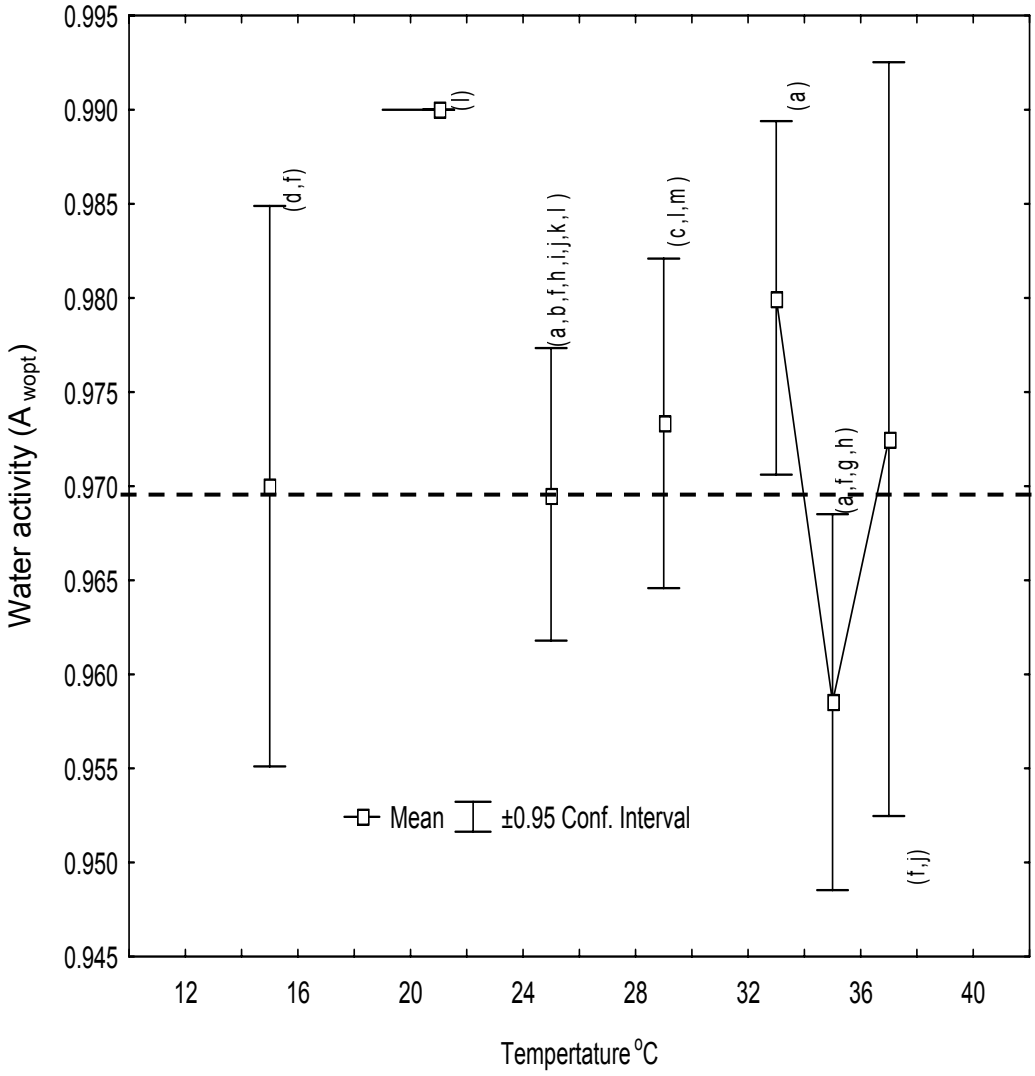

Figure 3.6. Optimal water activities (a_{wopt}) confidence intervals vs. reported temperature and forecasted a_{wopt} (—)(Eq. 4) for *A. niger* L11 and B1.

Key to references: (a) Ayerst, 1969; (b) Cuero *et al*., 1987; (c) Gibson *et al*., 1994; (d) Holmquist *et al*., 1983; (e) Horner and Anagnostopoulos, 1973 ; (f) Marin *et al*., 1998; (g) Mitchell *et al*.,2003; (h) Magan and Lacey, 1984; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari, 1983.

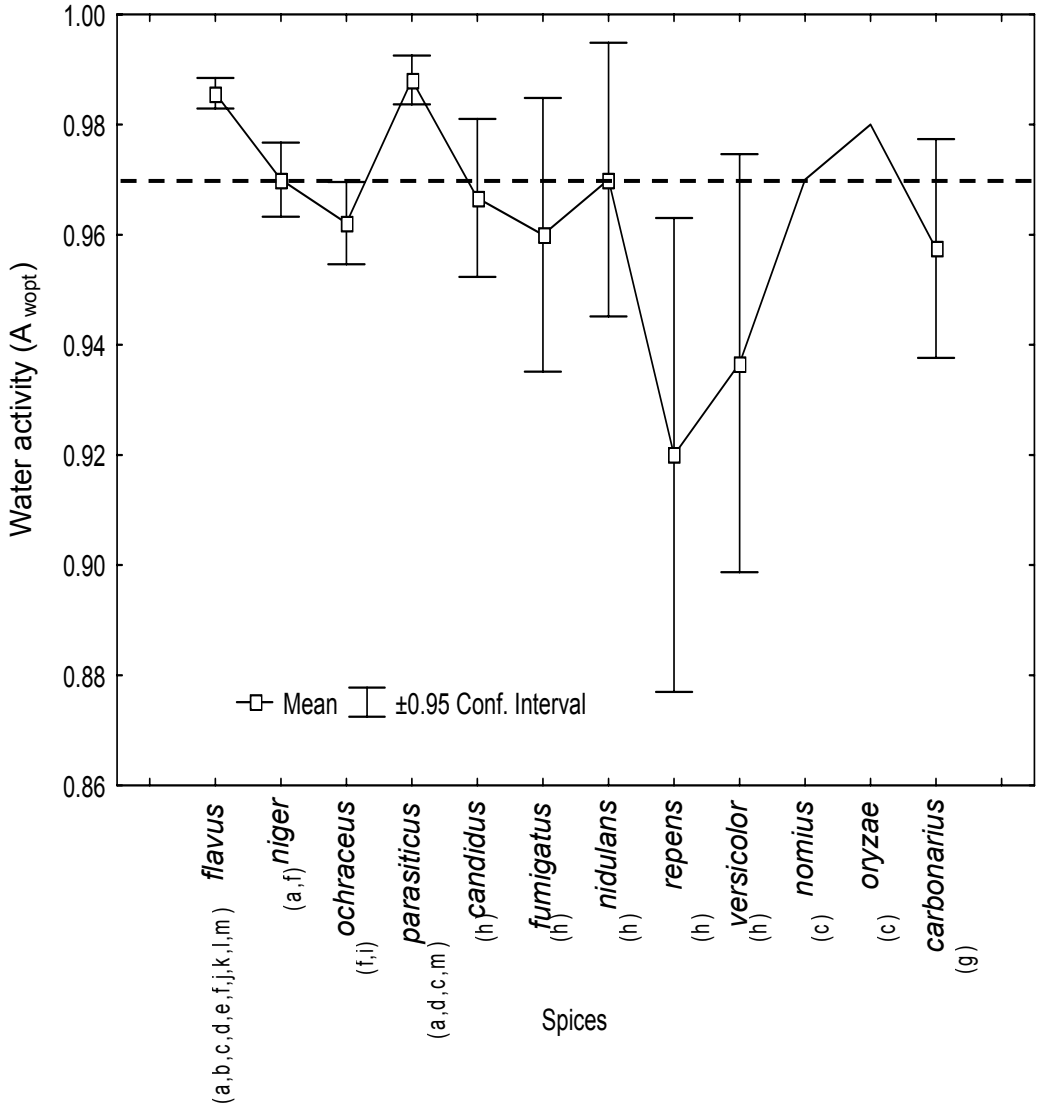

Figure 3.7. Optimal water activities (a_{wopt}) confidence intervals vs. reported *Aspergillus* species and forecasted a_{wopt} (—)(Eq. 4) for *A. niger* L11 and B1.

Key to referneces: (a) Ayerst, 1969; (b) Cuero *et al*., 1987; (c) Gibson *et al*., 1994; (d) Holmquist *et al*., 1983; (e) Horner and Anagnostopoulos, 1973; (f) Marin *et al*., 1998; (g) Mitchell *et al*.,2003; (h) Magan and Lacey, 1984; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari, 1983.

When a range of *Aspergillus* species were compared, an excellent correlation was found between the model values for optimum aw for growth and the reported values for *Aspergillus niger* (Ayerst, 1969; Marin *et al*., 1998) as shown in Figure 3.7. Similar values were obtained with *Aspergillus ochraceus* (Marin *et al*., 1998), *Aspergillus candidus* (Magan and Lacey, 1984), *Aspergillus carbonarius* (Mitchell *et al*.,2003), *Aspergillus fumigatus and Aspergillus nidulans (=Emericella nidulans)* (Magan and Lacey, 1984).

The minimal a_w values predicted by the model, the experimental values and the data published for several species of *Aspergillus* and related species all show good agreement (Figure 3.8), especially when glycerol was used as an humectant (Ayerst, 1969; Holmquist *et al*., 1983; Magan and Lacey, 1984; Marin *et al*., 1998; Ramos *et al*., 1998). Similarly, reasonable agreement was found where glucose or fructose was used to modify a_w (Pitt and Hocking, 1977; Wheeler *et al*., 1988; Gibson *et al*., 1994), and with some species of *A. carbonarius* where glucose or glycerol were used (Mitchell *et al*.,2003).

The model gave a good prediction of minimal a_w levels for growth in relation to a range of temperatures between 25-35^{o}C (Figure 3.9). An extrapolation of the model produced a good estimated of a_{wmin} at 15 (Marin *et al*., 1998) and 20^{o}C (Wheeler *et al*., 1988). At 37^{o}C the predicted value falls on the mean of the reported values (Marin *et al*., 1998). The best agreement of minimal water activity was obtained with species of *Aspergillus flavus, A. niger, A. ochraceus* and *A. parasitucus* at 25^{o}C. Moreover good agreement was found with *Aspergillus candidus*, *A. nidulans*, *A. versicolor* at 30^{o}C and *Eurotium repens* at 35^{o}C (Figure 3.10).

A range of published data describing the effect of a_w on radial growth rates of *A. flavus* was summarized in ICMSF (1993). Using the model described in the present work, growth rates for the two strains of *A. niger* were calculated for each a_w and temperature and compared with those listed in ICMSF (1993) (Table 3.3). For a_w values between 0.87 and 0.99, the predicted growth rates fall well within the range of those already published. It should be noted that data summarized in that publication were obtained from several authors using different species of *Aspergillus* grown on a variety of media using different humectants, pH levels and incubation temperatures.

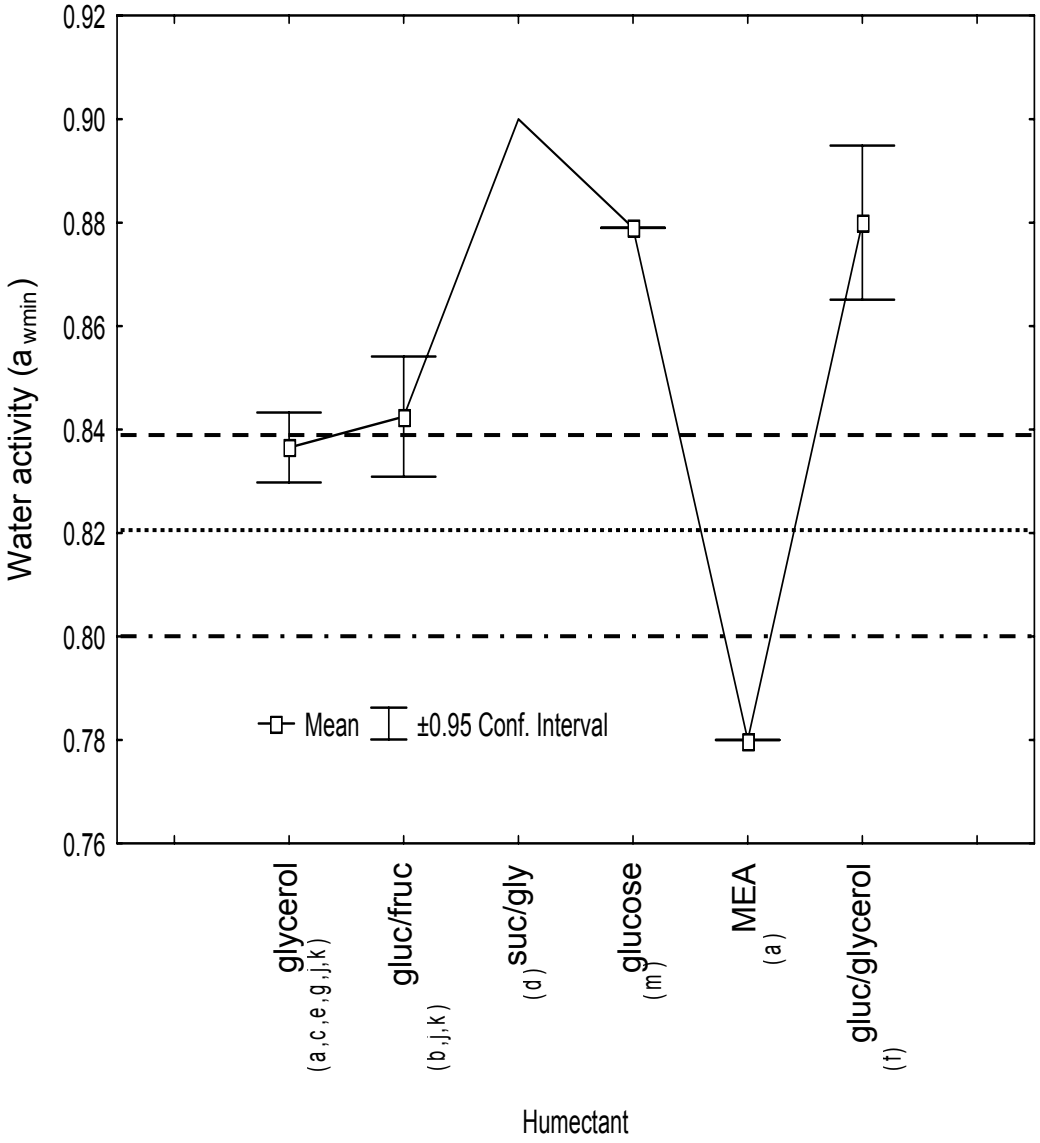

Figure 3.8. Minimal water activity (a_{wmin}) confidence intervals vs. reported humectant and forecasted a_{wopt} at 25(—), 30(···) and 35°C (—·—) (Eq. 4) for A. niger L11 and B1.

Key to references: (a) Ayerst, 1969; (b) Gibson *et al*., 1994; (c) Holmquist *et al*., 1983; (d) Horner and Anagnostopoulos ,1973; (e) Marin *et al*., 1998; (f) Mitchell *et al*.,2003; (g) Magan and Lacey, 1984; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari, 1983.

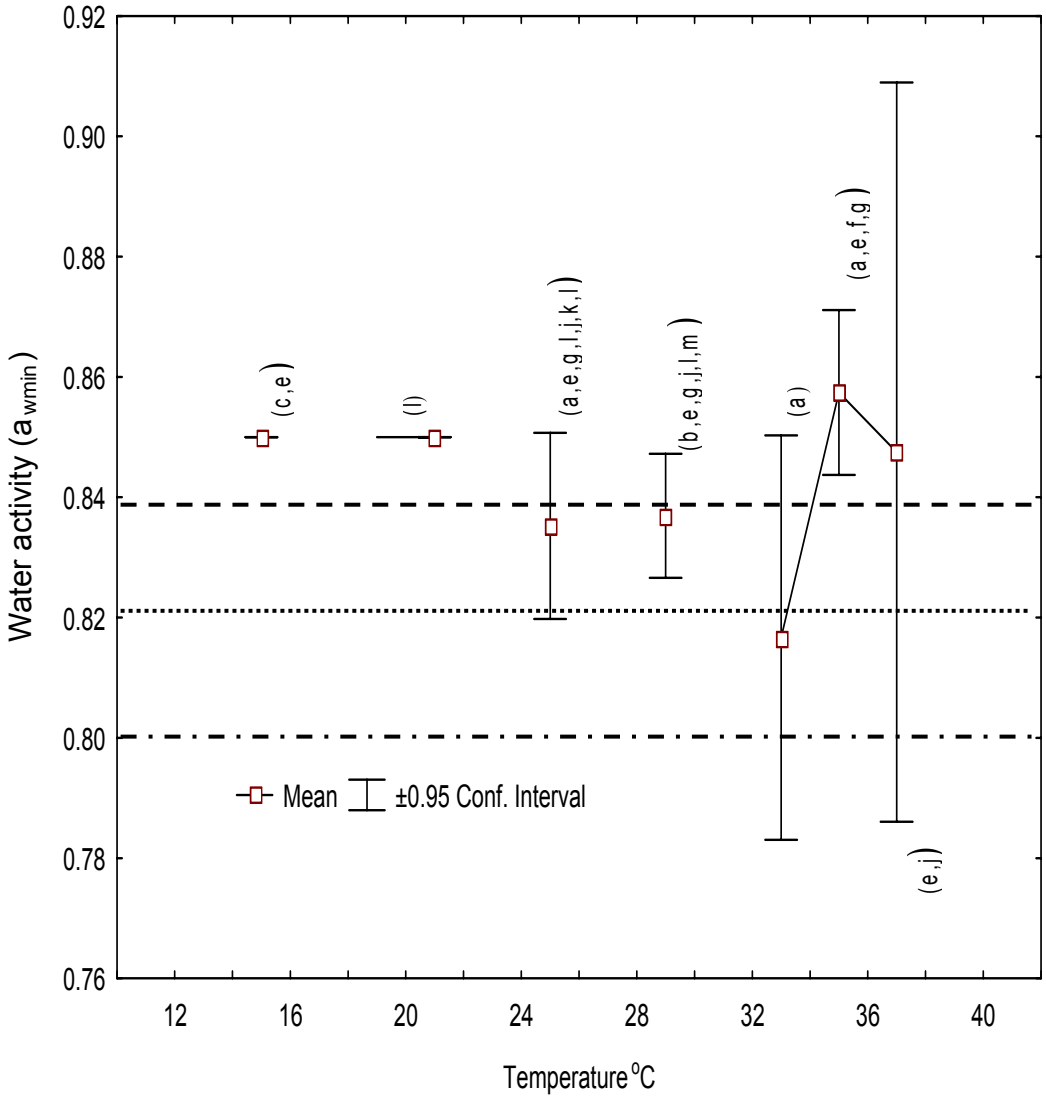

Figure 3.9. Minimal water activity (a_{wmin}) confidence intervals vs. reported temperature and forecasted a_{wopt} at 25(—), 30(···) and 35°C (—·—) (Eq. 4) for *A. niger* L11 and B1.

Key to references: (a) Ayerst, 1969; (b) Gibson *et al*., 1994; (c) Holmquist *et al*., 1983; (d) Horner and Anagnostopoulos, 1973 ; (e) Marin *et al*., 1998; (f) Mitchell *et al*.,2003; (g) Magan and Lacey, 1984; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari 1983.

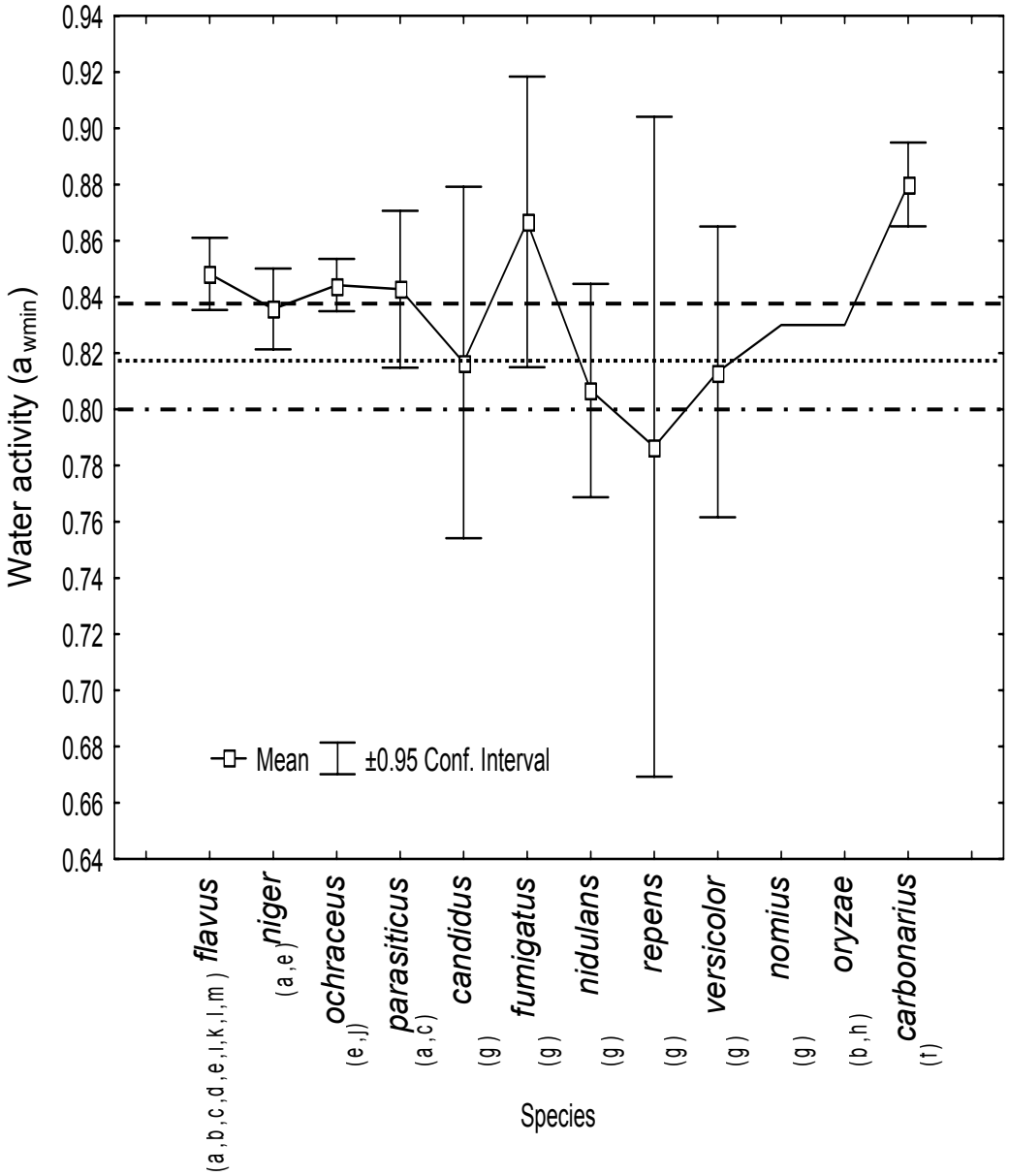

Figure 3.10. Minimal water activity (a_{wmin}) confidence intervals vs. reported data on *Aspergillus* and related species and forecasted a_{wopt} at 25(—), 30(···) and 35°C (— · —) (Eq. 4) for *A. niger* L11 and B1.

Key to references: (a) Ayerst, 1969; (b) Gibson *et al*., 1994; (c) Holmquist *et al*., 1983; (d) Horner and Anagnostopoulos, 1973; (e) Marin *et al*., 1998; (f) Mitchell *et al*.,2003; (g) Magan and Lacey, 1984; (h) Northolt and Bullerman, 1982; (i) Pitt and Hocking, 1977; (j) Ramos *et al*., 1998; (k) Sautour *et al*.,2001; (l) Wheeler *et al*., 1988; (m) Wiesner & Casolari, 1983.

Table 3.3. Comparison of published growth rates (μm h^{-1}) of *Aspergillus niger* B1 and L11 studied with published growth rates at various a_w's

a_w	Predicted radial growth rates								Published radial growth rates[a]
	20°C		**25°C**		**30°C**		**35°C**		
	B1	L11	B1	L11	B1	L11	B1	L11	
0.99	158	177	167	187	179	210	194	273	120-260
0.98	256	264	271	278	290	313	315	407	130-500
0.95	261	270	276	284	296	319	321	415	115-430
0.9	60	81	63	85	68	96	74	124	20-220
0.87	17	29	18	30	19	34	21	44	5-110

[a] data from several publications summarized in ICMSF (1993)

In the present work the model has been investigated as a tool for the interpretation of fungal growth rate data. Within the experimental boundary limits of temperature and a_w the model produced is able to predict accurately the colony radial growth rates (mm day^{-1}) and at the margins of the model it produces good forecast values. This type of model will assist in predicting conditions over which growth in food matrices may be a problem and also may assist in developing prevention strategies in food production processes. However, it also has applications as a bioprocess tool to assess the a_w and temperature conditions to achieve a specific growth rate according to the operational parameters in biotechnological applications. Potential exists to extend the model application to wider range of different humectants with different properties such as ionic solutes. Although data regarding the effect of a_w on mould growth is available, comparisons of results between investigators is often difficult because of differences in methodology or isolates used. Standardization of methodology across combinations of factors affecting growth can help to produce models to describe growth under any combination of conditions within the range tested.

CHAPTER IV

EFFECT OF WATER ACTIVITY, INOCULUM SIZE AND AUTOCLAVING PROCEDURE ON LYSOZYME PRODUCITION BY TWO GENETICALLY ENGINEERED *Aspergillus niger STRAINS*

Submitted to Biotechnology Progress

4.1 INTRODUCTION

Filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* are able to produce and secrete large concentrations of enzymes (e.g. amylases, proteases, cellulases), metabolites and organic acids into the environment. This property has been widely exploited by the food and beverage industries (Conesa *et al*., 2001). Increasingly, these species are being used to produce recombinant proteins. Filamentous fungi offer advantages for the production of recombinant proteins: they possess an efficient secretion system, are able to glycosylate proteins and have higher specific growth rates than plant, insect or mammalian cells.

The commercial use of the fungal secretory machinery and the limitations encountered in the production of heterologous proteins has stimulated research into genetics of the protein secretion in filamentous fungi, design of novel fermentation media, and optimization of the environmental conditions to enhance the heterologous protein production. Growth of filamentous fungi has been shown to be dependent on thermodynamic factors such as water availability and temperature (Scott, 1957; Gervais *et al*., 1988) and chemical factors such as glucose, O_2, and CO_2, concentrations (Trinci, 1969; Trinci and Collinge, 1973). The most important environmental stress parameter that influences fungal activity and secondary metabolite production is water availability (water activity, a_w). It has been demonstrated that significant improvements in pharmaceutical metabolite production can occur when a_w stress is imposed during fungal growth (Baxter *et al*., 1998; Aldred *et al*., 1999).

Sterilization of fermentation media can result in vital nutrients (e.g. vitamins, amino acids, and sugars) being destroyed, while the hydrolysis of complex substances (e.g. proteins and polysaccharides) may be enhanced. The formation of insoluble compounds may eliminate the availability of a nutrient, while inhibitory compounds resulting from the interaction of medium components may result in a medium which is not conductive to growth or to product formation (Anderson *et al*., 1986; Wang *et al*., 1979). Sterilization conditions have been shown previously to affect fermentation performance. For example, efrotomycin production by Nocardia lactamdurans was

greatly improved by sterilizing glucose together with the rest of the medium components (Jain and Buckland, 1988). Zaragozic acid production by *Leptodontidium elatius* was improved under different autoclaving conditions (Connors *et al*., 1995).
Proteolysis has been recognised as a major problem associated with recombinant protein production, not only in fungal, but also in bacterial populations (Enfors, 1992; Verdoes *et al*., 1995). Several strategies have been employed for reducing proteolysis of recombinant proteins. The primary strategy has been to use a protease deficient host strain to generate the transformant (van den Hombergh *et al*., 1995; Zheng *et al*., 1998).

In addition to the use of a protease deficient host, or when no such host is available environmental and morphological parameters can be manipulated to reduce proteolysis. The pattern of expression of proteases in *Aspergillus niger* can be modified by changes in the inoculum size (Papagianni and Moo-Young, 2002). Inoculum quality, in terms of size, type or age, is of prime importance in determining the outcome of filamentous fermentations (van Suijdam *et al*., 1980; Gencheva and Dimova, 1984; Vecht-Lifshitz *et al*., 1990 and Brückner and Blechschmidt, 1991).

The objectives of the present work were to evaluate the effect of (a) water availability, (b) inoculum size and (c) sterilization procedure on the production of the heterologous protein lysozyme by two genetically engineered *Aspergillus niger* strains (L11 and B1).

4.2 MATERIALS AND METHODS

4.2.1 Fungal Strains

See section 2.2.1

4.2.2 Activation and Strain Inoculation

Thawed 500 µl cryogenic stock spore suspension vials (Nalgene) ($1.8x10^7$ spores/ml ± 2%) of *Aspergillus niger* L11 and B1 were used. A 5 µl of the spore suspension was inoculated on glycerol-modified MEA ($0.97a_w$) Petri plates and incubated at 35^oC. a Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland) was used to check the a_w levels and found to be within 0.005 of the desired a_w level. Actively growing 8-day-

old cultures were harvested to produce the spore inoculum by pouring 10 ml of sterile Tween 80 (100 µl l^{-1}) solution onto the glycerol-modified MEA (0.97a_w) growing cultures and dislodged using a sterile microbiological loop. The extracted spores of each of the two strains were used to inoculate the liquid media modified with glycerol (0.98, 0.97 and 0.96a_w) with three spore concentration treatments ($2.7x10^5$, $2.7x10^4$, and $2.7x10^3$ spores ml^{-1}) using 3 autoclaving procedures for the fermentation medium: treatment A (all components autoclaved together); treatment B (medium autoclaved + maltose filtered); treatment C (medium autoclaved + maltose & soya milk filtered). The cultures (130 ml in 250-ml conical flasks) were incubated at 37 ºC on a shaker at 150 rev min^{-1} for 25 days.

4.2.3 Media Preparation and Incubation

A standard lysozyme production medium was prepared containing 150 g maltose (Sigma), 50 g tri-Na^+-citrate (Fisher Chemicals), 15 g $(NH_4)_2SO_4$ (BDH), 1 g $NaH_2PO_4 \cdot 7H_2O$ (BDH), 1g uridine (Sigma), 70 ml soya milk (Local supermarket, Tesco), 1 ml tween 80 (Sigma). This medium is a modification of the soya complex medium (SCM) by MacKenzie *et al*. (1994). The soya milk contained 3.6 % (w/w) protein, 0.6 % (w/w) carbohydrates, 2.1 % (w/w) fat. The soya milk and the maltose (aqueous solution) were filtered through sterile membranes (0.2 µm). The mixing of the soya milk and maltose with the autoclaved medium was performed under aseptic conditions. The a_w of this medium without modification was 0.98. The a_w of the fermentation medium was modified to 0.97 and 0.96 a_w with 0.44 and 0.79 molar glycerol.

4.2.4 Lysozyme Analysis

Lysozyme activity in culture was determined by measuring the decrease in absorbance at 600 nm with a double beam spectrophotometer (CAMPSPECT M350). The kinetics of lysis of *Micrococcus lysodeikticus* ATCC No. 4698 (Sigma) 0.4 mg ml^{-1} cell in phosphate buffer was carried out according to the method developed by Archer *et al*. (1990b).

4.2.5 Experimental Design and Data Treatment

The environmental conditions and inoculation levels were selected as being relevant and applicable to the lysozyme production. The combinations of environmental conditions used were selected using the experimental design module of Statistica 6.0 (SoftStat, Inc. 1984-2001, USA). The experimental design was 2^3 cube plus star central composite with three factors at three values: Aw, 0.98, 0.97 and, 0.96; spore concentration, $2.7x10^5$, $2.7x10^4$, and $2.7x10^3$ spores ml^{-1}; autoclaving procedure, treatment A, B and C as defined previously. The experiment was blocked by strains L11 and B1.

For the analysis, factors were given coded values between -1 (lowest value of environmental parameter) and +1 (highest value). Coded values for intermediate conditions are calculated as (actual value-factor mean)/(range of the factorial value/2). Thus, for the inoculum of spores, where the logarithm of the values were 7.9 ($2.7x10^3$), 1.02 ($2.7x10^4$) and 1.25 ($2.7x10^5$ spores ml^{-1}), the coded values are -1, 0, +1. A similar operation was done for the a_w values of 0.96, 0.97 and 0.98. The autoclaving procedure was set as (+1) A, (0) B, and (-1) C.

4.3 RESULTS

Table 4.1. summarises the amounts of protein actually produced and predicted for both *A. niger* strains L11 and B1 under the experimental design conditions at 37°C after 16.5 days. This time was selected based on economical and productivity balances. This shows the autoclaving procedure treatments A and B reached higher levels of lysozyme production than C. Strain B1 produced comparatively more lysozyme than L11 in all the cases in the treatments A and B under the same experimental conditions. However, L11 produced more lysozyme than B1 using nutritional treatment procedure B at 0.97 a_w. The best treatments for B1 were 1(15.2 mg l^{-1}), 3 (14.7 mg l^{-1}) and 9 (14.7 mg l^{-1}, B1), while for L11 this was 11 (14.7 mg l^{-1}). While strain B1 produced the highest concentration of lysozyme in the medium with all the components autoclaved, the maximum production of L11 was

obtained with autoclaving procedure B. Both strains produced the least lysozyme when autoclaving procedure C was used.

Figure 4.1 shows the kinetics of the best treatments for strain B1. The physical appearance of the fermentation medium was inevitably affected by the way the components of the medium were autoclaved. Different autoclaving procedures also resulted in different kinetics of protein production (data not presented). The production kinetics started with a lag phase (2-4 days), then a first cycle with a linear production and a stationary phase. The cycle was repeated, then the activity of the enzyme decreased. In the first cycle the production of lysozyme reached concentrations just above 10.0 mg l^{-1} at day 6. However, this concentration was only 60% of the concentration achieved at day 16.5. The medium prepared with autoclaving treatment A resulted in a very rapid and high production medium with a very dark brown colour.

These results show the importance of autoclaving all the components together. Although similar results could be obtained at day 20.5 for all the treatments, increasing the fermentation time is often not a practical consideration. Taking into account the cost-return relationship (minimal time-highest concentration), day 16.5 was the most efficient time for the harvesting of the protein.

4.3.1 Effect of Environmental Conditions on the Production of Lysozyme

The production of lysozyme from the two strains was very different depending on the autoclaving treatment of the medium and hence the two sets of data were separated for the statistical analysis, and two different models for B1 and L11 were produced using the experimental design. A second order (quadratic) polynomial equation fitted the experimental data for lysozyme production by *A. niger* B1 with a multiple correlation coefficient (R^2) of 0.997 (final loss: 0.01, variance explained: 99.5 %):

Table 4.1. Experimental design, observed and predicted values of lysozyme production by *Aspergillus niger* L11 and B1 under different environmental conditions

Treatment No.	Strain *A. niger*	Water activity (a_w)	Autoclaving procedure	Spore concentration (spores ml^{-1})	Observed (mg l^{-1})	Predicted (mg l^{-1})
1	B1	0.96	A	2.70E+03	15.2	14.9
2	B1	0.97	A	2.70E+04	11.8	12.3
3	B1	0.98	A	2.70E+05	14.7	14.4
4	L11	0.96	A	2.70E+03	5.9	05.9
5	L11	0.97	A	2.70E+04	7.8	7.8
6	L11	0.98	A	2.70E+05	14.2	14.2
7	B1	0.96	B	2.70E+03	10.7	11.0
8	B1	0.97	B	2.70E+04	11.3	10.7
9	B1	0.98	B	2.70E+05	14.7	15.0
10	L11	0.96	B	2.70E+03	9.8	10.3
11	L11	0.97	B	2.70E+04	14.7	13.6
12	L11	0.98	B	2.70E+05	12.7	13.3
13	B1	0.96	C	2.70E+05	11.3	11.3
14	B1	0.97	C	2.70E+04	3.9	3.9
15	B1	0.98	C	2.70E+03	1.0	1.0
16	L11	0.96	C	2.70E+05	6.0	5.8
17	L11	0.97	C	2.70E+04	4.0	6.0
18	L11	0.98	C	2.70E+03	2.9	2.4

A = all components autoclaved together

B = medium autoclaved + maltose filtrated

C = medium autoclaved + maltose & soya milk filtrated

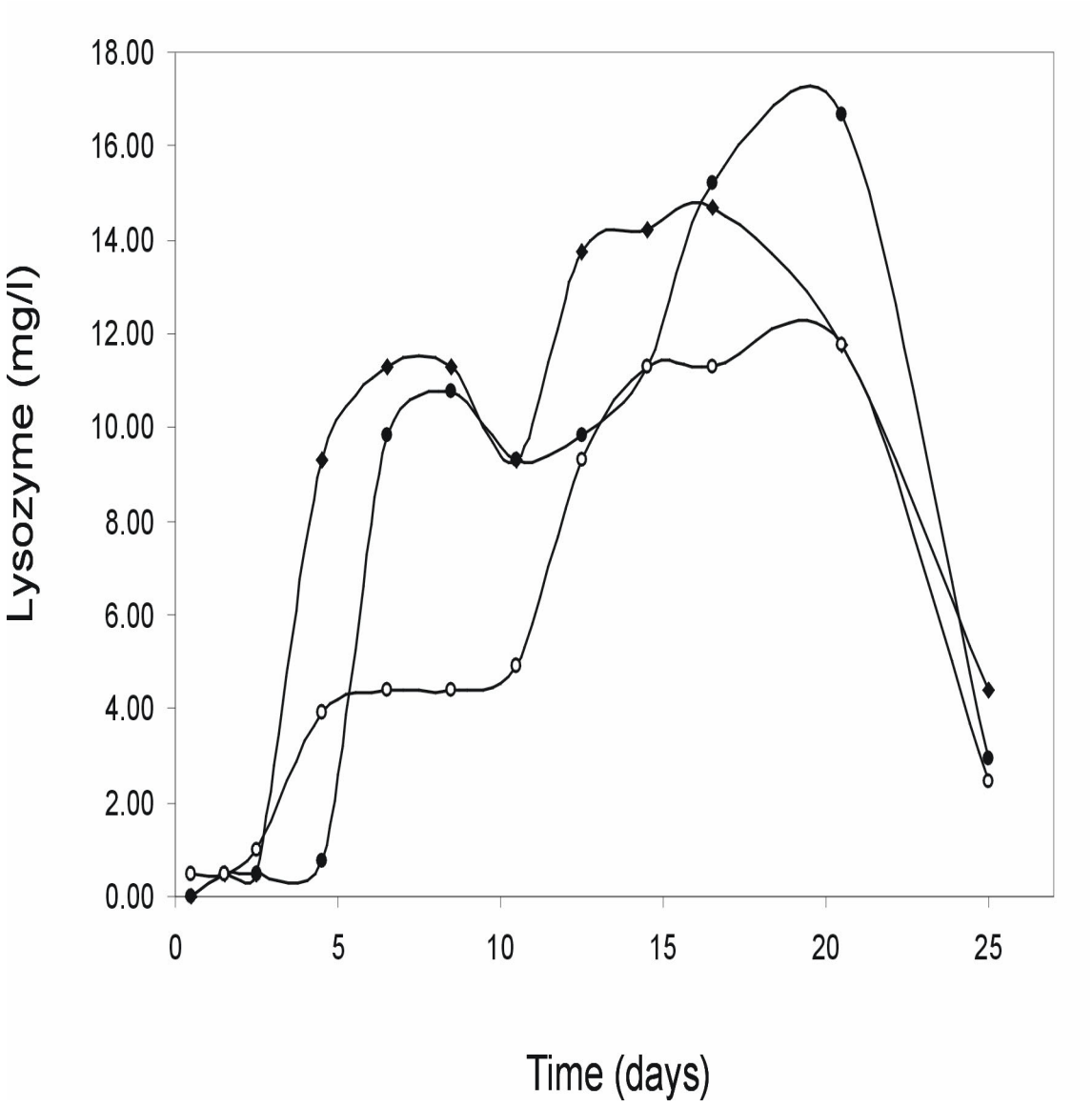

Figure 4.1. Kinetic of best treatments for lysozyme production by *A. niger* B1 on autoclaving procedure A treatment no. 1 (♦), and 3 (●) and procedure B treatment no. 9 (○)

$$Lysozyme\left(\frac{mg}{l}\right) = 10.7 - 2.7\,X_{a_w} + 1.1\left(X_{a_w}\right)^2 + 4.7X_{Inoculum} + 1.1\left(X_{inoculum}\right)^2 + 4.2X_{Auticlaving} - 2.6\left(X_{Auticlaving}\right)^2 + 0.055X_{a_w}X_{\text{Inoculum}} - 2.2X_{a_w}X_{Autoclaving}$$

(Eq. 4.1)

The polynomial equation fitted the experimental data for *A. niger* L11 with a multiple correlation coefficient (R^2) of 0.988 (final loss: 0.03, variance explained: 98.0%)

$$\text{Lysozyme}\left(\frac{\text{mg}}{\text{l}}\right) = 13.6 - 1.2\,X_{a_w}\;0.1\left(X_{a_w}\right)^2 + 0.25X_{\text{Inoculum}} + 0.1\left(X_{\text{inoculum}}\right)^2 + 0.093X_{\text{Auticlaving}}\;7.0\left(X_{\text{Auticlaving}}\right)^2 - 2.0X_{a_w}X_{\text{Inoculum}} + 3.2X_{a_w}X_{\text{Autoclaving}}$$

(Eq. 4.2)

Were X is the coded value (between -1 and +1) for the factor indicated by the attached subscript. The coefficients in the equation were significant $X_{Autoclaving}$ (P = 0.01) and the interaction $X_{Autoclaving}\,X_{aw}$ (P = 0.04) in the equation.

The relative influence of individual factors on the production of lysozyme (with other factors at their midpoints: 0.96a_w, 2.7x10^4 spores/ml, and with medium autoclaving treatment B) are shown in the perturbation plot for the two strains, B1 and L11 (Figure 4.2). A steep slope or curvature shows that the lysozyme production is sensitive to this factor. It is evident that the lysozyme production shows different patterns for each of the strains. B1 is more sensitive to changes of a_w, inoculum size and autoclaving procedure than L11. The autoclaving procedure shows the steepest slopes in both cases. At lower water activity (0.96 a_w) higher lysozyme production is expected than at higher water activity for B1. L11 was not sensitive to changes of a_w and inoculum size. In all cases the media with the soya milk and maltose autoclaved separately produced low concentrations of lysozyme.

4.3.2 Interactions Between Water Activity, Inoculum Size and Autoclaving Procedure

Higher concentrations of lysozyme were achieved with B1. The interactions of water activity and inoculum size under different procedures of autoclaving are presented in Figure 4.3. The highest production of lysozyme was obtained under high inoculum concentrations (2.7 x 10^5 spores ml^{-1}) and low a_w conditions (0.96) with autoclaving procedure A (Figure 4.3 (a)). The production was halved under the same conditions but using autoclaving procedure C (Figure 4.3 (c)).

The lysozyme produced can be ranked by autoclaving procedure from the highest to the lowest production as follows: autoclaving procedure A > autoclaving procedure B >autoclaving procedure C. An optimum of lysozyme production was found when combining autoclaving procedure A, lower water activities (0.96) and higher levels of initial inoculum (2.0x10^5 spores ml^{-1}) with the strain *A. niger* B1.

4.4 DISCUSSION

Statistical modelling for optimising parameters showed that for strain *A. niger* B1 the best conditions were: high water stress (0.96 a_w), high inoculum size (2.0 x 10^5 spores ml^{-1}) and autoclaving all the components of the medium together (autoclaving procedure A). Overall, the fermentation performance for the production of the heterologous protein lysozyme was significantly affected by the changes in autoclaving procedure used for medium sterilization, water activity and inoculum levels. These have all been identified as important parameters to maximise the production of lysozyme by *A. niger*. The results obtained in this study showed that the reduction of the water activity in the external environment affected the protein secretion by the fungal cells. Although similar concentrations achieved by both strains, the selection of B1 as the most suitable strain to be used in future work due to the process advantage of this strain to produce relatively higher level of lysozyme production (15.2 mg l^{-1} in comparison with L11 14.7 mg l^{-1}) on a medium with all the components autoclaved together.

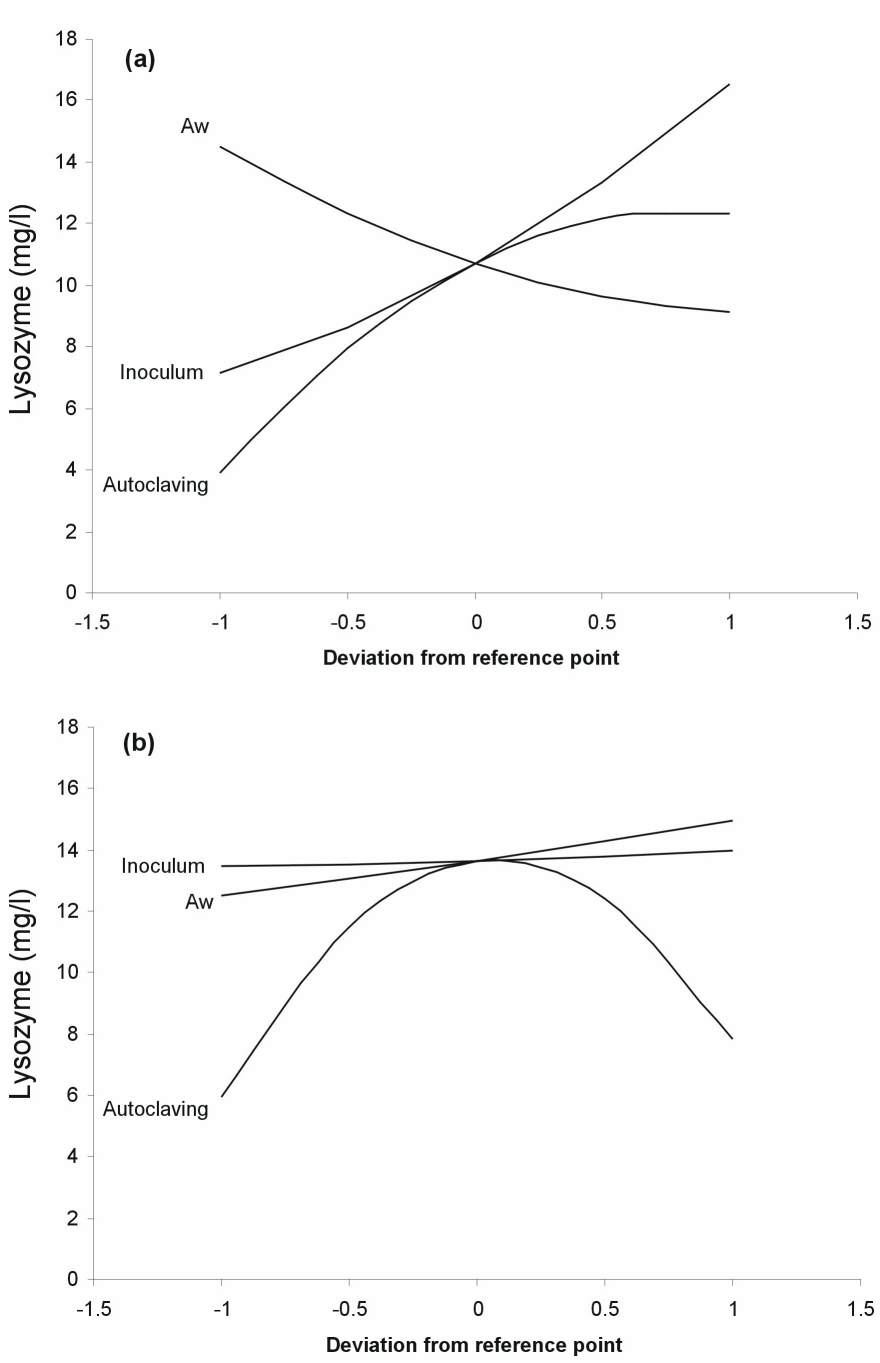

Figure 4.2. Perturbation plot showing the predicted effect of water activity, inoculum size and the autoclaving procedure on the production of lysozyme by *A. niger* B1 (a) and L11 (b) when other factors are at their midpoints ($0.97a_w$, $2.7x10^4$ spores ml^{-1} and maltose autoclaved apart). Water activity (-1.0=0.96, +1.0=0.98), inoculum size (-1.0=2.7 x 10^3, +1.0=2.7 x 10^5 spores ml^{-1}), autoclaving procedure (-1.0= autoclaving procedure C, +1.0= autoclaving procedure A)

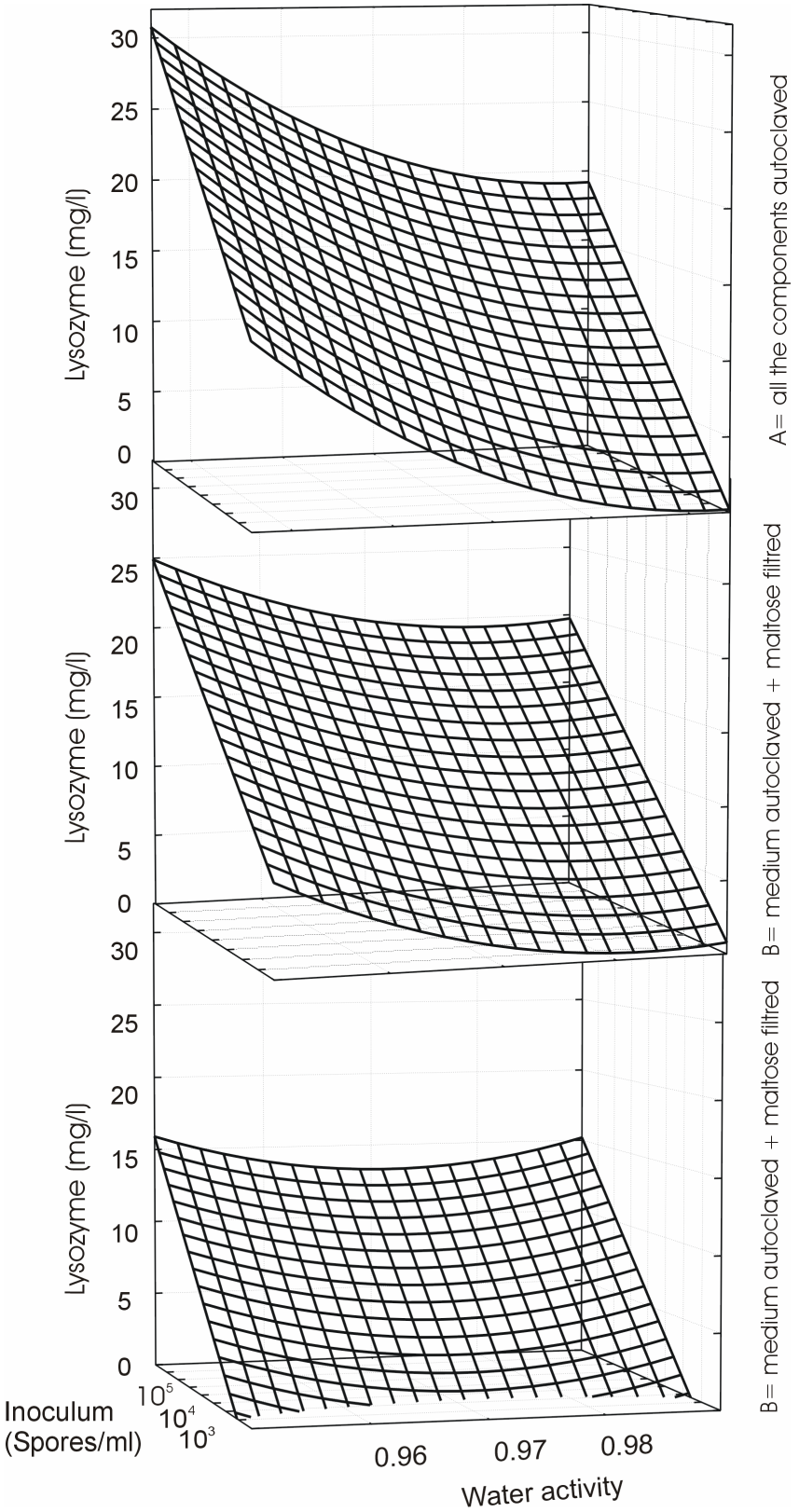

Figure. 4.3 Response surface showing predicted effect of water activity and the inoculum size on lysozyme production by *A. niger* B1 under different autoclaving procedure: A (a), B (b) and, C (c)

This represents a considerable reduction of contamination risk due to handling operations in a bigger scale. The choosing of B1 supports previous studies with the same strains (Archer *et al*., 1990b). MacKenzie *et al*. (1994) reported concentrations of 3-5 mg l^{-1} with L11 in the range of 30-37^{o}C. Levels up to 12 mg l^{-1} were reported with optimum levels of 10^6 spores ml^{-1} (Archer *et al*., 1990a). Moreover 9.3 mg l^{-1} of lysozyme was reported at pH 4.5 with B1 (Mainwaring *et al*., 1999). Occasionally, significantly higher concentrations of up to 30-60 mg l^{-1} were reported in a medium containing soya milk (Mackenzie *et al*., 1994). The comparatively low heterologous protein production achieved in this work is possibly due to the heterokaryosis of the recombinant DNA (Mainwaring *et al*., 1999; Swift *et al*., 2000), excised recombinant DNA from the genome (Mainwaring *et al*., 1999), rearrangements within the genome (Wiebe *et al*.,2003) or proteolysis (Enfors, 1992; Verdoes *et al*., 1995).

There are reports in the literature of the influence of osmolarity on the active transport of *Eucaryonta* (Roy and Sauve, 1987; Sanchez *et al*., 1991; Wills *et al*., 1991). Unfortunately, no data were found concerning the ion transport in cells of filamentous fungi in response to osmotic stimulation. Bobowicz-Lassociska and Grajek (1995) found that the water activity reduction considerably influenced the protein secretion resulting in a 60% increase over the non-modified medium.

The physicochemical and chemical properties of the medium were affected by the heat input, and the interactions between substrates (autoclaving procedure) at high temperatures. This resulted in an increase in lysozyme production when all the components of the medium were autoclaved together. Sterilization conditions have been shown previously to affect fermentation performance. Efrotomycin production by *Nocardia lactamdurans* was greatly improved by sterilizing glucose together with the rest of the medium components (Jain and Buckland, 1988). Moreover zaragozic acid production by *Leptodontidum elatius* increased by 26% due to the heat input during autoclaving (Connors *et al*., 1995). Possibly the high-heat input of all the components autoclaved results in a greater flux of carbon into acetate and succinate or there is a molecular uptake directly from the medium to the citric acid cycle (Connors *et al*., 1995). Similarly, an increase in the hydrolysis of protein substrates (as a result of heat input) could result in an increased supply of amino acids from the medium made available for lysozyme synthesis.

It should be noted that the medium formulation employed for the production of lysozyme contains maltose and during the sterilization will result in non-enzymatic browning Millard-type reactions between maltose and the free amino acids together with the conversion of maltose to glucose. The glucose can pass through the cell membrane by free diffusion but maltose requires active transport. This could become a limiting step in the lysozyme production in two ways: reduction of carbon as a structural molecule and as an energy source. Dominguez *et al*. (2000) reported that the media composition seems to influence morphogenesis and cellulose formation by *T. reesei*. The composition of the fermentation medium had a significant effect on morphology. Moreover the composition of the medium resulted critical for growth morphology and lovastatin production by *Aspergillus terreus* in submerged cultures (Long-Shan *et al*., 2003).

The methodology employed in the present study of using contour surface plotting has shown a useful method for the determination of relevant variables for process optimization. This made it possible to simulate conditions isolating the effects of marginal, factors avoiding increasing the number of runs and enhancing the information uptake. This information is essential in the optimization process. The regressed polynomials had a high regressed coefficient (R^2=0.997 and R^2=0.988) for the production of heterologous protein by B1 and L11. The use of the perturbation graph for clarifying the effects is a novel tool in the production of heterologous protein and can be useful in further optimization.

The statistical model generated by the contour surfaces is a very useful technique for forecasting and interpreting of the effect of different factors. The plots provide evidence for real differences between the strains. The autoclaving procedure may have generated a toxic or inhibitory environment for lysozyme production by L11. However, the results suggest a simple medium preparation system for B1 suitable for scale up. Under different a_w conditions the performance of the strains was also different. The effect on production of lysozyme by L11 was not significant. However, for B1 there were significant differences under the different a_w levels tested.

Previous studies have examined the effect of inoculum size alone, but not in relation to water availability or interaction with nutritional conditions. Archer *et al*. (1990a) used inocula of between 10^3-10^7 spores ml^{-1} and found optimum at 10^6. However, for commercial exploitation lower concentrations are preferred. The present study suggests that even at lower concentrations there is a significant effect of inoculum size on lysozyme production by these strains. The model predicted that, 2.0 x 10^5 spores ml^{-1} was needed to produce the maximum concentration of lysozyme. This may be due to different morphological states achieved by the different levels of inoculum (van Suijdam *et al*., 1980; Vecht-Lifshitz *et al*., 1990; Trucker and Tomas, 1992). This spore concentration for production of heterologous protein is similar to the lowest reported for extracellular protease activity by *Aspergillus niger* (Papagianni and Moo-Young, 2002). A more efficient fermentation systems with on-line control of pH and aeration coupled with novel culture techniques such as immobilization of the mycelium could provide alternative approaches to improve the production levels of heterologous proteins such as lysozyme.

CHAPTER V

A NOVEL IMMOBILIZED DESIGN FOR THE PRODUCTION OF HETEROLOGOUS PROTEIN LYSOZYME BY GENETICALLY ENGINEERED *Aspergillus niger* STRAINS

Applied Microbiology and Biotechnology. In Press

5.1 INTRODUCTIUON

The ability to secrete glycosylated proteins makes filamentous fungi attractive hosts for recombinant protein production (Lin *et al*., 1993). They have been widely used for the production of heterologous as well as homologous proteins. However the levels of production of heterologous proteins do not exceed a few tens of milligrams per litre (Gouka *et al*., 1997). Filamentous fungi are able to utilize a great variety of carbon and nitrogen sources by secreting a range of different enzymes into their environment. Unfortunately, degradation of heterologous proteins by extracellular proteases secreted by fungi into the culture media has been a major problem for the efficient production of recombinant proteins (Archer *et al*., 1992; Enfors, 1992).

Attempts to improve the concentration of heterologous proteins in fungal fermentations, include strategies such as gene fusion (Gouka *et al*., 1996), the use of protease deficient mutants (van den Hombergh *et al*., 1995), and environmental and morphological parameters which can be manipulated to reduce proteolysis. Papagianni and Moo-Young, (2002) have reported that a change of protease expression in *Aspergillus niger* can be achieved by changing the inoculum size.

A close relationship between a particular morphology and increased process productivities is characteristic of a number of industrially important fermentations (Papagianni, 1999; Calam, 1987).

Fermentative production of metabolites can be obtained in both solid state and submerged cultivation. While solid state produces higher metabolite concentrations released into the medium (Lin 1973; Lee *et al*., 1995), solid substrate fermentation also has several disadvantages: low mass transfer and often no control of the operation parameters such as pH, temperature and water activity. Immobilization of microbial cells gives the advantages of using solid substrate support in a submerged culture fermentation framework. This approach has received increasing interest in recent years (Wang *et al*., 1997).

The immobilization of microbial cells in different carriers leads to changes in their microenvironment (Shreve and Vogel 1993). Because of these changes, immobilized

cells show various modifications in physiological and biochemical composition when compared to suspended cells (Hilg-Rotmann and Rehm 1990; Adam *et al*.,2001). The use of polysaccharides has become one of the most important methods for fungal immobilization. This technique can be carried out in a single step process under very mild conditions and is therefore compatible with most viable cells. Polysaccharide matrices generated in gel beads are strongly dependent on several variables such as polymer type, concentration and ionic strength (Skjårk-Bræk *et al*., 1989; Nava-Saucedo *et al*., 1994; Gmeiner *et al*., 1996). Such modifications may also result in modifications of the water activity in the beads and influence rates of colonization and subsequent production of enzymes or secondary metabolites. This could have important implications in studies of xerotolerant or xerophilic microorganisms where this may have an impact on concentration.

Traditional methods of optimization involve changing one independent variable at a time while keeping the others fixed at a certain level. This single-dimensional search is laborious, time consuming, costly and incapable of reaching the optimum due to the interactions among variables. Response surface methodology is an important strategy for seeking the optimal conditions for multivariable systems. It has been successfully employed for optimizing medium ingredients and operating conditions in many bioprocesses (Kemp *et al*., 1989; Roseiro *et al*., 1992; Prapulla *et al*., 1992; Lee *et al*., 1997).

In order to carry out the optimization process various mathematical tools can be used. The traditional method is the use of multiple regression of the partial or full factorial experiments to come up with a model of the system. This involves fitting the data to a complex polynomial. This polynomial can be made simpler by ignoring non-significant effects using stepwise multiple regression. Response surface methodology can then be used to evaluate the optimal region (Prapulla *et al*., 1992).

The objective of the present study was to optimize, by response surfaces methodology, the physiological and immobilization conditions to enhance the production of the heterologous protein lysozyme, by a genetically engineered *A. niger* B1 strain. The factors examined in this study were (a) polymer type and concentration, (b) gel-inducer ($CaCl_2$) concentration and (c) inoculum levels.

5.2 MATERIALS AND METHODS

5.3 Fungal strains

The culture of *A. niger* B1 was maintained as described in section 4.2.1

5.4 Activation and inoculation

Thawed 500 µl cryogenic stock spore suspension vial (Nalgene) ($1.8x10^7$ spores ml^{-1} ± 2%), was used to reactivate the strain. A 5 µl of the spore suspension was inoculated on MEA modified by glycerol to 0.97 a_w and incubated at 35°C. The water activity (a_w) values of the media were determined by using a Humidat-IC II water-activity measuring apparatus (Novasina, Talstrasse, Switzerland) at 25°C. The water activity was found to be within ± 0.005 of a_w treatment values. An actively growing 8-day-old culture of *Aspergillus niger* B1 was used to produce the spore inoculum. The fungal spore inocula were prepared by flooding 10 ml glycerol-modified MEA with 10ml of sterile water + 0.1% of Tween 80. Each flask (250 ml) containing 100 ml of the seeding medium consisted of 4.0 % malt extract modified with glycerol to 0.96 a_w. Seeding medium was inoculated with 1 ml of a spore suspension (9.0×10^6 spores ml^{-1}). The cultures were placed in an orbital shaker incubator (L.H Engineering Co. Ltd, England) set to 150 rpm at 25 ºC for 5 days.

5.5 Immobilization

Growing mycelia of *Aspergillus niger* were used for immobilization. In aseptic conditions the seeding fungus were immobilized in Ca-pectate and Ca-alginate using a modified version of the method used by Escamilla *et al*. (2000). The mycelial pellets were homogenised in a blender (50 seconds) and filtered through a sinterglass funnel to remove all the liquid. The biomass was recovered on Whatman filter paper (No. 2). The inoculum was adjusted in a volumetric cylinder to either 1:2 (33 % v/v) or 1:4 (20 % v/v) inoculum:polymer ratio by using either polygalacturonic or alginic acid at two levels; 2 and 4% (w/v). The mixture was forced through a multi-needle template (18 gauge for 5 mm beads) with a peristaltic pump (Watson Marlon) flowing at 10 ml min^{-1} and the droplets were collected in sterile gel-inducer solutions of 2 %(w/v) $CaCl_2$ or 3.5 % (w/v) $CaCl_2$. After soaking for 3 h the liquid was decanted and the spherical beads were washed with sterile RO water and stored at 4ºC for 24 hours.

The water activity (a_w) of the decanted beads were evaluated with a Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland).

5.6 Media preparation and incubation

See section 4.2.3

5.7 Lysozyme analysis

See section 4.2.4

5.8 Experimental design and data treatment

The immobilized cell fermentation design conditions and inoculation levels were selected as being relevant and applicable in the lysozyme production. The combinations of environmental conditions were selected using an experimental design generated with Statistica 6.0 (SoftStat, Inc. 1984-2001, USA). The experimental design was based on a central composition with four factors at two levels each: polymer type (alginate, pectate); concentration of polymer (2% and 4% w/v); and inoculum-polymer ratio 1:2 (33% v/v), 1:4 (20% v/v); and concentration of gel-inducing agent $CaCl_2$ (2.0 % and 3.5 % w/v) (Table 5.1). The effect of pH in the medium and of water activity in the beads was evaluated by blocking the polymer type and perform a polynomial regression and response surfaces were generated.
For the analysis, each factor was given a code value between -1 (lowest value) and +1 (highest value). The central point (CP), for intermediate conditions was calculated as follows:

$$CP = \frac{[A - M]}{\left[\frac{RF}{2}\right]} \qquad \text{(Eq. 5.1)}$$

Where A = actual value, M = factor mean, RF = range of the factor value. The initial inoculum levels were 1:2 (33 % v/v) and 1:4 (20 % v/v), and the coded values were -1 and +1 respectively. Similarly, the concentrations for polymer were set at 2 % and 4 % w/v and for gel-inducer agent, at 2 % and 3.5 % w/v. The coded values for polymer type were set as follows: polygalacturonate +1 and alginate -1. The experiments were repeated three times and the results were statistically evaluated.

For the screening of the effect of the immobilization design and inoculation level on the production of the heterologous protein lysozyme, a response surface methodology was used. The above four factors and the fermentation time (10 days) were selected on the basis of previous experiments with *Aspergillus niger* B1 and published works with related systems (Kumar and Lonsane, 1988; Martinsen *et al*., 1989; Charlet *et al*., 2000; Escamilla *et al*., 2000; Becerra *et al*., 2001).

5.3 RESULTS

Figure 5.1 shows the kinetic of the best treatments and make a comparison with our previous work with free cell fermentations. Pectate resulted the best polymer to immobilized *Aspergillus niger* B1 for the production of lysozyme. Treatment 7 and 8 produced the maximum lysozyme concentration of 22.9 and 20.3 mg l^{-1} at day 10.

Table 5.2 shows the amounts of heterologous protein produced by *A. niger* strains B1 under the experimental design conditions at 37°C after 10 days. The importance of polymer selection and the impact on lysozyme production is demonstrated. Overall, immobilization in Ca-pectate resulted in higher amounts of lysozyme production compared to that in Ca-alginate. Maximum lysozyme production with Ca-pectate was 13-fold higher than that achieved with Ca-alginate. The highest productions of lysozyme was at a low polymer concentration (2% w/v) and a high inoculum level (33% v/v) with Ca-pectate. Regardless of the gel-inducing concentration, maximum lysozyme production was achieved when pectate (2% w/v) and a low ratio of inoculum:polymer (high inoculum level 33% v/v) was used in treatments 7 (22.8 mg l^{-1}) and 8 (20.3 mg l^{-1}).

A second order (quadratic) polynomial equation fitted the experimental data for lysozyme production by *A. niger* B1 with a multiple correlation coefficient (R^2) of 0.96 (residual: 0.045, variance explained: 95%):

$$Lysozyme\left(\frac{mg}{l}\right) = 6.2 - 5.5X_{Polymer} - 5.0X_{Polymer_{Conc.}} - 4.8X_{Inoculum} + 0.08X_{Gel-inducer}$$
$$- 0.48X_{Polymer}X_{Gel\text{-}inducer} - 0.18X_{Polymer_{Conc.}}X_{Gel-inducer} - 0.57\,X_{\mathrm{Inoculum}}X_{Gel-inducer}$$

(Eq. 5.2)

were X is the coded value (between -1 and +1) for the factor indicated by the attached subscript.

Table 5.3 presents the analysis of variance of the main factors (R^2 =0.96; R^2_{adj}=0.95). The coefficients in the linear equation for polymer type $X_{Polymer}$, polymer concentration $X_{Polymer_{Conc.}}$ and inoculum size $X_{Inoculum}$ were statistically significant (P = < 0.0000001) except for the coefficients for gel-inducer $X_{Gel\text{-}inducer}$ (P = 0.85) ($CaCl_2$). The interactions were not significant ($X_{Polymer} X_{Polymer_{Conc.}}$; P = 0.29), $X_{Polymer} X_{Inoculum}$;P = 0.68) and $X_{Polymer} X_{Gel\text{-}inducer}$;P = 0.20). However, the least significant terms were included in the equation to maintain the hierarchy in the model.

Figure 5.2 presents the parameter estimates based on their absolute importance in the production of lysozyme. Regardless of the concentration of gel-inducer, the most important factors in the immobilization which affected lysozyme production by *A. niger* B1 were: polymer type, polymer concentration and the inoculum-polymer interaction.

5.3.1 Interactions between polymer type and concentration, inoculum-polymer ratio and gel-inducer

Figure 5.3 (a) shows marginal means, and interactions between inoculum-polymer ratio and polymer concentration and the confidence intervals (0.95%). At high concentrations of polymer (4%) similar concentrations of lysozyme were obtained regardless of the inoculum-polymer ratio. However, at low polymer concentrations a more than 13 fold increase in the concentration of lysozyme was achieved at 1:2 inoculum:polymer ratio compared to the 1:4 inoculum:polymer treatment.

Figure 5.3 (b) shows the effect of inoculum-polymer ratio and polymer type. Both polymers produced comparable concentrations at low inoculation levels (20%). When a high immobilized inoculum concentration (33% v/v) was used, immobilization with pectate resulted in a 13 fold increase in the production of lysozyme.

Table 5.1. Experimental design, observed and predicted values of lysozyme production by *Aspergillus niger* B1 under different immobilization and inoculum conditions: 250 ml-shake flask with 100 ml operation volume at 25^{o}C and 150 rpm.

Treatment no.	Polymer	Concentration w/v	Inoculum v/v	$CaCl_2$ w/v
1	Alginate	4	33	3.5
2	Alginate	4	33	2.0
3	Alginate	2	25	3.5
4	Alginate	2	20	2.0
5	Pectate	4	20	3.5
6	Pectate	4	20	2.0
7	Pectate	2	33	3.5
8	Pectate	2	33	2.0

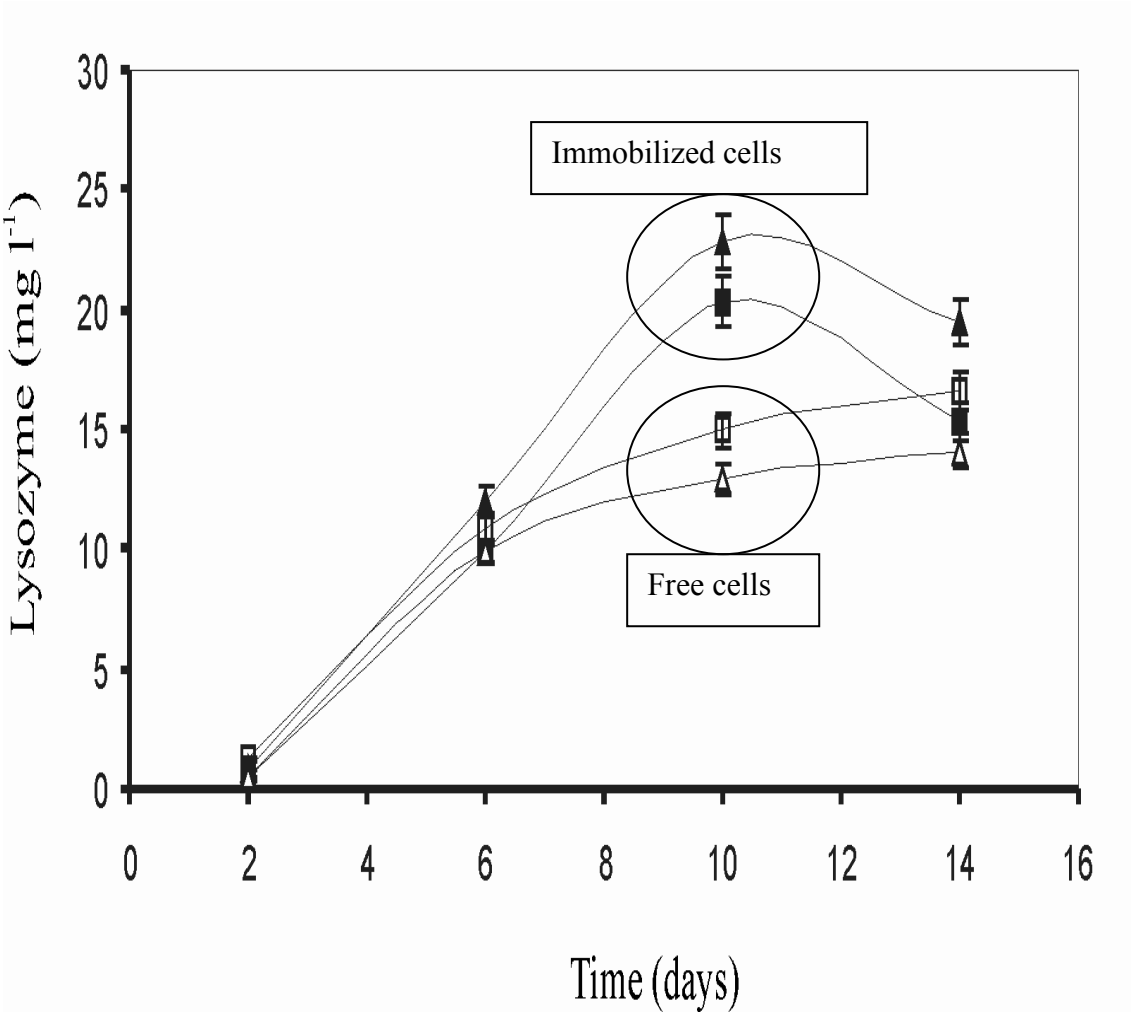

Figure 5.1. Kinetic of lysozyme production with free cell fermentations inoculated 2.7 x 10^3, 0.96 a_w (□) and 2.7 x 10^4 spores ml^{-1} 0.97 a_w (Δ) and with pectate immobilized cells 2 % w/v, 33 % v/v inoculum :support ratio, 0.94 a_w beads and gel-inducer 2.0 w/v (■)and 3.5 w/v (▲).

Table 5.2. Final pH of the fermentation medium, beads water activity, observed and predicted values of lysozyme production by *Aspergillus niger* B1 under different immobilization and inoculum conditions

Treatment no.	pH	a_w	Lysozyme production (mg l^{-1})	
			Observed	Predicted
1	6.5	0.85	0.50±0.03	0.49
2	6.6	0.85	0.50±0.00	0.49
3	7.1	0.90	0.20±0.02	0.20
4	5.3	0.90	1.7±0.10	1.7
5	4.5	0.91	1.6±0.07	1.6
6	4.8	0.91	2.0±0.03	2.0
7	4.4	0.94	22.9±0.27	22.9
8	4.3	0.94	20.3±0.18	20.3

Table 5.3. Analysis of variance of the effect of polymer type (Ca-pectate or Ca-alginate) and concentration, gel-inducer concentration and inoculum levels on the production of lysozyme

	SS	df	MS	F	p
Polymer type	7.27	1	7.23	158.14	0.00000*
Polymer concentration (w/v)	6.127	1	6.12	134.0	0.00000*
Inoculum level	5.60	1	5.60	122.0	0.00000*
Gel-inducer $CaCl_2$	0.0016	1	0.0016	0.04	0.85NS
Error	0.89	19	0.05		
Total SS	19.8	23			

* $P<0.00001$

NS = not significant

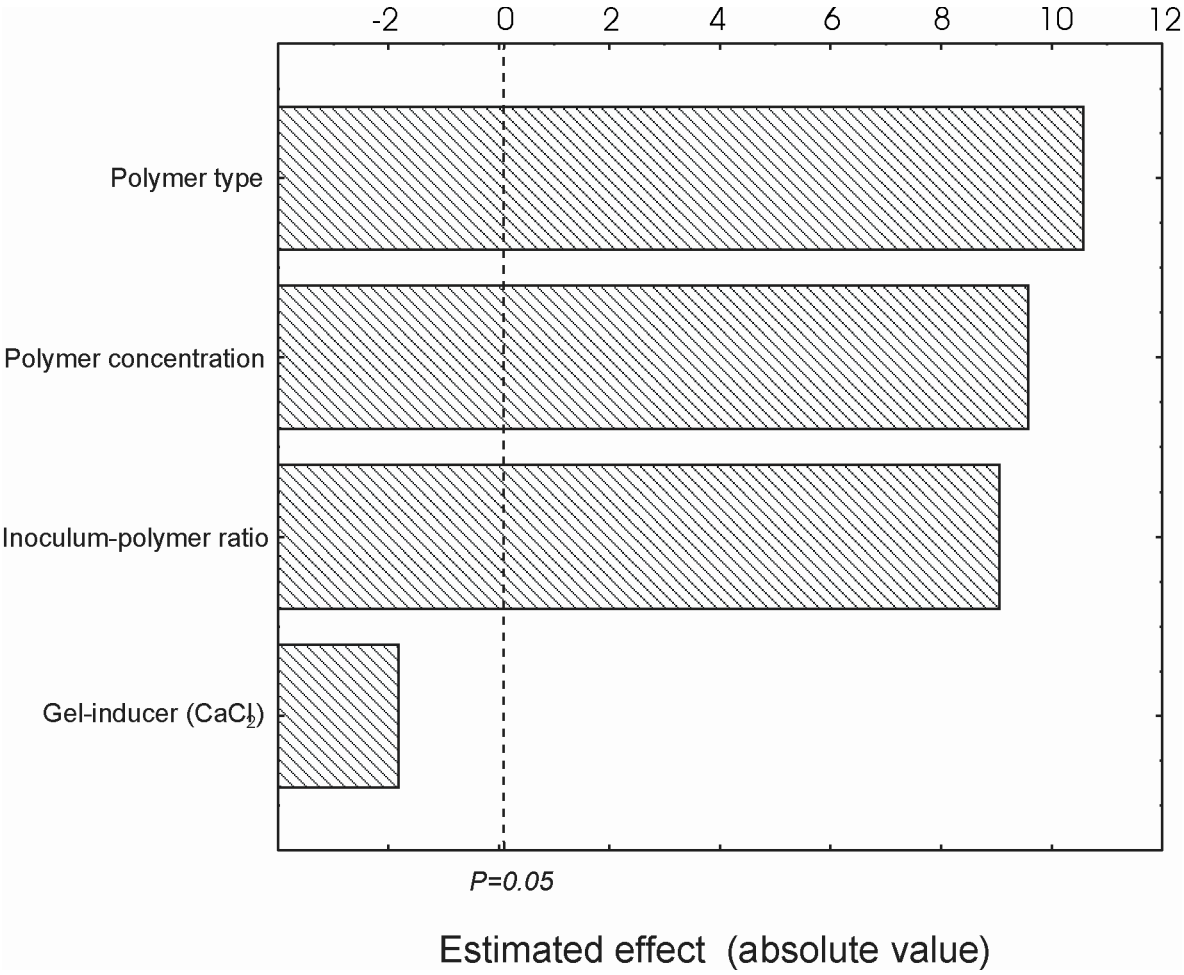

Figure 5.2. Standard Pareto distribution of polymer type and concentration, inoculum polymer ratio and gel-inducer agent ranked by importance on the production of lysozyme, dotted line (--) represents minimum of statistically significant difference.

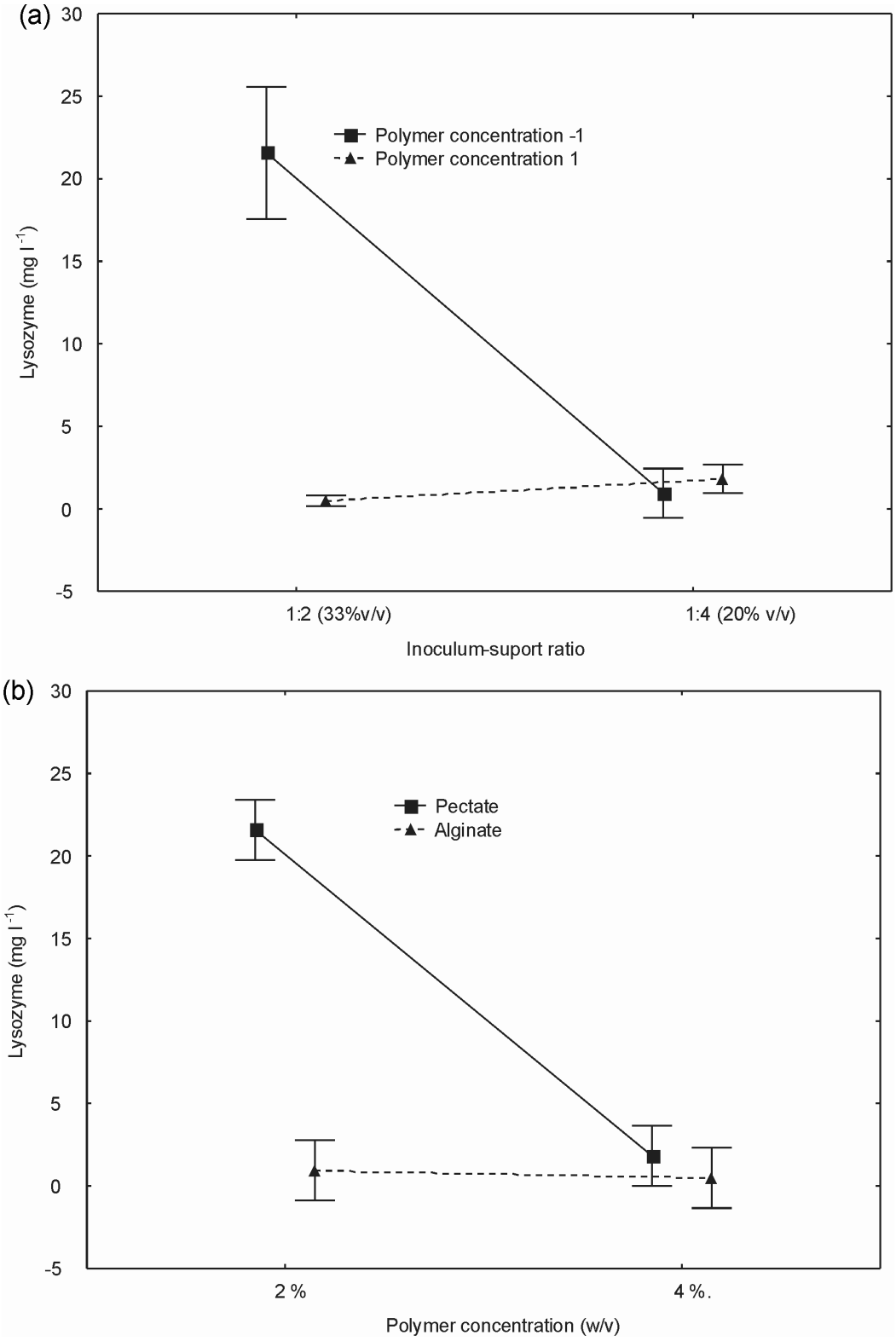

Figure 5.3. (a) Effect of inoculum:polymer ratio and polymer concentration on the production of heterologous lysozyme. (b) Effect of polymer type and polymer concentration on the production of lysozyme.

5.3.2 pH and water availability conditions in the final lysozyme concentration

It was noted that the pH and water activity in the beads were changed by the different treatments examined. Thus alginate beads with a range of pH 5.3-7.1 treatments had the lowest lysozyme production (0.2-1.7 mg l^{-1}) while pectate bead treatments had a pH range of 4.3-4.8 and produced higher lysozyme concentrations (1.6-22.9 mg l^{-1}). The pH reached by the medium at different fermentation times for all the experimental conditions is shown in Figure 5.4 (a). Interestingly, only a narrow range of pH at between 4.4 to about 5.0 is where the lysozyme was produced. A sharp decrease in lysozyme production was observed at >pH 5.

Figure 5.4 (b) shows the impact of bead water availability on lysozyme production. The immobilized cell fermentation with < 0.94 a_w produced only marginal lysozyme concentrations regardless of the polymer type. Average lysozyme produced at 0.94 a_w was 21.6 mg l^{-1} representing a 44 and 12-fold higher lysozyme concentrations compared to that at 0.85 a_w (alginate) and 0.91a_w (pectate).

A second order (quadratic) polynomial equation fitted the experimental data for lysozyme production by *A. niger* B1 with a multiple correlation of the coefficients of water activity, pH and polymer type (R^2=0.96, R^2_{Adj}=0.95):

$$\text{Lysozyme}\left(\frac{\text{mg}}{\text{l}}\right) = -11801.9X_{a_w} + 6742.5X^2_{a_w} + 2.0X_{\text{Polymer}} - 0.3X^2_{\text{pH}} + 2.3X_{a_w \times \text{pH}} + 1.6X_{\text{pH}} \quad \text{(Eq. 5.3)}$$

were X is the value of the factors not coded except for the polymer type, indicated by the attached subscript.

The linear (X_{a_w}) and quadratic ($X^2_{a_w}$) coefficients of water availability were statistically significant ($P < 0.000001$). The rest of the coefficients, polymer type ($X_{Polymer}$), linear (X_{pH}) and quadratic (X^2_{pH}) pH and interaction of $a_w \times pH$ ($X_{a_w \times pH}$) terms were not significant. However, the least significant terms were included in the equation to maintain the hierarchy in the model.

5.3.3 Lysozyme optimization

The experiments focusing on enhancing the lysozyme production are illustrated in Figure 5.5. These data were fitted to the model which included all the four main effects and three interactions. It presents the predicted heterologous protein concentrations under the different immobilization conditions. The arrow pinpoints the optimal immobilization conditions and the levels achieved under each specific condition. Maximum lysozyme production (22.8 mg l^{-1}) occurred when pectate was the immobilization polymer provided at 2% w/v combined with an inoculum level of 33% v/v. It is interesting to note that lysozyme production under these conditions was 10-fold higher than the highest lysozyme production achieved when alginate was used as an immobilization polymer. Table 5.4 summarized the optimal immobilization conditions achieved on the lysozyme concentration.

5.4 DISCUSSION

5.4.1 Immobilization, polymer type and inoculum level

This paper present the first comprehensive study of immobilization design and inoculum level on the production of the heterologous protein lysozyme. In this work Ca-pectate gel (CPG) was shown to be an important alternative to Ca-alginate gel (CAG) in the production of fungal enzymes. Although, Ca-alginate has been considered to be the most important material for cell immobilization (Kumar and Lonsane, 1988; Vassileva *et al*., 1998; Vílchez *et al*., 2001), it has been reported that CPG beads are less sensitive to chemical attack and have a higher mechanical stability (Berger and Rühlemann, 1988; Rühlemann *et al*., 1990; Gmeiner *et al*., 1996). Ca-alginate has a 2.3 times higher intrinsic viscosity than Ca-pectate (Tomáška *et al*., 1995). The higher viscosity of Ca-alginate represents additional mass transfer resistance and thus a much lower lysozyme production system.

Productions of the heterologous protein lysozyme in this experimental design showed a wide variation from only 0.20 mg l^{-1} to 22.9 mg l^{-1}. This variation reflects the importance of the immobilization design optimization to attain higher production of heterologous proteins.

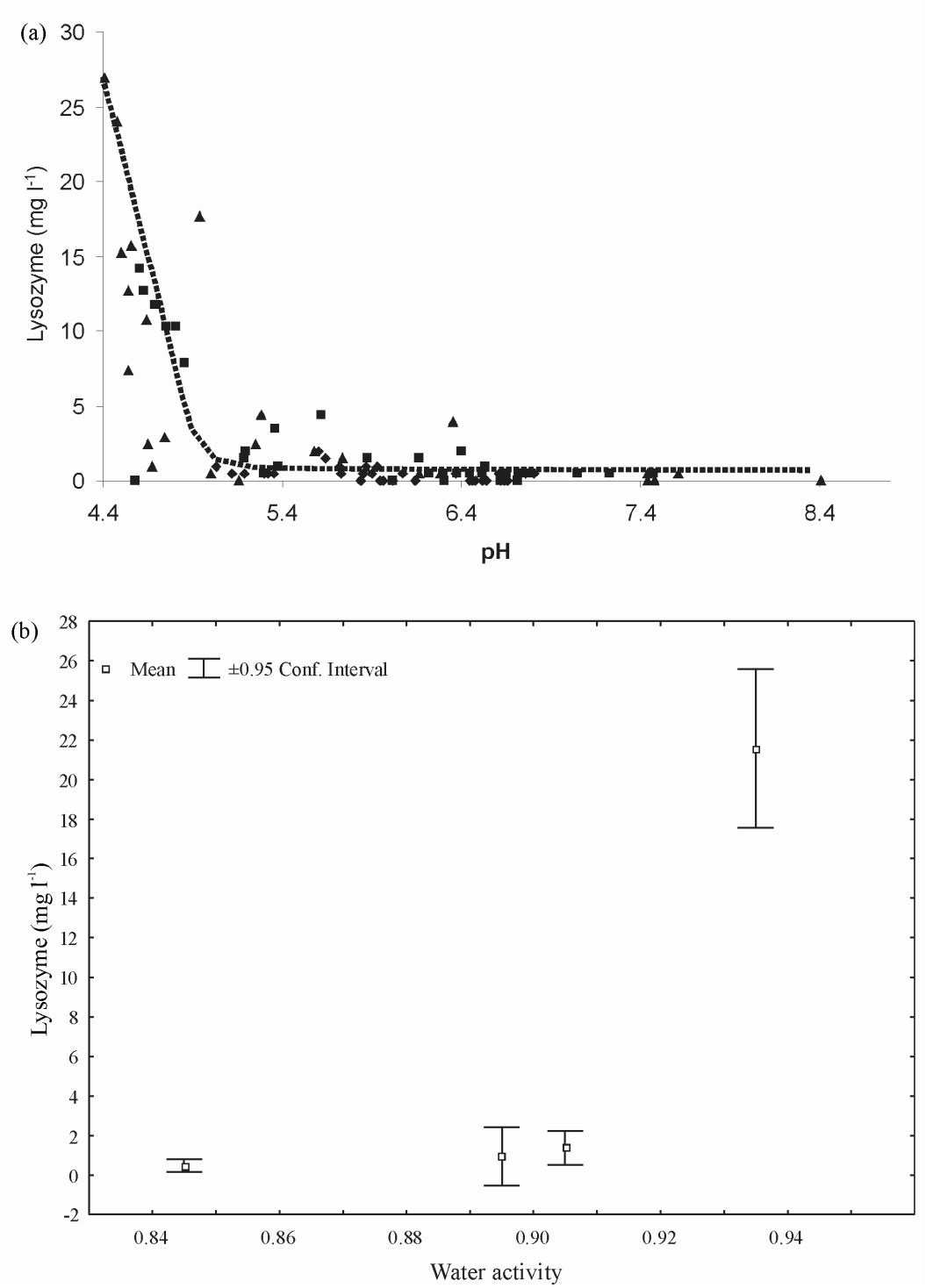

Figure 5.4. (a) Relation between pH of active culture and lysozyme production at different experimental conditions and at time: 2 (♦) 6 (■), and 10 days (▲), dots represent the tendency. (b) Effect of beads water activity on the production of lysozyme, the (□) represent the mean of three treatments and (├┤) confidence interval (95 %).

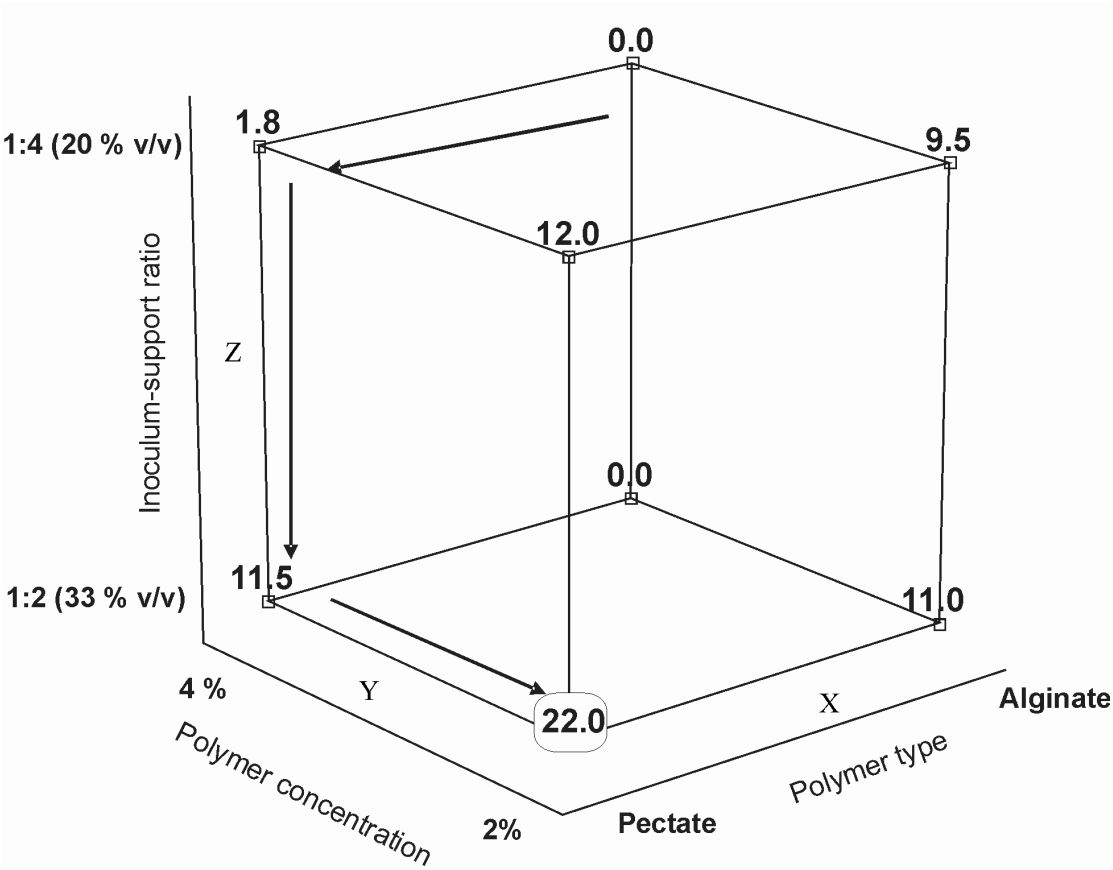

Figure 5.5. Predicted mean lysozyme production. The vertex represented by the codified conditions in the coordinated axes: (x) polymer type, (y) polymer concentration, (z) inoculum ratio.

Table 5.4. Factors, levels, predicted and confidence interval under the best immobilization conditions for maximal heterologous protein production

Immobilized design		Experimental mg l^{-1}	Predicted mg l^{-1}	-95% mg l^{-1}	+95% mg l^{-1}
Polymer type:	Ca-pectate	22.8	21.6	17.0	27.0
Concentration:	2 % (w/v)				
Inoculum level:	33% (w/v)				
Gel-inducer :	3.5%				

The main production with 2% Ca-pectate was 13-fold higher (21.6 mg l^{-1}) than with 4% (1.8 mg l^{-1}). The polymer concentration has the effect of enhancing the gel strength, and at 4% this is four times higher than at 2.0% (Martinsen *et al*., 1989). Previous studies have reported that the concentration of polymer altered the spectrum and activity of degrading enzymes (Pashova *et al*., 1999). This could be due to the water activity of the hydro-gel (Mizrahi *et al*., 1997). Changes in polymer concentration affects the a_w which has an important effect on the pattern of protein secretion in *Aspergillus niger* (Bobowicz-Lassocika and Grajek, 1995). The optimal concentration found in this work agrees with previous reports with alginate used as the polymer (Angelova *et al*., 1998; Jianlong *et al*., 2001).

The optimal inoculum:polymer ratio 1:2 (33% mycelium immobilized v/v) represent a 10% (22.9 mg l^{-1}) improvement in comparison with the production of lysozyme at a higher inoculum:polymer ratio 1:4 (25% mycelium immobilized v/v) (20.3) with Ca-pectate. The effect observed could be attributed to the increase biomass available to bio-convert substrates and products.

It was noted that the treatments reaches different final pH levels had an impact on the produced. The lysozyme concentration was maximum at pH 4.5, and a dramatic reduction occurred as the pH increased up to 5. An optimal range of 4.0-5.0 was reported for glucoamylase production in batch cultures of two different *A. niger* isolates (Aguero *et al*., 1990). MacKenzie *et al*. (1994) reported an improvement that the production of lysozyme was due to a reduction in medium acidification (sodium acetate buffer pH 4.5). They reported an improvement in lysozyme production by *A. niger* strain L11 when a pH 4.5 buffer was added to the fermentation medium. Furthermore, maximum lysozyme production was reported at 4.5 with *A. niger* B1 (Mainwaring *et al*., 1999).

The main factors in the experiment: polymer type, concentration and inoculum:polymer ratio had a marked effect on the water availability of the produced beads. Ca-alginate at the same concentration of Ca-pectate had a much lower a_w. Thus, at 2% inoculum:polymer ratio level, Ca-alginate has an a_w of 0.90 compared to 0.94 a_w of Ca-pectate. The a_w linear and quadratic terms were significant in the statistical analysis.

The secondary analysis performed to separate the effect according to a_w, pH blocked by polymer type. Interestingly, the analysis showed that the most significant term was the a_w. Polymer type, pH and interactions are no significant terms. This is the first time, to our knowledge, that the a_w of immobilized cell fermentation a_w is considered as an important parameter in the production of heterologous proteins. Interestingly, the water availability for the production of lysozyme is different to that for optimal growth (0.96 a_w) (Parra and Magan, 2004; Parra *et al*., 2004a). The water activity coupled with the polymer type in the beads may change the homologous and heterologous protein expression resulting in different levels of lysozyme production.

5.4.2 Comparison between immobilized and free cells

Immobilized cells shows various modifications in physiology and biochemical composition when compared to suspended cells. Altered morphological forms as well as higher or lower metabolic activity, growth rates and product concentrations have been detected (Yadwad *et al*., 1996). The production of lysozyme produced in immobilized cell fermentations resulted in much higher concentration than in free cell fermentations by *A. niger*. The maximal lysozyme concentration achieved in free cell fermentations was 15.2 mg l^{-1} (Parra *et al*., 2004b). Under the optimized immobilized cell conditions the production of lysozyme was 22.0 mg l^{-1} and represent a 46 % increase in the total lysozyme production in a free cell fermentation.

It could be that immobilization of the fungal cells leads to changes in their microenvironment (Shreve and Vogel, 1993). Immobilized cells produce various advantageous modifications in physiological and biochemical composition when compared to suspended cells (Hilge-Rotmann and Rehm, 1990). For example the activity level of the defence enzyme superoxide dismutase (SOD) against reactive oxygen species, an important part of the cells' defensive repertoire in immobilized fungi, was reported to be 1.5-fold higher than free cells (Angelova *et al*., 2000).

For future work in improving the production of heterologous protein lysozyme, it is recommended to scale-up the immobilized cell fermentation developed into a

bioreactor which permits higher mass transfer coefficients and assures pH monitoring and control.

As far as known, there are no reports of heterologous lysozyme produced in a optimized immobilized cell fermentation. The results strongly support the use of immobilized cells in the lysozyme production. The optimization of the immobilized cell fermentation not only improve the production under the experimental conditions tested but also represents a 46 % higher concentrations than the achieved in previous work with free cell fermentations.

The immobilization based on the properties solid bead succeed in finding optimal conditions to improve production of heterologous protein lysozyme by the genetically engineered *A. niger* B1. Statistical methods like polynomial models to evaluate optimal conditions were useful for the optimization of lysozyme production by *A. niger*. The effect of a_w and pH are parameters important to take into account in the scaling up of the biotechnological process with immobilized cell system for the lysozyme production

CHAPTER VI

MEDIUM OPTIMIZATION FOR THE PRODUCTION OF THE SECONDARY METABOLITE SQUALESTATIN S1 BY A *Phoma* sp. COMBINING ORTHOGONAL DESIGN AND REPONSE SURFACE METHODOLOGY

Submitted to Applied and Environmental Microbiology

6.1 INTRODUCTION

The treatment of hypercholesterolemia with pharmaceutical agents reduces the risk of developing arteriosclerosis. Several therapies such as bile acid sequestrants or cholesterol biosynthetic inhibitors are available. In the isoprenoid biosynthetic pathway, the first step to the biosynthesis of cholesterol involves the dimerization of farnesyl pyrophosphate to squalene. This step is catalyzed by squalene synthase and is a potential drug target. Substrates analogous of the farnesyl pyrophosphate have been synthesized and found to be inhibitors of this enzyme. Squalestatins are a potent inhibitor of the squalene synthase (Blows *et al*., 1994). Among the various fungal species that produce the family of squalestatins as secondary metabolites, a *Phoma* sp. is particularly important for high concentration capacity (Baxter *et al*., 1998, Dawson *et al*., 1991).

Some key intermediates of primary metabolism serve as branching points of biosynthetic pathways leading to end products of primary and secondary metabolism. Secondary metabolism is regulated by precursors, carbon sources, nitrogen sources, phosphate, trace elements, induction of enzymes of secondary metabolism, catabolic repression and inhibition, feed back repression and inhibition, and control by auto-regulators (Betina, 1994).

The available cell precursor levels may regulate antibiotic/secondary metabolite production, especially when the specific synthase is already active in the cells (Sathosh *et al*., 1976). There are differences between the carbon sources for growth and secondary metabolism. For example, glucose is usually an excellent source for growth but may interfere with secondary metabolism. Glucose has also been shown to interfere with the biosynthesis of actinomycin, benzodiazepine alkaloids, cephalosporin, chlorotetracycline, enniantin, ergot alkaloids, erythromycin, kanamycin, oleandomycin, penicillin, puromycin, tetracycline and tylosin (Demain *et al*., 1992). The synthesis of pectinolytic enzymes is considerably influenced by the glucose content of the cultivation medium containing mixed carbon sources in a predetermined optimal ratio (Panda *et al*., 2004).

The effect of nitrogen sources on secondary metabolism is conditioned by several factors including the type of metabolic pathway, the producing organism, the type and concentration of the nitrogen sources and whether cultures are stationary or submerged. Very often, secondary metabolic pathways are negatively affected by nitrogen sources favourable for growth (Betina, 1994). Negative effects of ammonium salts have been reported in the production of cephalosporine, penicillin, erythromycin, tylosin, leucomycin, chloramphenicol, macbecin, rifamycin, streptomycin, streptothricin and tetracycline (Demain, 1992).

It is known that several trace elements are essential for microbial growth because of their involvement in metalloenzymes or as enzyme activators. In secondary metabolism, zinc, iron and manganese are the most important trace elements. Several reports have been published on the importance of these three elements in aflatoxin production (Betina, 1994). It has been shown that zinc is an essential element for aflatoxin biosynthesis. The omission of zinc resulted in no detectable versicolorin production by a blocked mutant of *Aspergillus parasiticus* (Betina, 1994). Inorganic phosphate is also known to exert a suppressive effect on the synthesis of many secondary metabolites.

In developing a biotechnology-based industrial process, designing the fermentation media is of critical importance. The fermentation medium affects the product concentration and volumetric productivity. It is also important to reduce the cost of the medium as much as possible, as this does affect the overall process economics. Medium screening studies are very time consuming and expensive. This is because the number of possible media combinations that can be tested and the number of fermentation substrates that are available are also very large. Thus, for economy of effort and scale different approaches have been used to rapidly identify the variables which need to be controlled for optimising production of useful metabolites.

Traditional screening approaches are time consuming and involve testing each medium component to examine which combination gives the best results. While this is extensively used it is very time consuming (Furuhashi and Takagi, 1984). Others have used mapping approaches to identify the optimization space. This enables a better picture of the possible effects of each component in the medium. Orthogonal and Plackett Burman designs are important methodologies that can reduce the number

of runs to an absolute minimum (Castro *et al*., 1992; Escamilla *et al*., 2000; Xu *et al*., 2003). The main disadvantage of these designs is that they consider only first order effects and ignore interactions. However, while a full factorial design (testing every combination possible) provides the most complete information, they often require such a large number of runs that they are impractical to carry out. Optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial to obtain a model of the production system, usually involving fitting of data to a polynomial equation, often using stepwise multiple regression. Response surface methodology has also been used to investigate the optimal regions of production of useful product (Papulla *et al*., 1992; White *et al*., 1990). Detailed analyses of the optimized region using cetroidal or simple designs have also been applied for optimization processes (White *et al*., 1990). However, several interactions of the experimental design and optimization of models are required for effective application to product formation in fermentation systems.

The purpose of this study was to optimize thirteen substrates, including those reported in the literature, at three different levels in submerged fermentation to produce squalestatin S1 by a *Phoma* species. This approach used a combination of statistical strategies involving the use of an orthogonal design, response surfaces and polynomial regression to find the best medium for maximising concentrations in a free fermentation system.

6.2 MATERIALS AND METHODS

6.2.1 Fungal strains

Stock spore suspension of the *Phoma* sp. (IMI 332962) were maintained in 0.5-ml cryogenic vials (Nalgene). The culture was maintained as aliquots of the spore suspension. (1.6×10^7 spores ml^{-1} ± 3%) with Tween 80 (100 µl l^{-1}) and 5%(w/v) glycerol stored at -70ºC (Connors *et al*., 1995). This was kindly supplied by GlaxoSmithKline R & D, Medicines Research Centre, Stevenage, Herts SG1 2NY, U.K.

6.2.2 Activation and strain inoculation

Thawed 500 µl cryogenic stock spore suspension vials (Nalgene) of *Phoma* sp. were used. A 5 µl of the spore suspension was inoculated on glycerol-modified MEA ($0.98a_w$) Petri plates and incubated at 25^oC (Baxter *et al*., 1998). a Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland) was used to check the a_w level. Actively growing 5-day-old cultures were harvested to produce the spore inoculum by pouring 10 ml of sterile Tween 80 (100 µl l^{-1}) solution onto the glycerol-modified MEA ($0.98a_w$) growing cultures and the spores dislodged using a sterile microbiological loop. Two ml of the extracted spore suspension were used to inoculate the 100 ml liquid medium according to the experimental design. The cultures (in 250-ml conical flasks) were incubated at 25 ºC on a shaker at 150 rev min^{-1} for 10 days.

6.2.3 Media composition

The medium composition was based on a combination of substrates: some reported in the literature and some new ones. The medium design included simple substrates such as a nitrogen source due to the importance of this element (C:N ratio) in secondary metabolism and also trace elements (Mg^{2+} and Fe^{3+}) (Table 6.1).

Table 6.2 presents the trace element concentration of a standard solution (*TE*) used to add to the fermentation medium. The trace elements were prepared in a standard solution and 10 ml used to make up 1 litre of solution.

6.2.4 Sample preparation and analyses for squalistatin S1

Sample preparation

The samples were prepared by placing 250 µl of the samples in 1 ml Eppendorf tubes with lids (Sartorius) and adding 750 µl of extractive solvent described below. This was mixed for 5 min. in a shaker at 250 rpm (L.H Engineering Co. Ltd, England) and centrifuged in a Microcentaur (MSE) for 15 min. at 13,000 rps. The supernatant was filtered through a 0.2 µm nylon syringe filter (BDH) and transferred to 2 ml HPLC vials (Anachem) and sealed with septa (0.010" PTFE, Anachem) and lids (Anachem). The samples were kept at -80 ºC and thawed before analysis. The reagents used in HPLC analysis are: Extraction solvent 80% methyl-acetonitrile (MeCN) containing

H_2SO_4 (0.3 ml l^{-1}) and mobile phase 50% MeCN containing H_2SO_4 (0.15 ml l^{-1}). A stock solution of 120 µm ml^{-1} squalestatin (S1) was prepared as a standard and kept frozen at -80 ºC. Aliquots were thawed and diluted in the range of 0.012 -120 µm ml^{-1} and placed in 2 ml HPLC vials. Dilutions of the samples were made if necessary.

HPLC system

A Gilson 715 HPLC system was used to quantify squalestatin (S1) from the fermentation broth. Aliquots of 50 µl were automatically injected with a Gilson 231XL sampling injector onto a 5µl C6 Spherisob 150 x 4.6 mm column (Phenomenex), with a guard column of the same material as the extractive column. Separated metabolites were detected with a Gilson 117 UV detector at 210 nm. Running conditions of the analysis were: 1 ml min^{-1}, time 30 min per sample. The concentration of S1 in the samples was analyzed against the standard and S1 standards were included randomly with the samples to detect changes in the retention time of S1. The retention time of S1 was between 8-9 min.

6.2.5 Experimental design and data treatment

To investigate the relationship between substrate medium components and their concentration to optimize the production of squalestatin S1 by the *Phoma* species, an orthogonal design L_{27} (3^{13}) was used. The medium composition was selected according to the experimental design. The combinations of substrate and concentrations were selected using the experimental design module of Statistica 6.0 (SoftStat, Inc. 1984-2001, USA).

The levels of substrates were allocated under three categories: (1) low, (2) medium and (3) high. The dummy variable of glucose in the experimental design was used as the measure of experimental design variability. It gives a direct estimate of the standard error of a factor effect.

For the analysis, the factors were given coded values between 1 (lowest concentration of substrate) and 3 (highest concentration of substrate). For the response surface methodology and the polynomial regression the coded values were changed as follows -1=1, 0=2 and 1=3 and the analysis was performed with the experimental design module of Statistica 6.0 (SoftStat, Inc. 1984-2001, USA).

Table 6.1. Experimental design combinations for the factors and levels used for the optimization of squalestatin production based on substrate and concentration reported.

Substrate abbreviation	Substrate	Design	Levels		
			Low	Medium	High
		Orthogonal design	1	2	3
		Response surfaces	-1	0	1
SC 1	Glycerol [1, 4, 5, 6]		30 g l^{-1}	50 g l^{-1}	70 g l^{-1}
SC 2	Glucose [2, 3, 5]		5 gl^{-1}	10 g l^{-1}	15 g l^{-1}
SC 3	Glucose [Replica]		5 g l^{-1}	10 g l^{-1}	15 g l^{-1}
OP 1	Soybean oil [1, 4, 5]		25 ml l^{-1}	30 ml l^{-1}	35 ml l^{-1}
OP 2	Cottonseed oil		5 ml l^{-1}	10 ml l^{-1}	15 ml l^{-1}
OP 3	Cottonseed flour [1, 5]		5 g l^{-1}	10 g l^{-1}	15 g l^{-1}
P 1	Yeast extract [2, 3, 6]		5 g l^{-1}	7.5 g l^{-1}	10 g l^{-1}
P 2	Soya milk		25 ml l^{-1}	75 ml l^{-1}	125 ml l^{-1}
P 3	Malt extract [1, 2, 3, 7]		15 g l^{-1}	21 g l^{-1}	27 g l^{-1}
TE	Trace elements [4]		5 ml l^{-1}	10 ml l^{-1}	15 ml l^{-1}
NaCit	Sodium citrate [4, 5, 6]		8 g l^{-1}	11 g l^{-1}	14 g l^{-1}
N	$(NH_4)SO_4$		1 g l^{-1}	2 g l^{-1}	3 g l^{-1}
Lac	Lactose [6]		35 g l^{-1}	50 g l^{-1}	65 g l^{-1}
Pep	Peptone [1, 3, 7]		5 gl^{-1}	10 gl^{-1}	15 g l^{-1}
Mg	$MgSO_4$ [1, 2, 3, 4]		0.01 g l^{-1}	0.5 g l^{-1}	0.9 g l^{-1}
Fe	$FeSO_4 \cdot 7H_20$ [2, 4]		0.5 g l^{-1}	1.0 g l^{-1}	1.5 g l^{-1}

[1]Dawson *et al*. (1991); [2]Dufresne *et al*. (1992); [3]Bergstrom *et al*. (1993); [4]Dufresne *et al*. (1993); [5]Blows *et al*. (1994); [6]Connors *et al*. (1995); [7]Aldred *el al*. (1999).

Table 6.2. The trace elements solution was made with 10 ml l^{-1} as reported by Dufresne *et al*. (1992).

*Trace elements mixture (*TE*)	Litre
$MnSO_4 \cdot 4H_20$	1.0g
$CuCl_2 \cdot 2H_2O$	0.025g
$CaCl_2 \cdot 2H_2O$	0.1g
H_3BO_3	0.056g
$(NH_4)_6MoO_{24} \cdot 24H_20$	0.019g
$ZnSO_4 \cdot 7H_20$	0.2g
Adjust pH	7

6.3 RESULTS

6.3.1 Effect of medium constituents on the S1 production

Table 6.3 summarises the S1 production obtained from the experiment and forecasted by contour surfaces under the different treatment conditions. Overall, glucose as an additional carbon source produced a higher S1 production in comparison with glycerol. Levels of S1 with added glucose ranged from 0 to 273 mg l^{-1}. Interestingly, cottonseed flour gave the highest S1 production.

Table 6.4 shows the analysis of variance (ANOVA) of the results after the fifth day of fermentation with an orthogonal design analysis. These indicated that S1 production could be enhanced using a combination of solutes at different concentrations in the fermentation medium. The levels of sources of carbon (*LSC*), oily precursors (*OP*), nitrogen (*N*) and peptone (*Pep*) were the most significant factors in the production of S1 ($P<0.001$) followed by precursors (*P*) and trace elements (*TE*) ($P<0.05$).
Using the orthogonal design L_{27} (3^{13}) approach the relationships between medium component variables and their concentrations on S1 production could be calculated. The factors could be ranked by importance (magnitude in parentheses). They were: levels of carbon sources (*LSC*=43.621) > nitrogen (*N*=41.782) > precursors (*P*=38.663) > levels of precursors (*LOP*=31.455) > trace elements (*TE*=29.289) > Oily precursor (*OP*=28.526) > peptone (*Pep*=25.271) > levels of precursors (*LP* =23.393) > sodium citrate (*NaCit*=18.949) > iron (*Fe*=18.768) > sources of carbon (*SC*=15.934) > magnesium (*Mg*= 2.702) > lactose (*Lac*=0.948). This pointed to the sources of carbon levels, nitrogen and precursors being the most important factors in the production of S1. Lactose was the least important nutrient.

Table 6.3. Experimental conditions and average concentrations of S1 produced with the orthogonal design L_{27} (3^{13}), standard error and predicted concentration with response surfaces (polynomial regression)

														Squalestatin S1 (mg l^{-1})		
Dsn. Id.#	*SC*	*LSC*	*OP*	*LOP*	*P*	*LP*	*TE*	*NaCit*	*N*	*Lac*	*Pep*	*Mg*	*Fe*	Experimental	SE	Predicted
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	17.1	0.0	17.1
2	-1	-1	-1	-1	0	0	0	0	0	0	0	0	0	153.2	0.0	153.2
3	-1	-1	-1	-1	1	1	1	1	1	1	1	1	1	34.7	0.0	34.7
4	-1	0	0	0	-1	-1	-1	0	0	0	1	1	1	1.7	0.0	1.7
5	-1	0	0	0	0	0	0	1	1	1	-1	-1	-1	7.4	0.0	7.4
6	-1	0	0	0	1	1	1	-1	-1	-1	0	0	0	0.0	0.0	0.0
7	-1	1	1	1	-1	-1	-1	1	1	1	0	0	0	0.0	0.4	0.0
8	-1	1	1	1	0	0	0	-1	-1	-1	1	1	1	26.7	0.6	26.7
9	-1	1	1	1	1	1	1	0	0	0	-1	-1	-1	89.7	0.7	89.7
10	0	-1	0	1	-1	0	1	-1	0	1	-1	0	1	273.4	1.4	273.4
11	0	-1	0	1	0	1	-1	0	1	-1	0	1	-1	118.4	2.0	118.4
12	0	-1	0	1	1	-1	0	1	-1	0	1	-1	0	1.6	2.1	1.6
13	0	0	1	-1	-1	0	1	0	1	-1	1	-1	0	128.1	2.4	128.1
14	0	0	1	-1	0	1	-1	1	-1	0	-1	0	1	55.1	2.6	55.1
15	0	0	1	-1	1	-1	0	-1	0	1	0	1	-1	162.9	2.9	162.9
16	0	1	-1	0	-1	0	1	1	-1	0	0	1	-1	15.7	3.0	15.7
17	0	1	-1	0	0	1	-1	-1	0	1	1	-1	0	0.7	3.5	0.7
18	0	1	-1	0	1	-1	0	0	1	-1	-1	0	1	0.0	3.7	0.0
19	1	-1	1	0	-1	1	0	-1	1	0	-1	1	0	253.0	4.7	253.0
20	1	-1	1	0	0	-1	1	0	-1	1	0	-1	1	118.3	5.4	118.3
21	1	-1	1	0	1	0	-1	1	0	-1	1	0	-1	35.5	5.4	35.5
22	1	0	-1	1	-1	1	0	0	-1	1	1	0	-1	0.0	5.7	0.0
23	1	0	-1	1	0	-1	1	1	0	-1	-1	1	0	0.0	6.2	0.0
24	1	0	-1	1	1	0	-1	-1	1	0	0	-1	1	0.0	6.4	0.0
25	1	1	0	-1	-1	1	0	1	0	-1	0	-1	1	271.5	8.0	271.5
26	1	1	0	-1	0	-1	1	-1	1	0	1	0	-1	49.3	8.2	49.3
27	1	1	0	-1	1	0	-1	0	-1	1	-1	1	0	23.7	8.3	23.7

(SC 1) Glycerol, (SC 2) Glucose (SC 3), Glucose Replica, (OP 1) Soybean oil, (OP2) Cottonseed oil, (OP 3) Cottonseed flour, (P 1)Yeast extract, (P 2) Soya milk, (P3) Malt extract, (TE) Trace elements, (NaCit) Sodium citrate, (N) $(NH_4)SO_4$, (Lac) Lactose, (Pep) Peptone, (Mg) $MgSO_4$, (Fe) $FeSO_4 \cdot 7H_2O$.

Table 6.4. The analysis of variance $L_{27}(3^{13})$ orthogonal test on optimization of culture medium in shake flask culture

	SS	df	MS	F	*P*-value
SC	26542.6	2	13271.3	4.592	0.019
LSC	53033.59	2	26516.79	9.174	0.001**
OP	52674.18	2	26337.09	9.112	0.001**
LOP	27383.44	2	13691.72	4.737	0.017
P	44001.77	2	22000.88	7.612	0.002*
LP	25657.82	2	12828.91	4.439	0.022
TE	46360.23	2	23180.12	8.020	0.002*
NaCit	14670.61	2	7335.303	2.538	0.098
N	59431.78	2	29715.89	10.281	0.000**
Lac	77.0779	2	38.53895	0.013	0.987
Pep	38852.08	2	19426.04	6.721	0.004**
Mg	706.447	2	353.2235	0.122	0.885
Fe	9968.942	2	4984.471	1.725	0.197
Residual	78038.03	27	2890.298		

*$P<0.005$

**$P<0.001$

Table 6.5 presents the optimal combination of substrates and concentrations to achieve the highest concentration of S1. A search was carried out to identify the optimum components for maximising concentration. The expected concentration of S1 that this optimization could produce was 387 mg l^{-1}. Thus an improvement of 42% could be obtained with the optimization from the highest concentration (273 mg l^{-1}) achieved in the experiment.

Figure 6.1 presents a graphical analysis based on the effect of the average production of squalestatin S1 under the sets of conditions and levels tested in the present study. The optimal conditions can be easily selected from the factors with the highest levels of production. This describes the important factors in determining S1 production and shows the effect of changing treatment levels on production.

In order to find interactions and optima between factors a re-codification of the experimental design according to response surface methodology was performed. A first order polynomial model with interactions was selected. The robustness and reproducibility of the experiment is confirmed by Figure 6.2 as the surface response is uniform.

6.3.2 Model development

The analysis of the model produced an R^2 of over 99% and overall model significance of $P<0.0001$. The model from the analysis consisted of an intercept, 13 main factor terms, 11 interaction terms and interception terms, thus including a total of 25 terms. The effect of factors, standard error, *P*-values and 95% confidence levels of parameter variability (parameter certainty) are presented in Table 6.6. The effects are ranked by *P*-values from the most to the least significant. In the polynomial analysis S1 concentration was shown to be affected principally by the interaction between level of carbon sources, oily precursor levels and type ($P<0.000001$).

The model expressed in Eq. (6.1) was generated with coded factors (X) between -1 to +1, to predict the S1 production.

$$\begin{aligned} Sl(mgl^{-1}) = {} & 238.7\, X_{SC\times OP} + 154.1 X_{LOP} + 124.7 X_{LSC} - 69.4 X_{OP} - 184.3 X_{P\times LP} + \\ & 223.8\, X_{NaCit} + 172.4 X_{LOP} + 253.4 X_{LSC\times NaCit} + 204.9 X_{SC\times P} + 196.9 X_{SC\times N} + \\ & 106.5\, X_{LOP} - 193.0 X_{SC\times LP} + 60.0 X_{LSC\times OP} + 136.2\, X_{Pep} + 77.2\, X_{Lac} + 71.5\, X_{TE} - \\ & 98.7 X_{SC\times Mg} - 53.0 X_{SC\times Pep} - 18.2\, X_{N} - 69.9\, X_{Fe} - 32.8\, X_{SC} - 13.6\, X_{Mg} - 10.7 X_{OP} \\ & + 6.0 X_{SC\times LSC} \end{aligned} \qquad \text{(Eq. 6.1)}$$

The concentration of S1 had a high correlation coefficient (R^2=0.99) with the predicted values presented previously in Table 6.3. Analyses of the observed versus predicted concentrations are shown in Figure 6.3. Points above or below the diagonal line represent areas of over or under prediction. This showed that no significant violations of the model were found in the analysis, with a good correlation of the model with the experimental data obtained.

Illustrations of the critical effects and interactions between factors are shown in the form of contour graph plots in Figure 6.4. The contour diagrams which represent the production of S1 (mg l^{-1}) as a function of substrate type or concentration of two nutrients, with the other factors being at constant levels. This shows the reproducibility and robustness of the polynomial model based on the experimental data.

To determine the most adequate operating conditions and analyze the process for concentration, the contour diagrams were plotted using Eq. (6.1) for all the combinations possible. Over all conditions glucose was the best sources of carbon used (see Figure 6.4). However, the optimal conditions for a combined substrate with the sources of carbon was different. The best substrate combinations for the sources of carbon was glucose. Optimum oily precursor (OP) was cottonseed flour (1) gave concentrations of 180-190 mg l^{-1} S1 production (Figure 6.4a).

For nitrogen addition, to obtain maximum S1 concentration, an intermediate values (2.0 g l^{-1}) on the contour surfaces produced 160-170 mg l^{-1} S1 (Figure 6.4b). A narrow region of optimal conditions was found at the trace elements level 0 (10 ml l^{-1}) with levels of 160-170 mg l^{-1} S1 production (Figure 6.4c). Optimal peptone concentration conditions were around the centre point (8.5 g l^{-1}) (Figure 6.4 d) producing S1 in the range 160-170 mg l^{-1}.

Similar results were found with oily precursors and interaction with other substrates. Overall the oily precursor (1), cottonseed flour, was the best producer of S1 in combination with: trace elements (0) 10 ml l^{-1} (180 mg l^{-1} S1) (Figure 6.5 a), peptone (0) 5 g l^{-1} (180 mg l^{-1} S1) (Figure 6.5 (b)), nitrogen (0) 2 g l^{-1} (170 mg l^{-1} S1) (Figure 6.5c) and precursor (-1) yeast extract (240 mg l^{-1} S1). The interaction between cottonseed flour and yeast extract produced a remarkably high concentration of 240 mg l^{-1} (Figure 6.5d).

Overall, trace elements at level 0 (10 ml l^{-1}) was optimal for production of S1 concentration in combination with: nitrogen (0) 2 g l^{-1} (160 mg l^{-1} S1) (Figure 6.6a), peptone (0) 5 g l^{-1} (160 mg l^{-1} S1) (Figure 6.6c). No interaction was found between peptone and nitrogen and the optimal concentrations were peptone (0) 5 g l^{-1} and nitrogen (0) 2 g l^{-1} with levels of 160 mg l^{-1} S1 (Figure 6.6b). However, a significant interaction between iron and trace elements produced a concentration of 190 mg l^{-1} S1 (Figure 6.6d).

To confirm the optimal conditions (Table 6.5) a set of four replicate experiments with the optimal combinations of substrates and concentrations were used as confirmatory to the forecasted production of S1. The confirmatory experiments produced 434 mg l^{-1} of S1. This represented a concentration 12% higher than the forecasted amount (387 mg l^{-1}). This medium resulted in 24% more than the highest concentration of squalestatin reported in the literature of 350 mg l^{-1} (Bergstrom *et al*., 1993) (Figure 6.7).This represented a productivity twice as high as the highest value reported in the literature (Figure 6.8).

Table 6.5. Optimal medium design for the production squalestatin S1 and forecasted production.

Terms	Substrates	Levels
SC	Glucose	
LSC	1	2.5 g l^{-1}
OP	Cottonseed flour	
LOP	1	2.5 g l^{-1}
P	Yeast extract	
LP	3	5 g l^{-1}
TE	Trace elements	5 ml l^{-1}
NaCit	Sodium citrate	4 g l^{-1}
N	$(NH_4)SO_4$	1 g l^{-1}
Lac	Lactose	32.5 g l^{-1}
Pep	Peptone	5 g l^{-1}
Mg	$MgSO_4$	0.45 g l^{-1}
Fe	$FeSO_4 \cdot 7H_20$	0.15 g l^{-1}
Expected	Squalestatins S1	387 mg l^{-1}

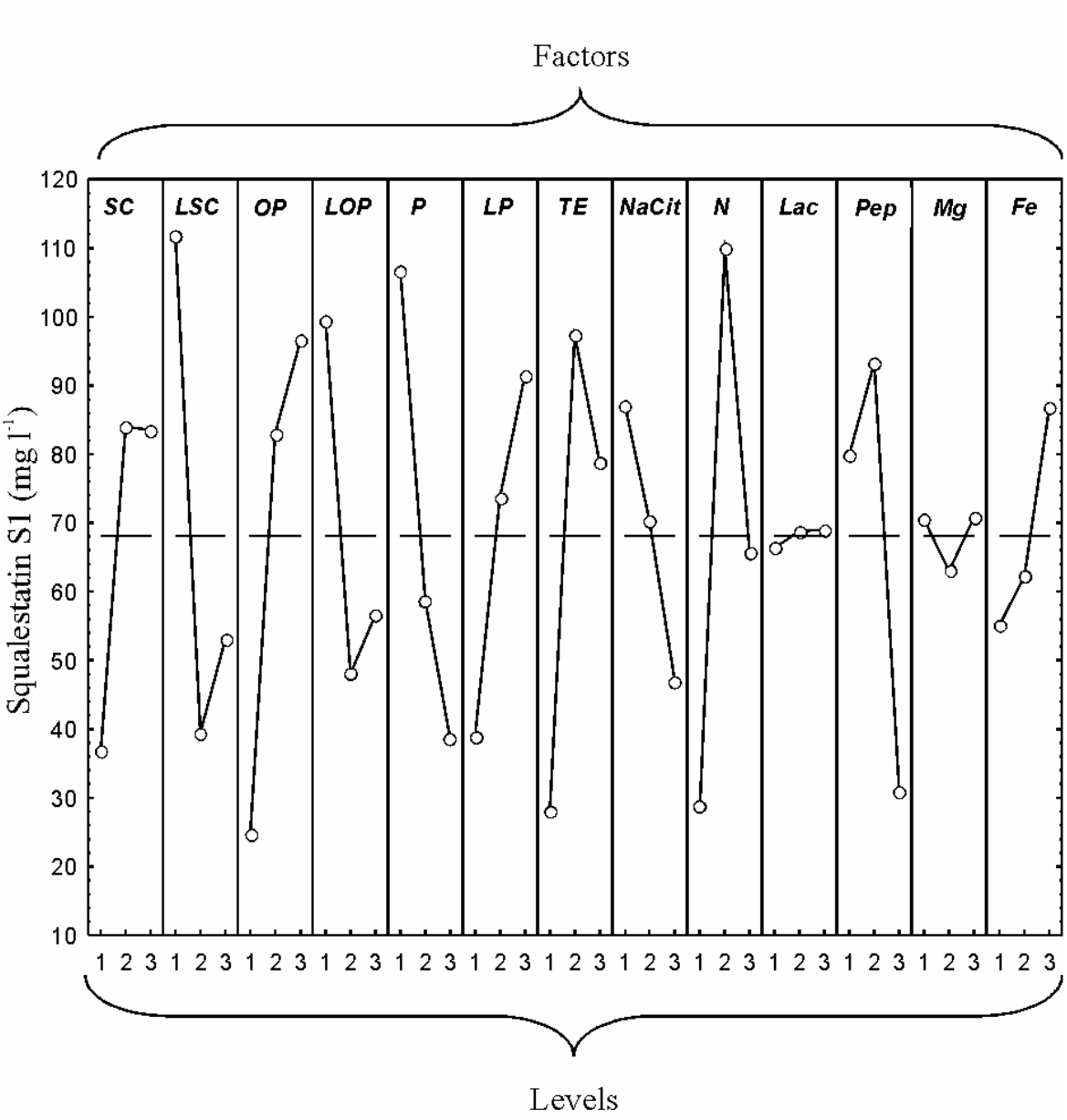

Figure 6.1.Graphical analysis of the relationship between media formulation and squalestatin S1 production. Each factor had three coded levels and the (○) represent mean squalestatin S1 produced. The factors levels coded are: low (1), medium (2) and high (3) as presented in Table 6.1.

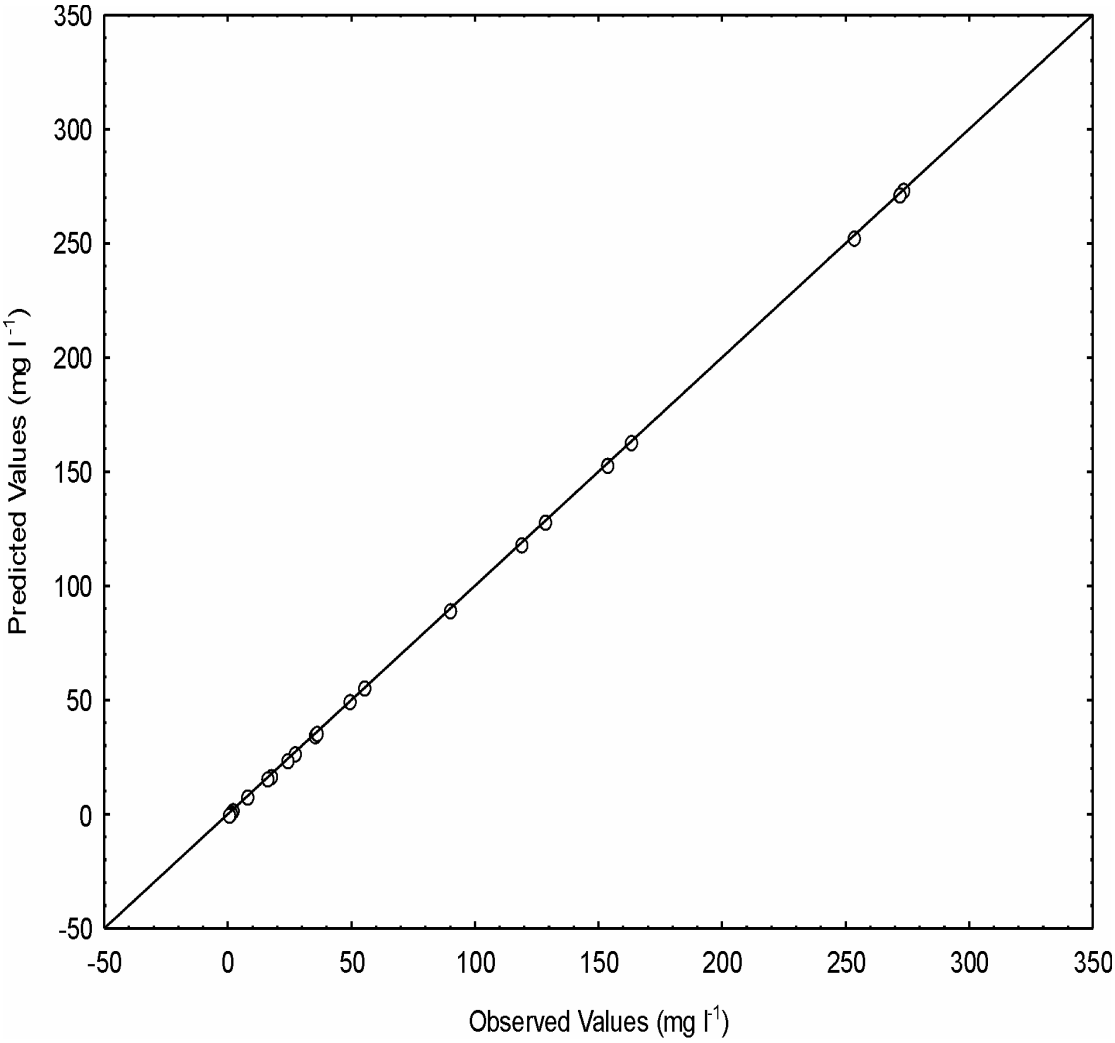

Figure 6.2. Residual diagnostics of contour surface of the quadratic model of the predicted vs. observed by replicated of squalestatin S1 production (mg l^{-1}).

Table 6.6. Estimated linear and interaction effects of the model ranked by magnitude from the orthogonal experiment statistical analysis

Term	Effect	S.E.	t(18)	*P*-value	-95.%	+95.%
Mean/Interc.	93.01	6.37	14.61	0.000000	79.6	106.4
SC × *OP*	477.47	44.01	10.85	0.000000	385.0	569.9
LOP	308.25	32.28	9.55	0.000000	240.4	376.1
LSC	249.31	30.36	8.21	0.000000	185.5	313.1
OP	-138.73	17.90	-7.75	0.000000	-176.3	-101.1
P × *LP*	-368.52	50.63	-7.28	0.000001	-474.8	-262.2
NaCit	447.60	64.06	6.99	0.000002	313.0	582.2
LSC × *P*	344.79	50.06	6.89	0.000002	239.6	450.0
LSC × *NaCit*	506.83	76.96	6.59	0.000003	345.1	668.5
SC × *P*	409.84	62.52	6.56	0.000004	278.4	541.2
SC × *N*	393.82	61.60	6.39	0.000005	264.4	523.2
LOP	212.98	35.84	5.94	0.000013	137.6	288.3
SC × *LP*	-385.98	66.92	-5.77	0.000018	-526.6	-245.4
LSC × *OP*	120.08	23.26	5.16	0.000065	71.2	168.9
Pep	272.40	56.23	4.84	0.000130	154.2	390.5
Lac	154.38	33.42	4.62	0.000213	84.2	224.6
TE	142.94	41.87	3.41	0.003094	54.9	230.9
SC × *Mg*	-197.42	59.66	-3.31	0.003904	-322.7	-72.1
SC × *Pep*	-105.92	37.45	-2.83	0.011140	-184.6	-27.2
N	-36.35	15.22	-2.39	0.028126	-68.3	-4.4
Fe	-139.77	59.77	-2.34	0.031102	-265.3	-14.2
SC	-65.70	28.42	-2.31	0.032838	-125.4	-6.0
Mg	-27.15	17.43	-1.56	0.136665	-63.8	9.5
OP	-21.33	29.39	-0.73	0.477380	-83.1	40.4
SC × *LSC*	12.02	39.67	0.30	0.765333	-71.3	95.4

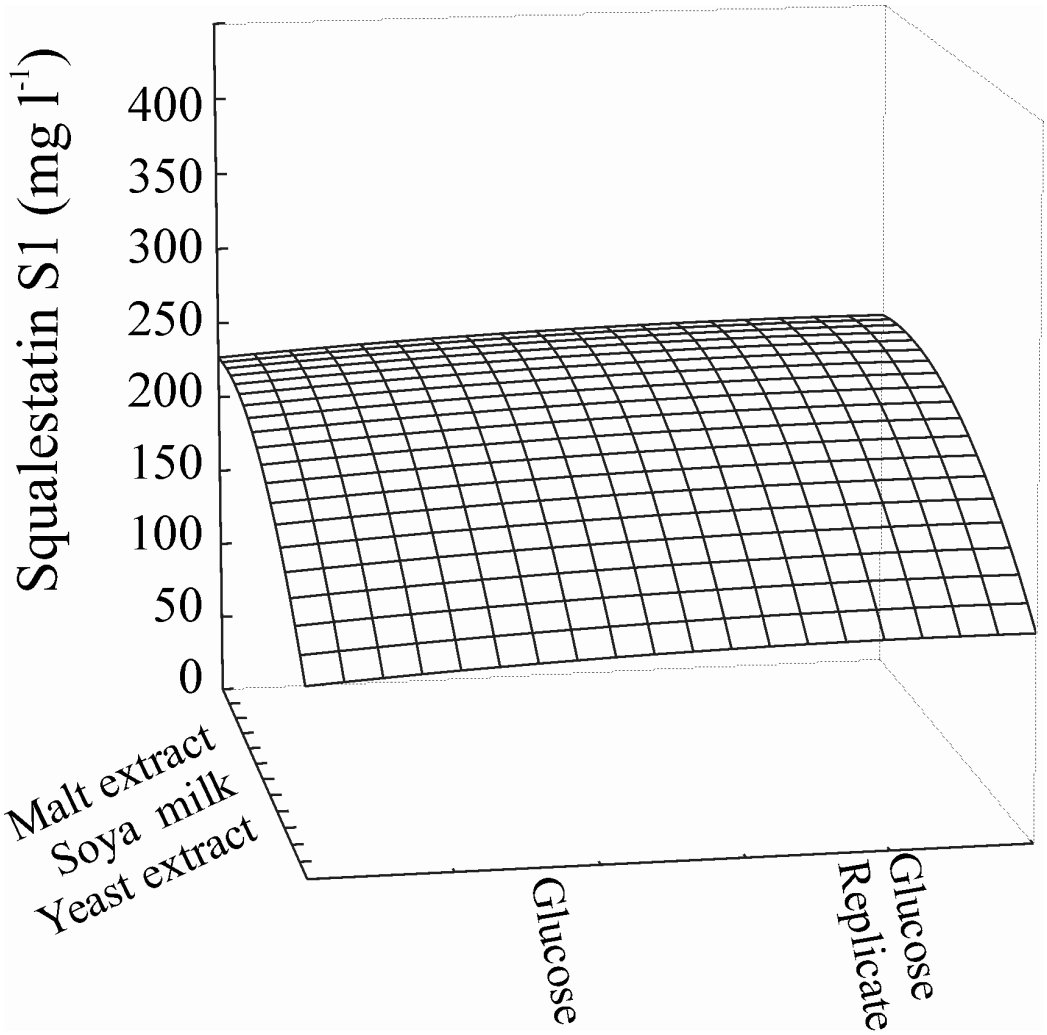

Figure 6.3. Response surfaces fitted to the experimental data points corresponding to the production of squalestatin S1 under different precursors (P) (-1=yeast extract, 0=soya milk and 1=malt extract) and glucose (0=glucose and 1=glucose replicated).

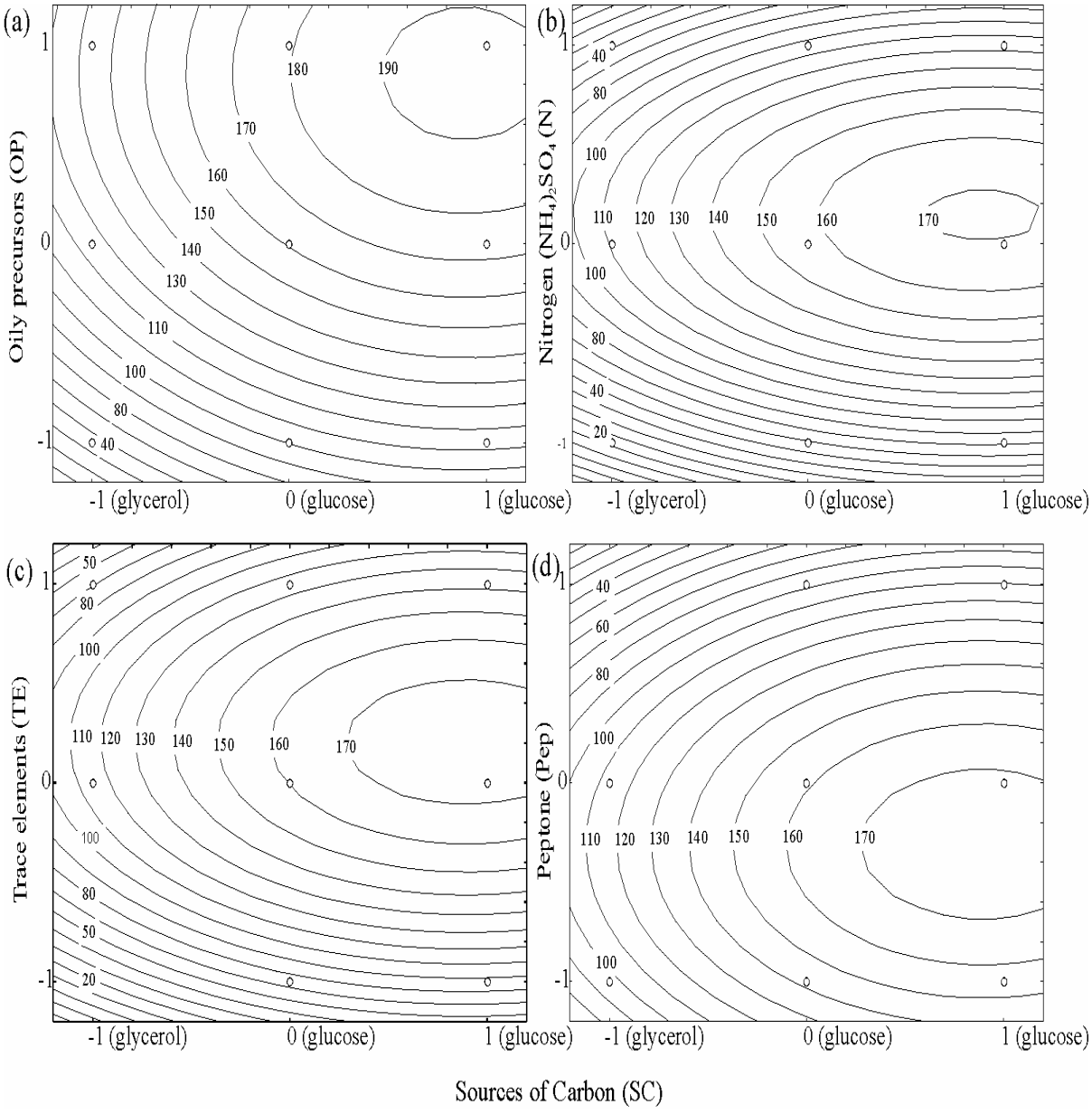

Figure 6.4. Contour diagram of squalestatin S1 generated by the model as a function of: (a) oily precursors, (b) nitrogen, (c) trace elements, and (d) peptone versus carbon sources. The coded values for oily precursor (*OP*): soybean oil (-1), cottonseed oil (0) and cottonseed flour (+1); trace elements (*TE*): 5 ml l^{-1} (-1), 10 ml l^{-1} (0) and 15 ml l^{-1} (+1); nitrogen (*N*): 1 g l^{-1} (-1), 2 g l^{-1} (0) and 3 g l^{-1} (+1); peptone (*Pep*): 5 g l^{-1} (-1), 10 g l^{-1} (0) and 15 g l^{-1} (+1) and sources of carbon (*SC*): glycerol (-1), glucose (0) and glucose replicate (+1) respectively

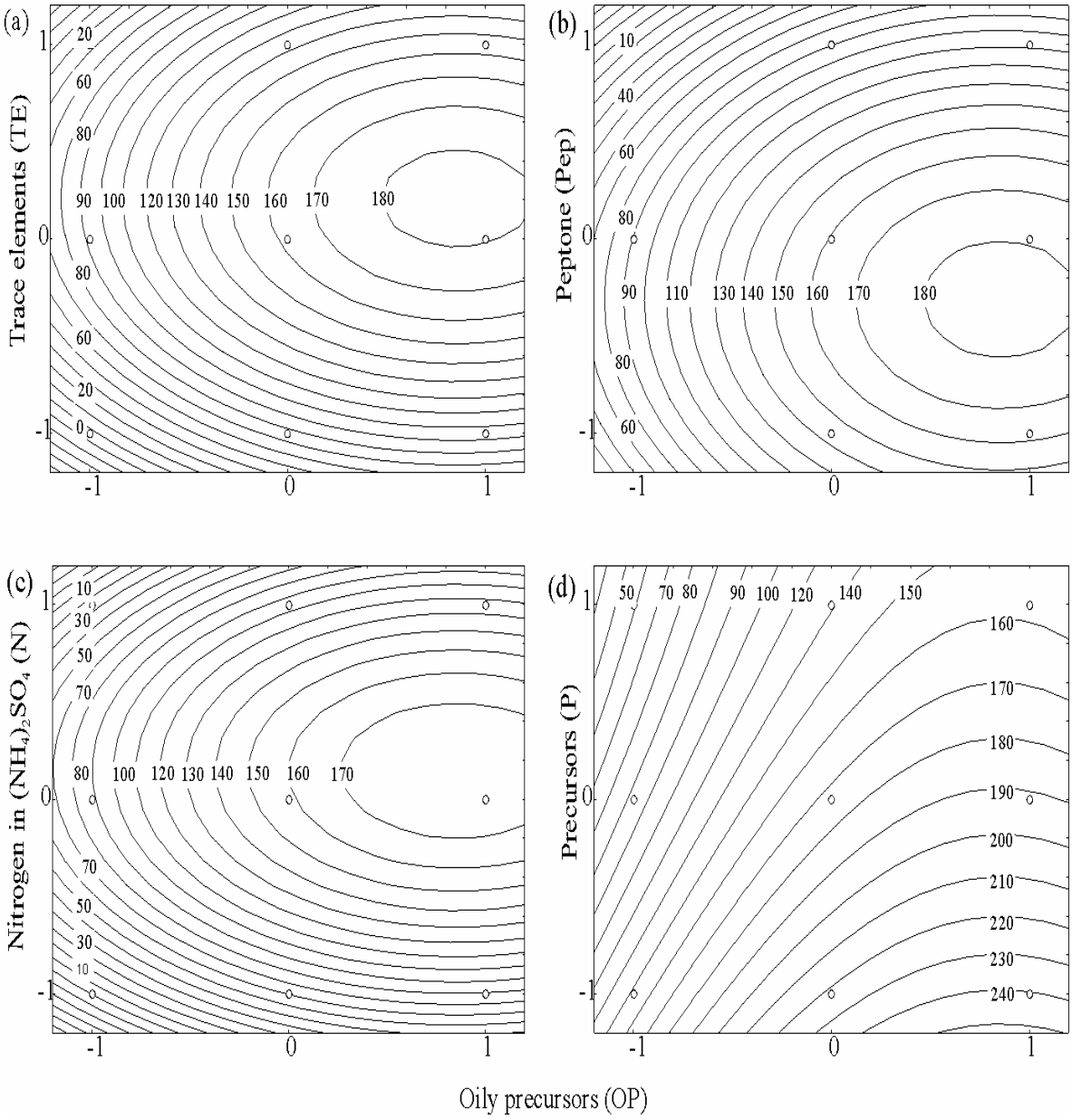

Figure 6.5. Contour diagram of squalestatin S1 generated by the model as a function of: (a) trace elements, (b) nitrogen, (c) peptone and (d) precursors against precursors (OP). The coded values for: trace elements (*TE*): 5 ml l^{-1} (-1), 10 ml l^{-1} (0) and 15 ml l^{-1} (+1), nitrogen (*N*): 1 g l^{-1} (-1), 2 g l^{-1} (0) and 3 g l^{-1} (+1), peptone (*Pep*): 5 g l^{-1} (-1), 10 g l^{-1} (0) and 15 g l^{-1} (+1), precursors (*P*): soya milk (-1), yeast extract (0) and malt extract (+1) and oily precursors (*OP*): soybean oil (-1), cottonseed oil (0) and cottonseed flour (+1) respectively

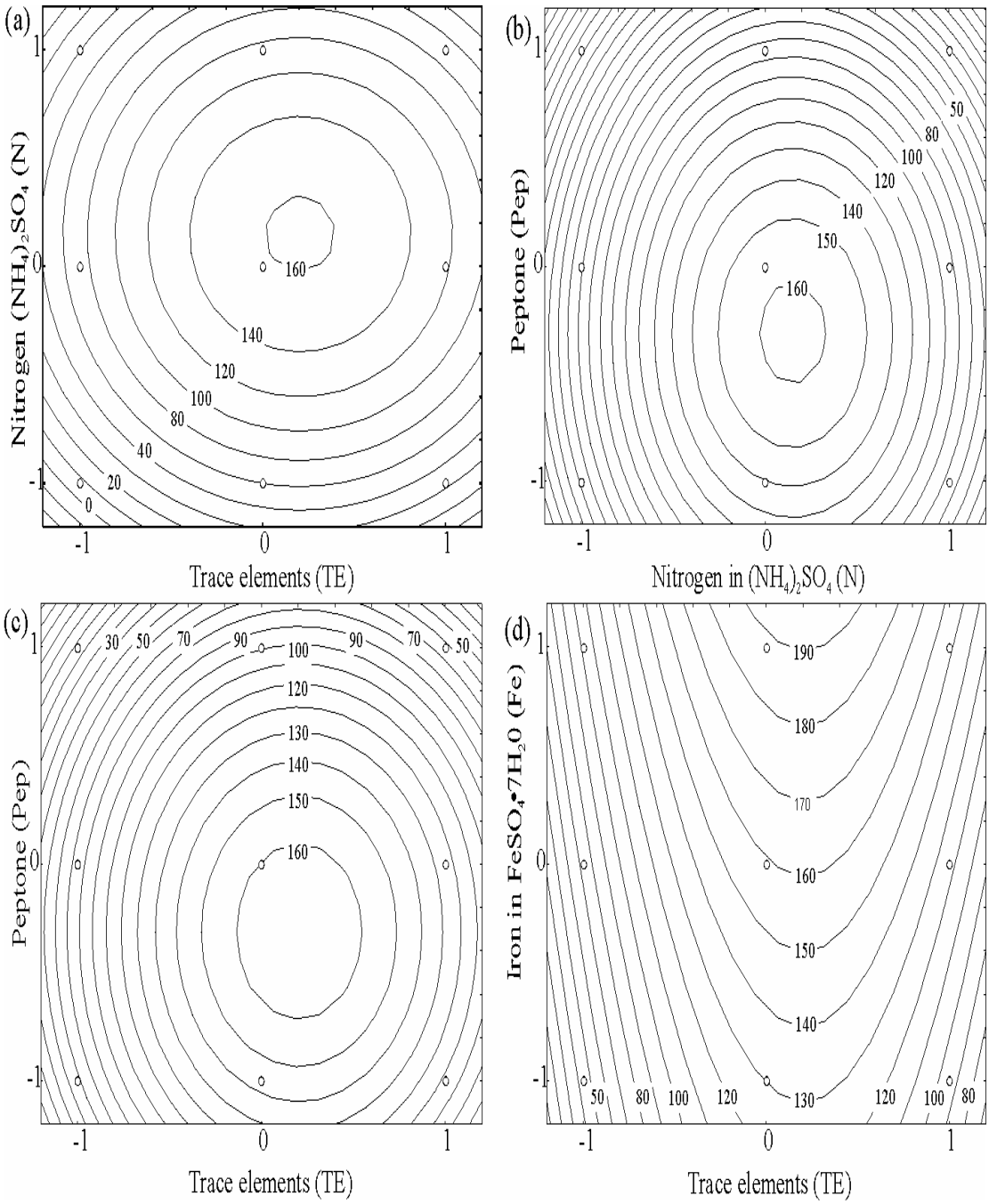

Figure 6.6. Contour diagram of squalestatin S1 generated by the model as a function of; (a) nitrogen and trace elements, (b) peptone and trace elements, (c) peptone and nitrogen, (d) iron and trace elements. The coded values for: nitrogen (*N*): 1 g l^{-1} (-1), 2 g l^{-1} (0) and 3 g l^{-1} (+1), trace elements (*TE*): 5 ml l^{-1} (-1), 10 ml l^{-1} (0) and 15 ml l^{-1} (+1), peptone (*Pep*): 5 g l^{-1} (-1), 10 g l^{-1} (0) and 15 g l^{-1} (+1), and iron (Fe): 0.5 g l^{-1} (-1), 1.0 g l^{-1} (0) and 1.5 g l^{-1} (+1)

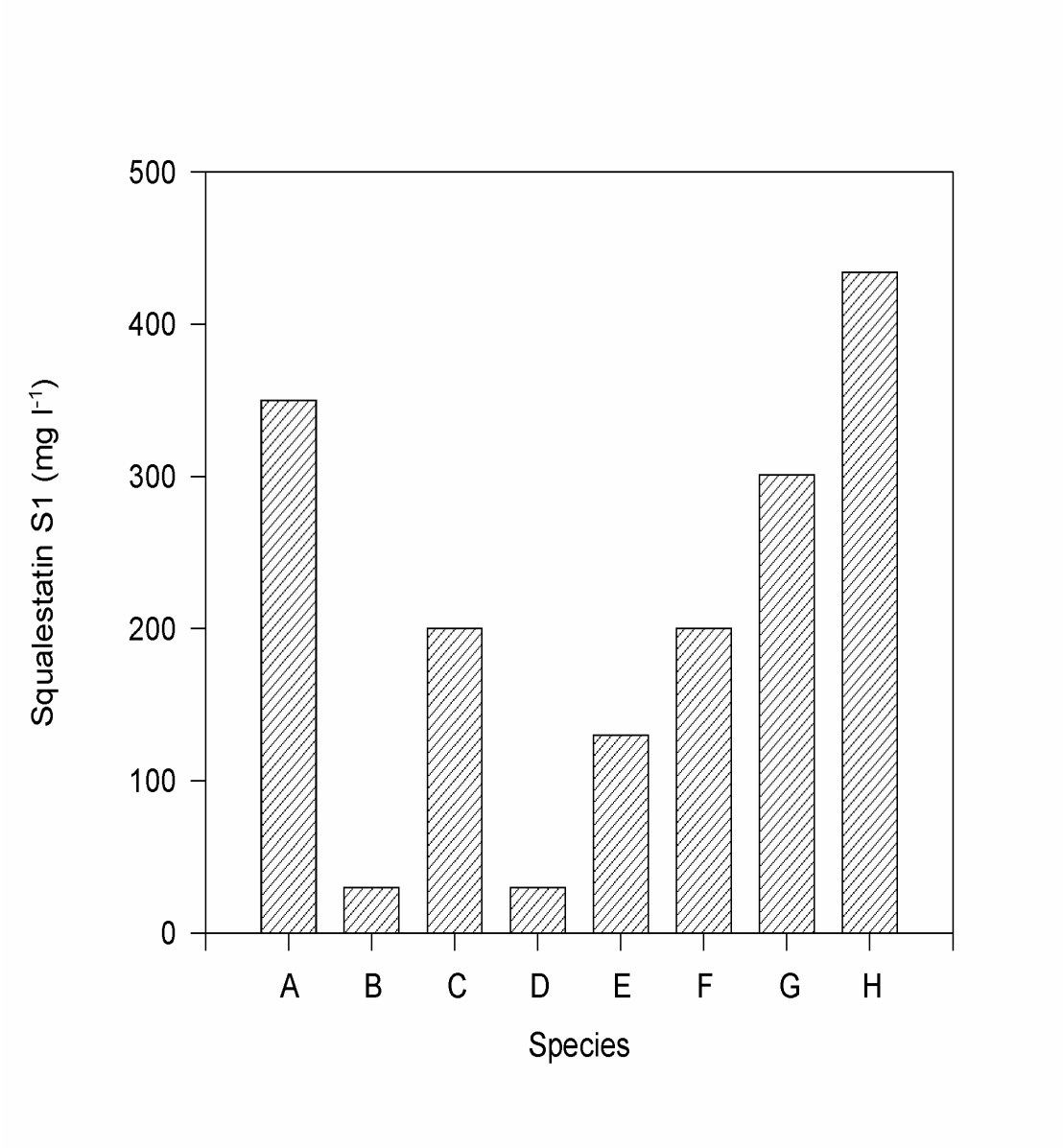

Figure 6.7. S1 concentrations reported in the literature for various species of fungi. (References in brackets): A) *Phoma* sp. (Bergstrom *et al*. (1993)), B) ATCC 20986 (fungus) (Huang *et al*. (1992)), C) *Curvularia lunata* (Bergstrom *et al*. (1993)), D) *Exserohilum rostratum* (Huang *et al*. (1992)), E) *Drechslera bisoplata* (Bergstrom *et al*. (1993)), F) *Phoma* sp (Aldred *et al*. (1999)), G) *Phoma* sp. (Baxter *et al*. (1998)), H) *Phoma* sp.(this work)

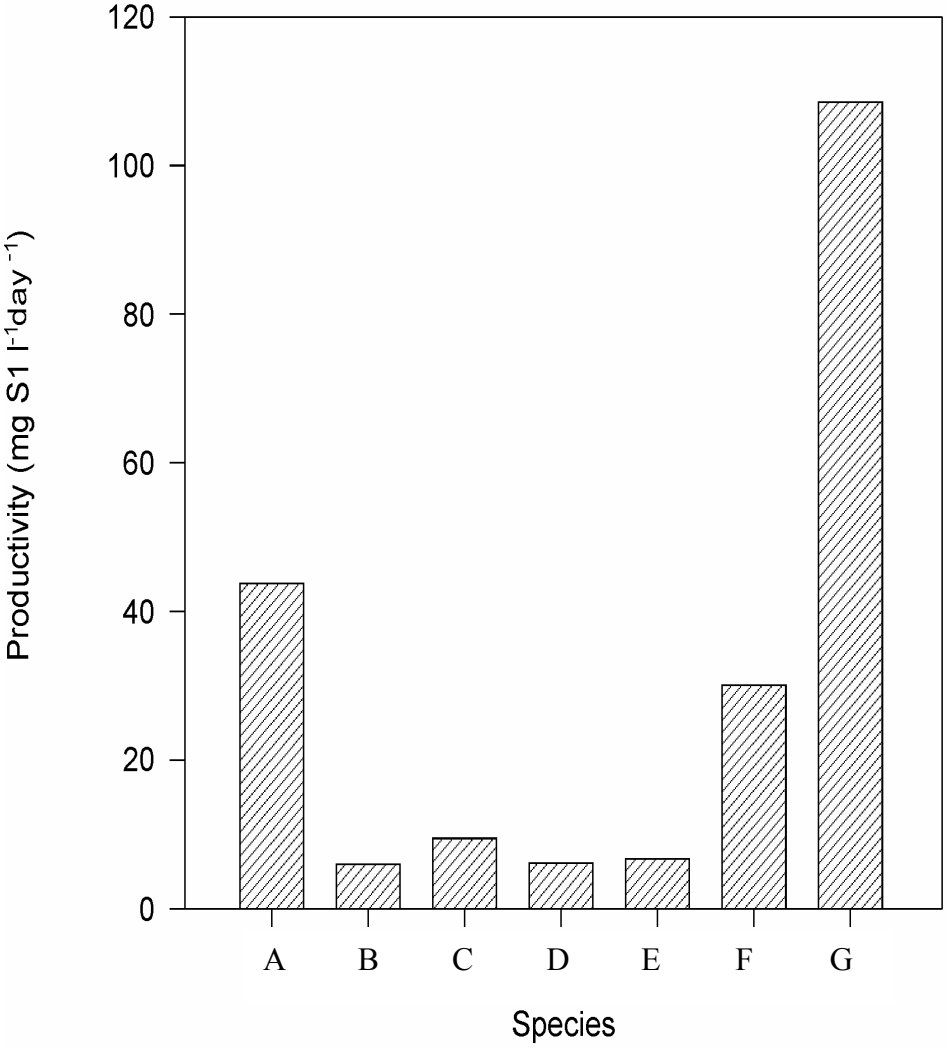

Figure 6.8. S1 productivity reported in the literature for various species of fungi. Key to references: A) *Phoma* sp. (Bergstrom *et al*. (1993)), B) ATCC 20986 (fungus) (Huang *et al*. (1992)), C) *Curvularia lunata* (Bergstrom *et al*. (1993)), D) *Drechslera bisoplata* (Bergstrom *et al*. (1993)), E) *Phoma* sp (Aldred *et al*. (1999)), F) *Phoma* sp. (Baxter *et al*. (1998)), G) *Phoma* sp.(this work).

6.4 DISCUSSION

To achieve the results obtained in this study using a full factorial design would have required 3^{13} x 2 replicates (3.2 x 10^6) experiments taking into account all the variables involved. However, by using the orthogonal matrix design, a significantly smaller combination of factors and levels could be used for effectively examining the effect of interacting factors on final concentration. Thus, with only 54 experiments an optimal medium composition was found that represented a 42% increase in product concentration compared to the non-optimized medium. The concentration of S1 achieved in this work of 387 mg l^{-1} represented a 24 % improvement when compared to the highest reported in the literature (Bergstrom *et al*., 1993). Moreover a considerable improvement in productivity (50%) was achieved because the new medium was designed to achieve a more rapid S1 production with suitability for industrial applications.

These kind of designs have been successfully applied to improving media formulation for the production of primary and secondary metabolites in fermentation processes (White *et al*., 1990; Lee *et al*., 1997; Escamilla *et al*., 2000; Li *et al*., 2001). For example, concentrations of another cholesterol-lowering drug, lovastatin, produced by *Aspergillus terreus,* was improved with Plackett-Burman screening factorial designs (Lai *et al*., 2003). However, there are no reports of a combined orthogonal design and contour surfaces methodology as far as we know, in the optimization of cholesterol lowering drugs.

The combined statistical strategies of orthogonal design and surface response succeeded in predicting the optimal condition and interactions between substrate type and concentrations. This work demonstrated the efficacy of the combined methodology achieving the maximum squalestatin S1 production reported in liquid medium. It produced a rapid screening and reliable methodology for the optimization of medium design. The contour surface methodology was a good tool to separate the effect of interactions and to find the optimal conditions between two factors. Evaluating the contour surface methodology provided a better understanding of the interactions between the fermentation medium substrates and their effects on the concentrations of S1. The R^2 values of all parameters showed a good fit between the

model and the experimental data. Contour plots were useful to locate the optimal points easily. This approach for medium optimization of S1 production by the *Phoma* species using this combined approach has not been reported previously.

This work was also an attempt to demonstrate the applicability of the statistical design to optimization of S1 production using a large number of medium combinations and levels by including dummy factors to find reliability and robustness of the experimental design. The use of a dummy factor is an *in situ* evaluation of the reproducibility and robustness of the experiment. Thus, in the experiment we found consistency of glucose and the glucose replicate. Glucose was a better substrate for the production of squalestatin S1 in comparison with glycerol. However, only at low concentrations did glucose produce high concentrations. This suggests that catabolic repression of glucose occurs at higher levels (Pan and Xu, 2003). Thus, under optimal conditions it was possible to surpass the highest squalestatin S1 concentration so far reported in liquid fermentation.

Together, this leads to the conclusion that a combined strategy of orthogonal design and contour surface methodology is an excellent means for fermentation medium optimization in the production of pharmaceutical compounds and enzymes. From an economic point of view the most important parameters for screening and optimizing media for the production of pharmaceutical compounds are time and cost. The combined strategies demonstrated advantages in comparison with traditional methods.

The precursor yeast extract, and the oily precursor cottonseed flour were very important components of the medium for the production of squalestatins and this is in agreement with previous reports (Bergstrom *et al*., 1993; Blow *et al*., 1994; Connors *et al*., 1995; Dawson *et al*., 1991; Dufresne *et al*., 1992). Trace element, sodium citrate, lactose concentration requirements were at levels similar to those in the literature (Dufresne *et al*., 1992). However, the medium required the addition of important new components such as nitrogen, magnesium and iron as part of the improved medium for enhance S1 production. In addition to establishing optimal fermentation medium composition for scale up, the present work makes it possible to predict both concentrations and productivity under different conditions by means of the contour surfaces and the polynomial model. This is useful not only for the

additional knowledge supplied about the process, but also for the potential in medium engineering and evaluation under economic constrains of medium composition, concentration and productivity. It is also possible to examine the economics of scale up of the process from flask to bioreactor. Future work on scale-up of the squalestatin S1 fermentation process in bioreactors will be evaluated to examine larger scale production and the impact on concentration/productivity.

CHAPTER VII

DESIGN OF A BUBBLE COLUMN BIOREACTOR BASED ON THE VOLUMETRIC MASS TRANFER COEFFICIENT AND SCALE UP OF SQUALESTATIN S1 PRODUCED BY *Phoma sp.*

7.1 INTODUCTION

The cholesterol lowering agent squalestatin produced by several fungal species has attracted attention in recent years. It is considered an important active ingredient for the poly-pill which prevents several deadly conditions, such as heart attacks. Among the various species that produce squalestatins as secondary metabolites, *Phoma* sp. is particularly important for its high concentrations of squalestatins (Dawson *et al*., 1991; Baxter *et al*., 1998).

In the scale up process it is vital to investigate the type of bioreactor suitable according to the physical and physiological requirements of the microorganism used before starting further optimizations. In general, stirred tank reactors are the most widely used bioreactors in the production of pharmaceutical compounds. This type of reactor offers some advantages, such as independent control of mixing conditions and aeration rate. The main disadvantages are the high energy consumption and high shear stress affecting such fragile cells such as fungal mycelium. Bubble column bioreactors (BC) are a good alternative to the stirred tank bioreactor (STR). The main advantages of bubble column reactors include the relatively simple construction and absence of mechanically moving parts enabling easy maintenance and low operating costs. With regard to the internal flow and efficiency, they offer a large interfacial area and transport rates, leading to excellent heat and mass transfer characteristics and more suitable shear conditions for fungal growth and secondary metabolite/enzyme production.

The objective of this study was to examine the effect of the liquid height and the height-diameter (H_D/D_r) ratios (11.1, 7.4 and 3.7) on the total volumetric mass transfer coefficient, k_La, at different temperatures. The experimental data were also compared with that reported in the literature for different geometries and configurations. Once the optimal height-diameter (H_r/D_r) ratio was determined a fermentation experiments were carried out in the bubble column bioreactors (BC) for the production of squalestatin S1 by a *Phoma* sp.

7.2 MATERIALS AND METHODS

7.2.1 Volumetric mass transfer $k_L a$ experiment

These Figure 7.1 shows the configuration of the bubble column bioreactor designed and used in experiments. Three bioreactors were set up in parallel to facilitate replication (Figure 7.2). An inside diameter (D_r) of 0.07 m and heights (H_L) of 0.26, 0.52 and 0.78 m were used to load 1, 2 and 3 litre volumes for the different experimental runs. A sintered glass no 2 (Soham Scientific, United Kingdom) sparger distributor was placed at the base of each column. The bubble column bioreactor had a water jacket to keep the temperature constant. Water was circulated through the jacket with a heating water bath and pump (Grant Instruments, Cambridge, UK) at three set temperatures of 15, 25 and 30^{o}C.

The liquid phase used in the experiments consisted of demineralised water. Air was used as the gas phase. The sparger used was sintered glass plate No. 2. The airflow into all columns was regulated by the use of rotameters set at different flow rates (2, 4, 6 and 8 l min^{-1}), while nitrogen flow (used for stripping out the dissolved oxygen) was controlled by a manually operated control valve. For the determination of gas hold up, pressure taps were installed along the columns.

To determine the gas hold up, the gas flow rate in the columns was adjusted to the desired value using the corresponding previously calibrated rotameter. Sufficient time was allowed to achieve a steady state in each column after which the increase in liquid pressure at the manometer was recorded.

The overall gas hold up ε in the bioreactor was determined by visual measurements of the static liquid height H_L and the aeration height H_E. The gas hold up ε was calculated from the following equation:

$$\varepsilon = \frac{H_E - H_L}{H_E}. \qquad \text{(Eq. 7.1)}$$

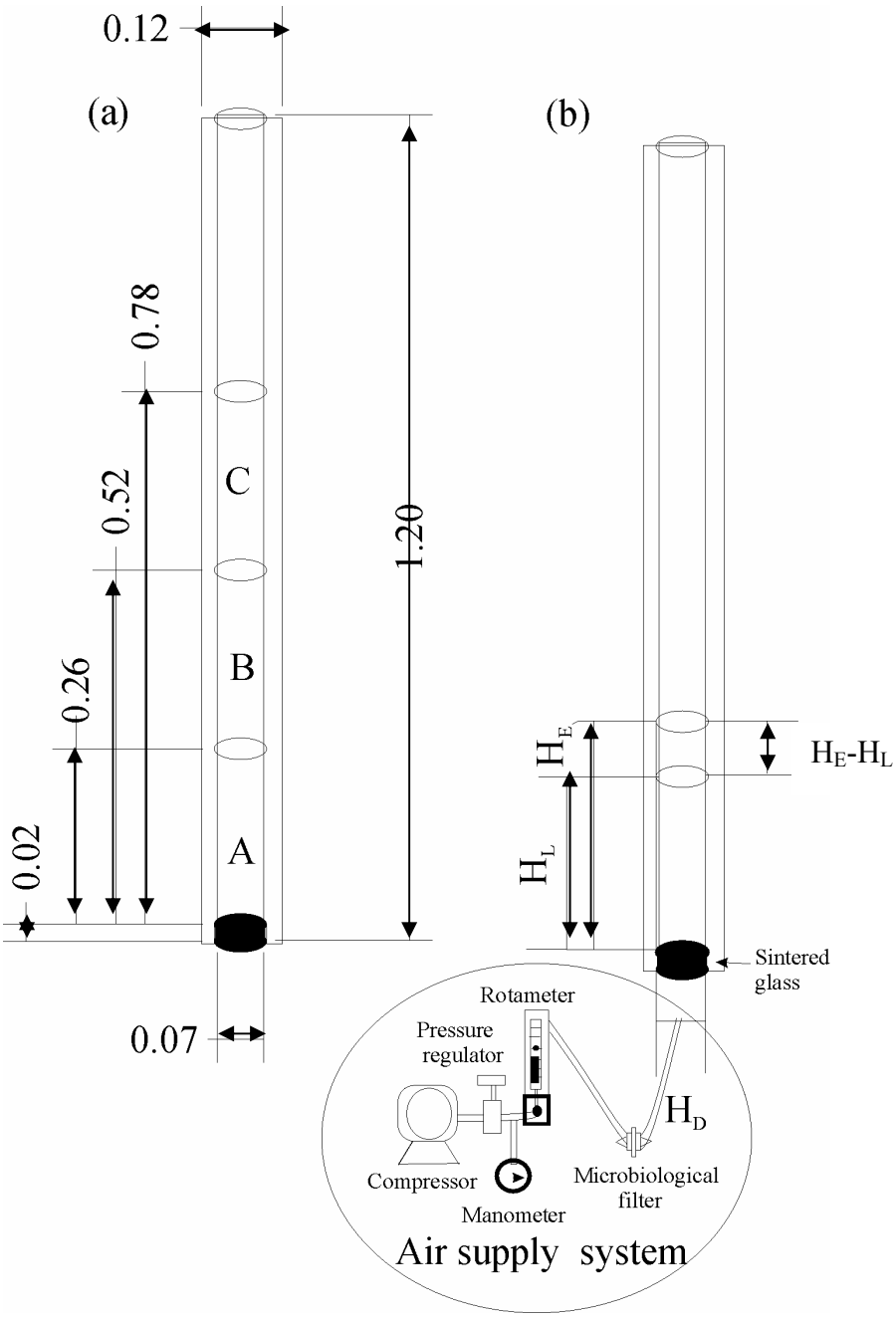

Figure 7.1. (a). Schematic diagram of the bioreactor dimensions (a) (measurements in m): 1 litre (A), 2 litres (B) and 3 litres (C). Air supply system and nomenclature dimensions (b): height of the liquid with (H_E) and without (H_L) aeration, column diameter (D_r), and gas hold up (ε).

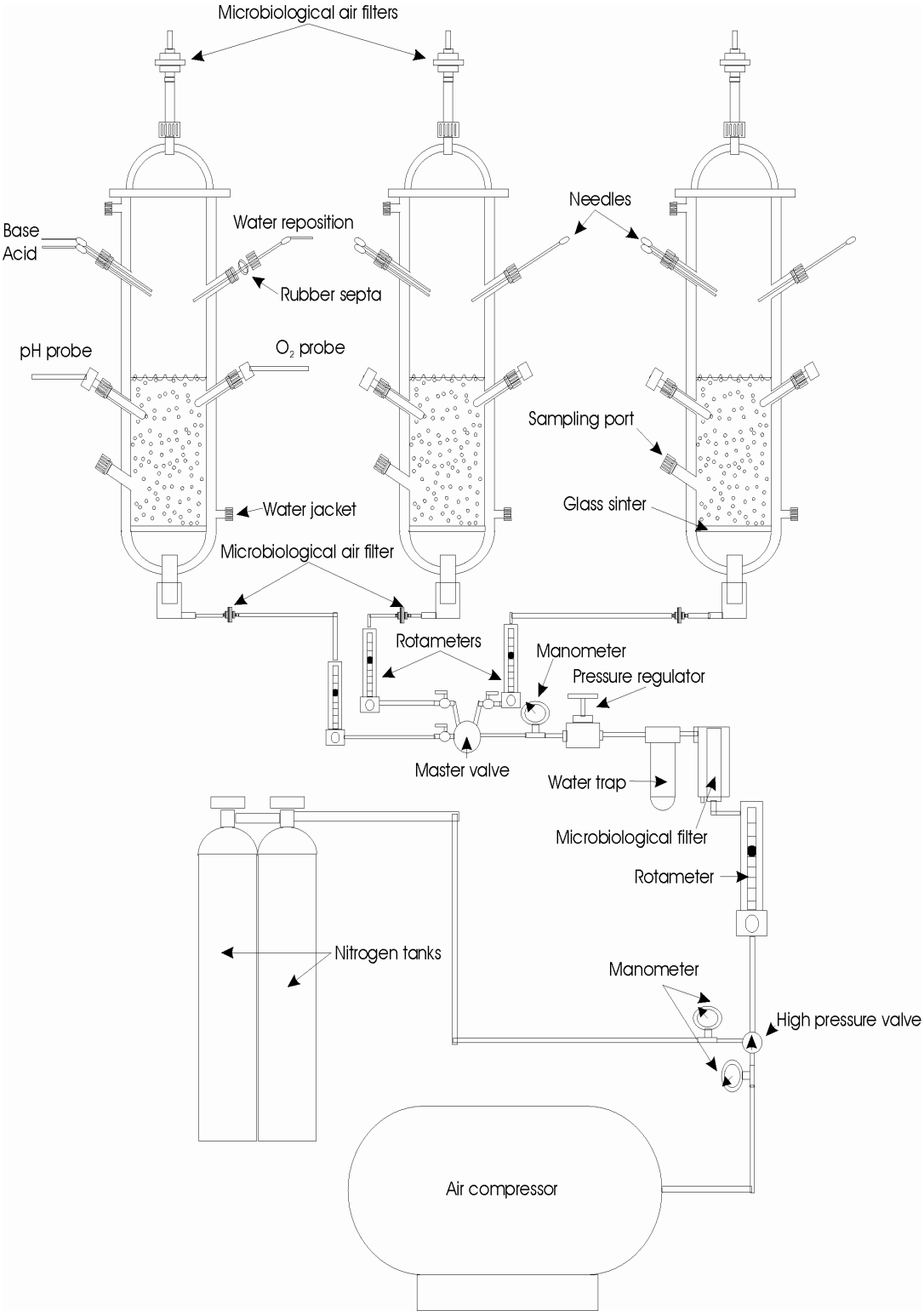

Figure 7.2. Diagram of the bioreactor configuration and connections set.

The superficial gas velocity (*U*) as a function of the characteristics of the reactor was calculated on the bases of the gas and liquid properties and the hydraulic conditions as follows:

$$U = \frac{Q_m RT}{V_L \rho_L g} \ln\left(1 + \frac{\rho_L g H_L}{P_h}\right) \qquad \text{(Eq. 7.2)}$$

Where Q_m = air molar flow (kmol s^{-1}), P_h = reactor head-space pressure (Pa), H_L = height of the liquid without aeration (m), g = acceleration due to gravity (m s^{-2}), ρ_L= water density (kg m^{-3}), R = gas constant (8.134 J K^{-1} mol^{-1}) and T = temperature (K). The overall volumetric oxygen transfer coefficient k_La was determined by the static gassing-in method (Bandyopadyay *et al*., 1967). A dissolved oxygen probe was set at 0.2 m above the diffuser. The dissolved oxygen concentration was measured by means of a polarographic dissolved-oxygen electrode (Thermo Russell: PDOC122/30K/WP, Auchtermuchty, UK) with integral temperature compensation connected to a dissolved-oxygen meter (Thermo Russell: RL425. Auchtermuchty, United Kingdom). The oxygen electrode was placed in the bubble column at a distance of 0.2 m above the gas distributor.

Firstly, the water was stripped of oxygen almost completely by the bubbling of pure nitrogen through the distributor. When all the oxygen had been stripped out, air was sparged into the column and the oxygen uptake into the liquid phase was monitored continuously by means of the oxygen sensor. The total measurement time was long enough to reach the oxygen saturation concentration, C^*.

The concentration change of the dissolved oxygen was measured using an oxygen meter. The k_La was calculated with reference to total reactor volume from the following expression:

$$k_L a = \frac{1}{t} \ln\left(\frac{C^* - C_{L0}}{C^* - C_L}\right). \qquad \text{(Eq. 7.3)}$$

where t is the time (s) and C_{L0} and C_L are the initial oxygen concentration and the concentration at different times. The volumetric mass transfer coefficient per unit volume of liquid in the bubble column ($k_L a_L$) is calculated from the following expression:

$$k_L a_L = \frac{k_L \mathrm{a}}{(1-\varepsilon)} \qquad \text{(Eq. 7.4)}$$

7.2.2 FERMENTATION

Fungal strains

See section 6.2.1

Activation and strain inoculation

See section 6.2.3

Media composition

See section 6.2.3

7.2.3 BIOREACTOR

The bubble column bioreactor (BCR) (Soham Scientific, United Kingdom) with the geometric ratio H_L/D_r= 3.7 equivalent to 1 l fermentation medium was loaded into the BC. A sintered glass plate of porosity 30 μm and 10 mm thickness was used as aerator in the columns. From the optimal k_La experiment the fermentations were performed at the superficial gas velocity of U = 0.120 m s^{-1} and a process temperature of T = 25°C.

All reactors were equipped with sterilizable pH (PHT20**)** (Thermo Russell) and oxygen (Bioengineering, Switzerland) probes, connected to a Bioengineering central controller (Bioengineering, Switzerland) for on-line measurements. Oxygen partial pressure measurements were measured by a polarographic electrode. Samples to determine biomass and S1 concentration were taken from a sterile suction port. Biomass was determined by filtering 4 ml onto filter paper (Whatman no. 2), dried in an oven at 90°C and weighed.

7.2.4 ANALYSIS OF SQUALESTATIN S1 PRODUCITION

See section 6.2.4

7.3 RESULTS AND DISCUSSION

In our experiments, the diameter of the column (D_r) was 0.07 m and the heights of liquid (H_L) were 0.78 m, 0.52m and 0.26 m. The H_L/D_r ratios were: 11.4, 7.4 and 3.7. Figure 7.3 shows the relationship between gas hold up ε and superficial gas velocity U. The gas hold up increased linearly as the superficial gas velocity increased. The gas hold up ε was found to decrease slightly with the increase in the liquid height H_L due to increased liquid recirculation with increasing column height at the different temperatures of the experiment (Figure 7.3 a, b, c). This result is in agreement with various previously reported studies (Ruzicka *et al*., 2003; Ruzicka *et al*., 2001).

A number of investigators have studied the influence of H_L/D_r on ε and $k_L a$ in pneumatic bioreactors. Ruzicka *et al*. (2001) suggested that ε decreased as H_L/D_r increased. Chisti (1989) revealed that for solid-free liquid, the hold up declined slightly with the height of split-cylinder bioreactors. The experiments of Weiland (1984) were carried out using three different ratios H_L/D_r of 12.8, 10.2 and 8.5. Their optimal H_L/D_r value was 10.2 at which there was a maximum value of ε.

No significant effect of the temperature on the hold up (ε) was observed in the experiment. However, the highest temperature of the experiment, 30^{o}C, produced a slightly higher hold up (0.105) in comparison to the other temperatures at the ratio H_L/D_r 3.4 at a high superficial gas velocity (0.120 m s^{-1}). Interestingly, at the same superficial gas velocity, with a ratio H_L/D_r 7.4 and 25^{o}C, a higher hold up was found (0.111).

This change in the gas hold up is due to increased liquid recirculation with the increase of the height of the column and the hydrostatic pressure (Krishan *et al*., 1999). The effect of the rheological properties (viscosity and mixing) with the increase of the ratio (H_L/D_r) affects mass transfer rates, reducing the oxygen concentration in the bulk.

The volumetric mass transfer coefficient k_La increased as the gas velocity increased (Figure 7.4). The k_La was highly influenced by the H_L/D_r ratio. For example, at 15°C and with the highest superficial gas velocities, the H_L/D_r ratio of 3.7 had a k_La 60% higher than at 11.7. Overall the H_L/D_r ratio of 3.7 produced the highest mass transfer coefficients at all superficial gas velocities. Thus, a greater liquid height resulted in lower mass transfer coefficients. This can be interpreted in terms of the different sizes of the bubble swarms. At the lower H_L/D_r ratio of 3.7 small bubbles could be formed, giving more efficient mass transfer conditions. However, increasing the H_L/D_r ratio produced an increase in bubble coalescence and a decrease in superficial area. Only a slight temperature effect on the k_La was observed. The reactor operation volume represented by the H_L/D_r ratio was the most important factor regardless of the temperature.

Figure 7.5 shows the k_La/ε as a function of the superficial gas velocity (U) for the three column H_L/D_r ratios. There are two distinct components of these plots. Firstly, a steep k_La/ε decrease occurs. Secondly, the k_La/ε reaches a constant value independently of the superficial gas velocity. There were important differences at which the constant k_La/ε was achieved as shown in Figure 7.5a. The k_La/ε started at a high value, then decreases to reache a constant value. For a superficial gas velocity below 0.08 m s^{-1}, the k_La/ε value was 2.5 s^{-1} initially and then decreased to a constant value of 1.5 s^{-1} for the H_L/D_r 3.7 and 7.4 ratios at 15°C. However, at the lower H_L/D_r ratio of 11.4, a much lower constant k_La/ε of 0.76 s^{-1} was achieved. Moreover the minimum U changed from 0.8 m s^{-1} at ratios of 3.7 and 7.4 to 0.05 m s^{-1}. A similar trend was obtained at 25 and 30°C (Figs. 7.5b and c) with a constant value of ~1.0 s^{-1} at all H_L/D_r ratios. The parameter k_La/ε represents the volumetric mass transfer coefficient per unit volume of bubbles. One interpretation of the constant k_La/ε is that the effective bubble diameter d_b is independent of the gas velocity. The k_La/ε is constant in both heterogeneous and homogeneous flow regimens (Vermeer and Krishna, 1981; Vandu and Krishna, 2004).

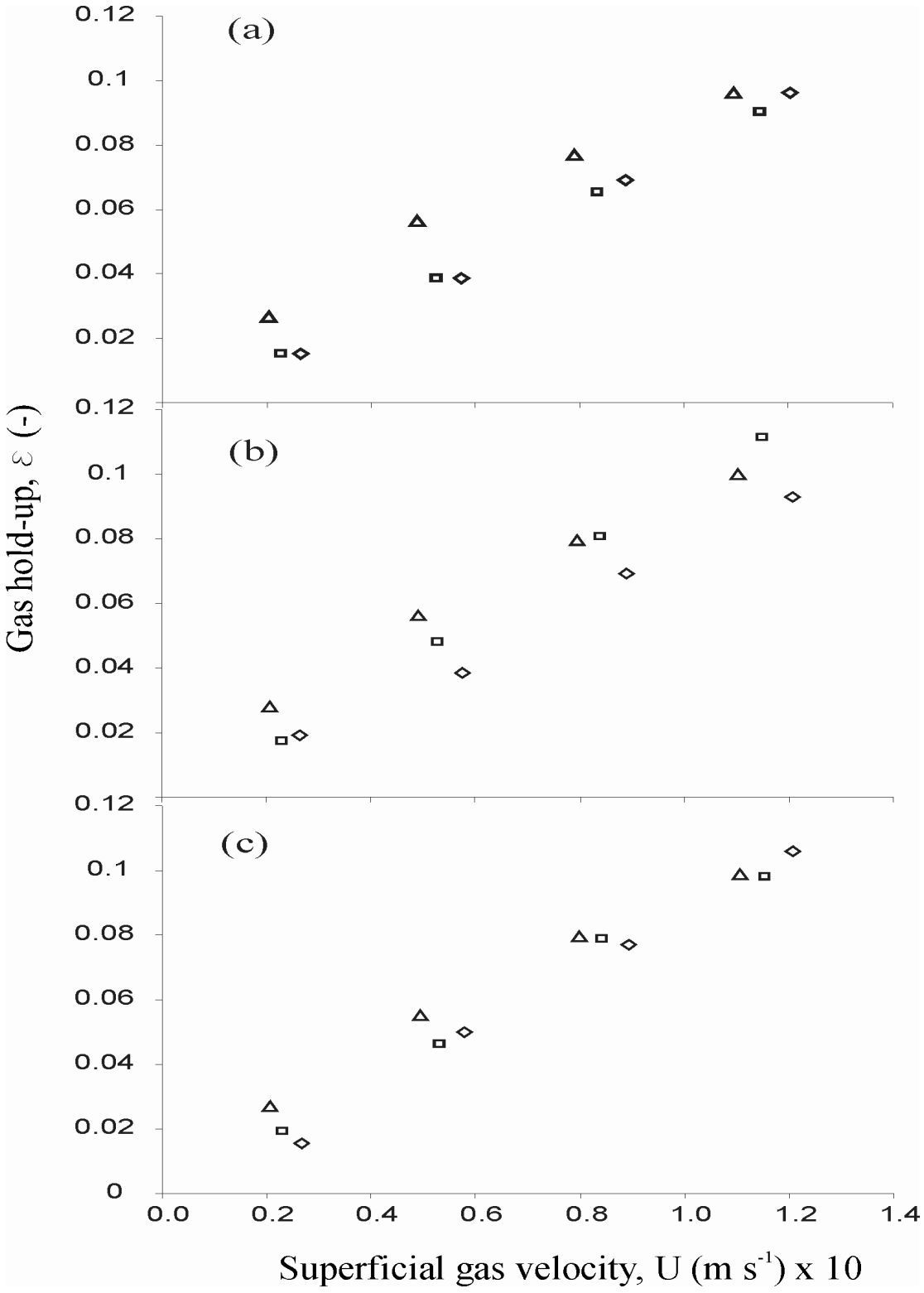

Figure 7.3. Gas hold up ε as a function of superficial gas velocity U, obtained in three columns with height/diameter ratio (H_L/D_r): 11.4 (◊), 7.4 (□) and 3.7 (Δ) at three different temperatures 15 °C (a), 25 °C (b) and 30°C (c).

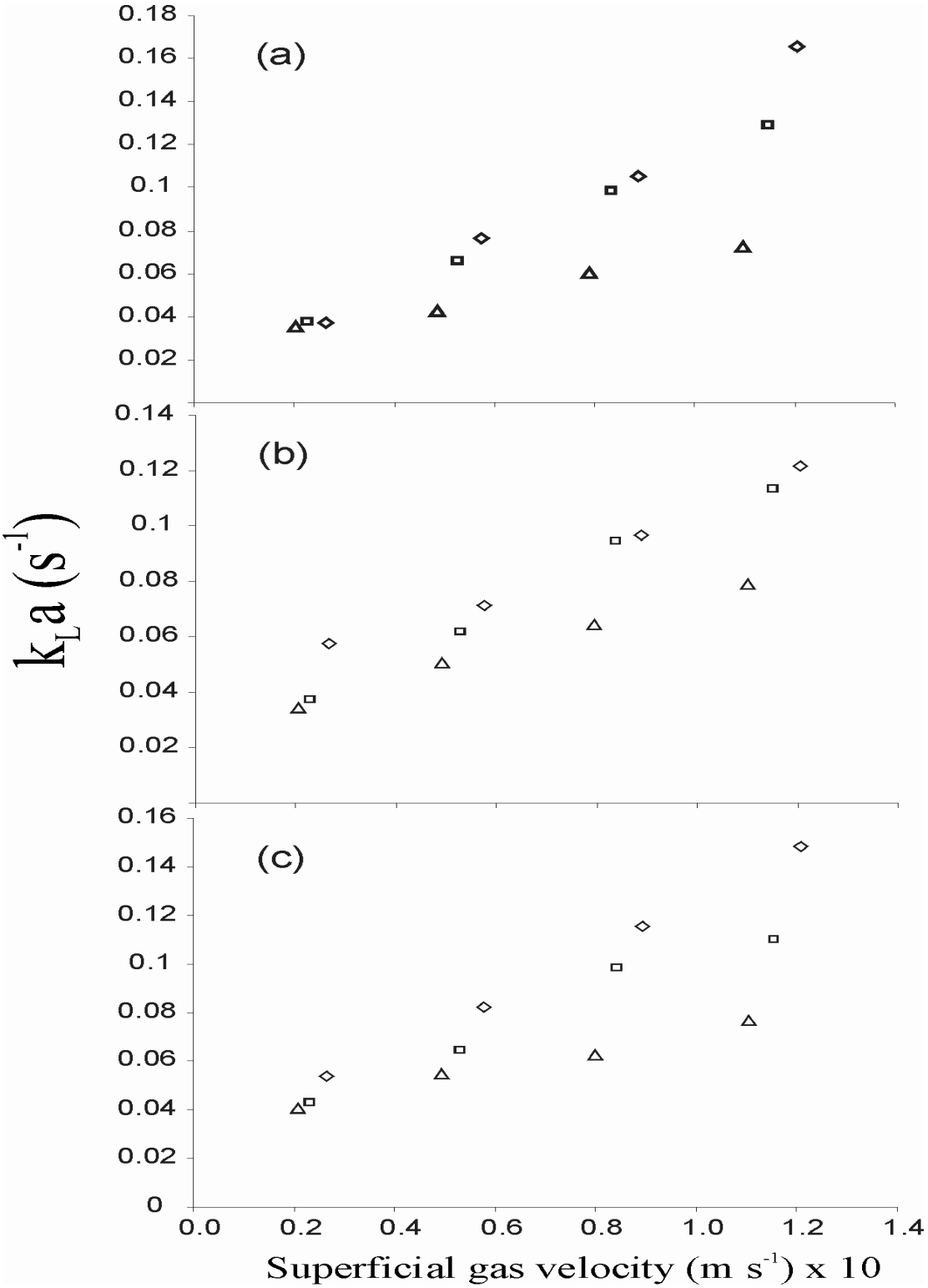

Figure 7.4. Variation of the volumetric mass transfer coefficient k_La with superficial gas velocity U at different column ratios (H_L/D_r): 11.4 (Δ), 7.4 (□) and 3.7 (◊) at three different temperatures 15 ^oC (a), 25 ^oC (b) and 30^oC (c).

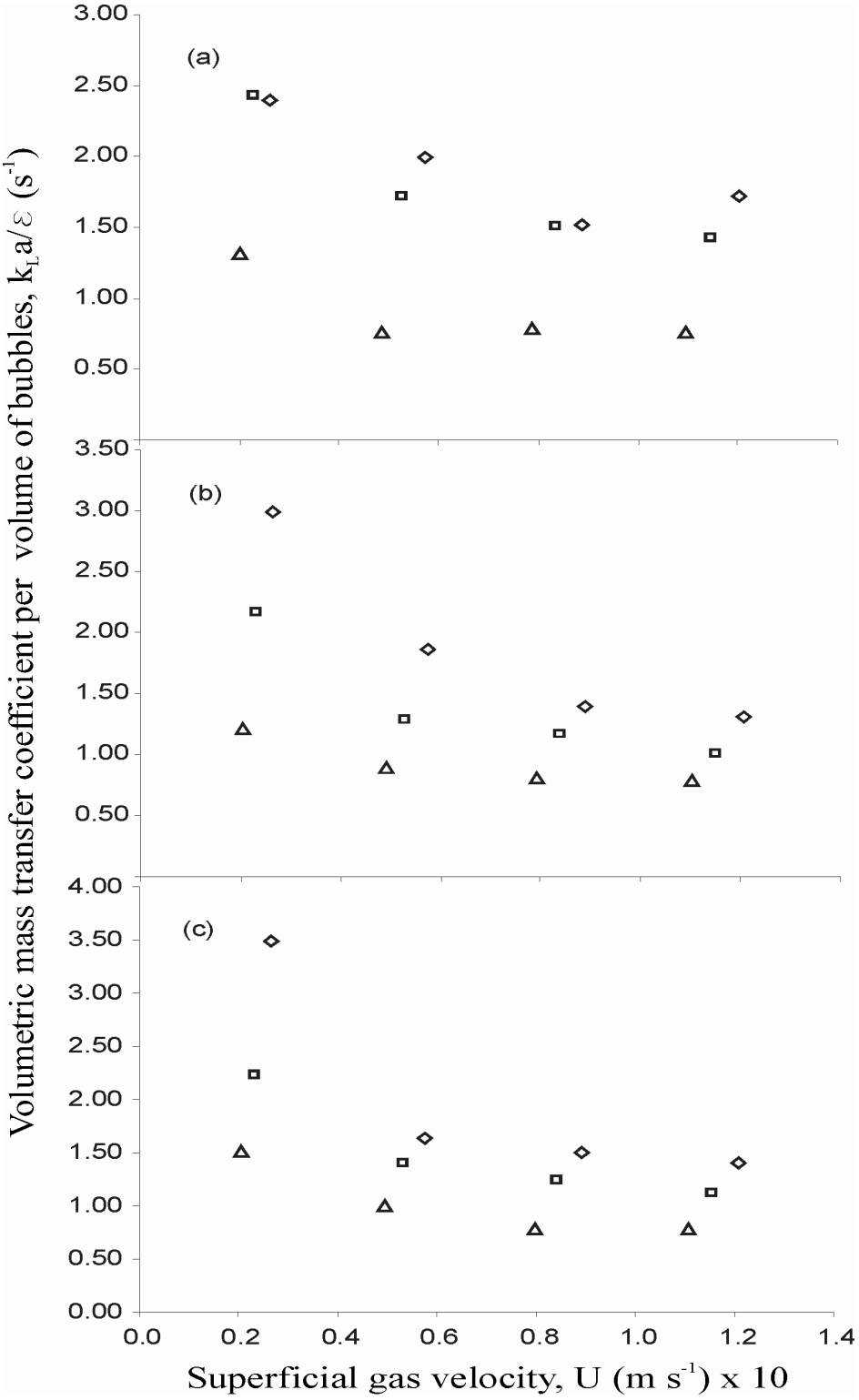

Figure 7.5. Variation of the volumetric mass transfer coefficient k_La/ε with superficial gas velocity U at different column ratios (H_L/D_r): 11.4 (Δ), 7.4 (□) and 3.7 (◊) at three different temperatures 15°C (a), 25°C (b) and 30°C (c).

Figure 7.6 shows the effect of the H_L/D_r ratio on the k_La at different superficial gas velocities. Regardless of the temperature an excellent volumetric mass transfer coefficient was achieved at high flow rates (1.3×10^{-4}, 1.0×10^{-4} m^3 s^{-1}). The k_La difference within the range of temperatures was only 0.0086 s^{-1} with the H_L/D_r ratio of 11.4 at 15°C. In contrast, significant differences (k_La ; 0.0372 s^{-1}) were found between the maximal and minimal flow rates. The effect of the H_L/D_r ratio on the k_La was not observed at the lowest superficial gas velocities. This result points to the importance of the high superficial gas velocities needed in a bubble column in order to gain a efficient mass transfer coefficient under the set geometry. The H_L/D_r ratios of 3.7 and 7.4 represent the best options for optimal mass transfer. At 25°C no significant differences were observed between these H_L/D_r ratios. Figure 7.7 shows the linear relationship between the hold up and the power consumption (P_G/V_L). The curves for the different ratios (H_L/D_r) related to the power consumption and the gas hold up are similar. Previously, Chisti (1989) reported no effect of the liquid height on gas hold up. The commonly used volumetric mass transfer coefficient per unit volume of (gas + liquid) dispersion against the power consumption is shown in Figure 7.8. The increase of k_La was directly related to the power consumption (exponentially). Interestingly, at low temperatures a higher k_La was achieved under the different geometric ratios and with a similar power consumption. This result points out the effect of oxygen solubility at lower temperatures and the mixing effect related to the viscosity as a function of temperature and shear stress. The H_L/D_r ratio of 11.7 under similar power consumption led to a more static and viscous medium, causing lower mass transfer coefficients. No differences were found between the geometric ratios 3.7 and 7.4 at 15 and 25°C. This result could be interpreted as the influence of the column hydrodynamics on the rheological properties of the liquid, which is influenced by the volume of liquid. An increase in the liquid volume caused a reduction of mass transfer under the same superficial gas velocities. Overall, superficial gas velocities and temperatures and a H_L/D_r ratio of 3.7 produced a better mass transfer in comparison with higher ratios of 7.4 and 11.7. Chisti (1989) reported no influence of the liquid height in a bubble column (H_L) on the k_La_L in any fluid. In the present study no influence was found between the ratios 3.7 and 7.4. However a (H_L/D_r) ratio of 11.4 showed significant differences. This result emphasizes the importance of selecting a suitable H_L/D_r ratio in the scale up process.

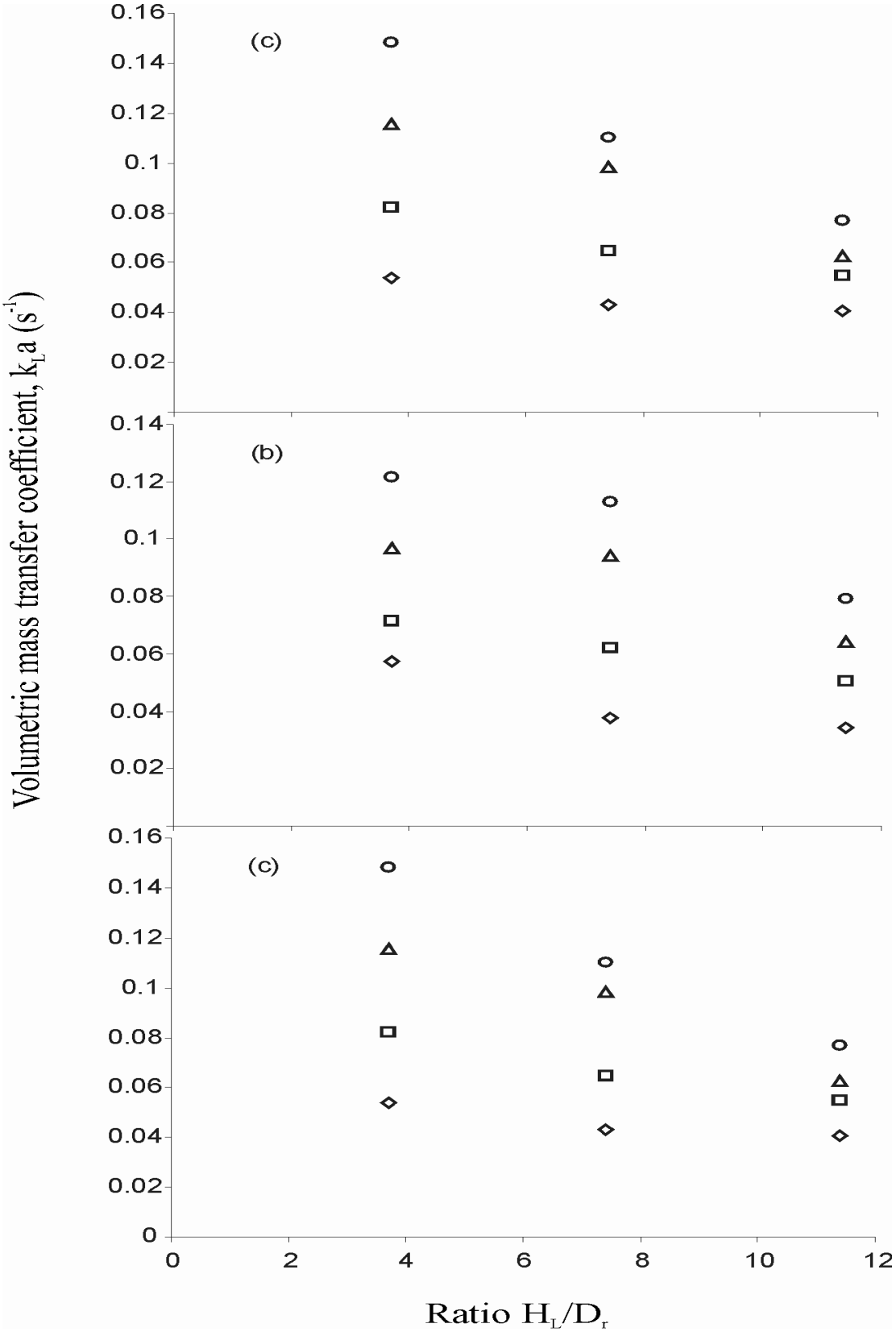

Figure 7.6. Effect of H_L/D_r on the k_La under different average superficial gas velocities U x 10^2 (m s^{-1}): 12 (○), 8.4 (Δ), 5.3 (□) and 2.3 (◊)

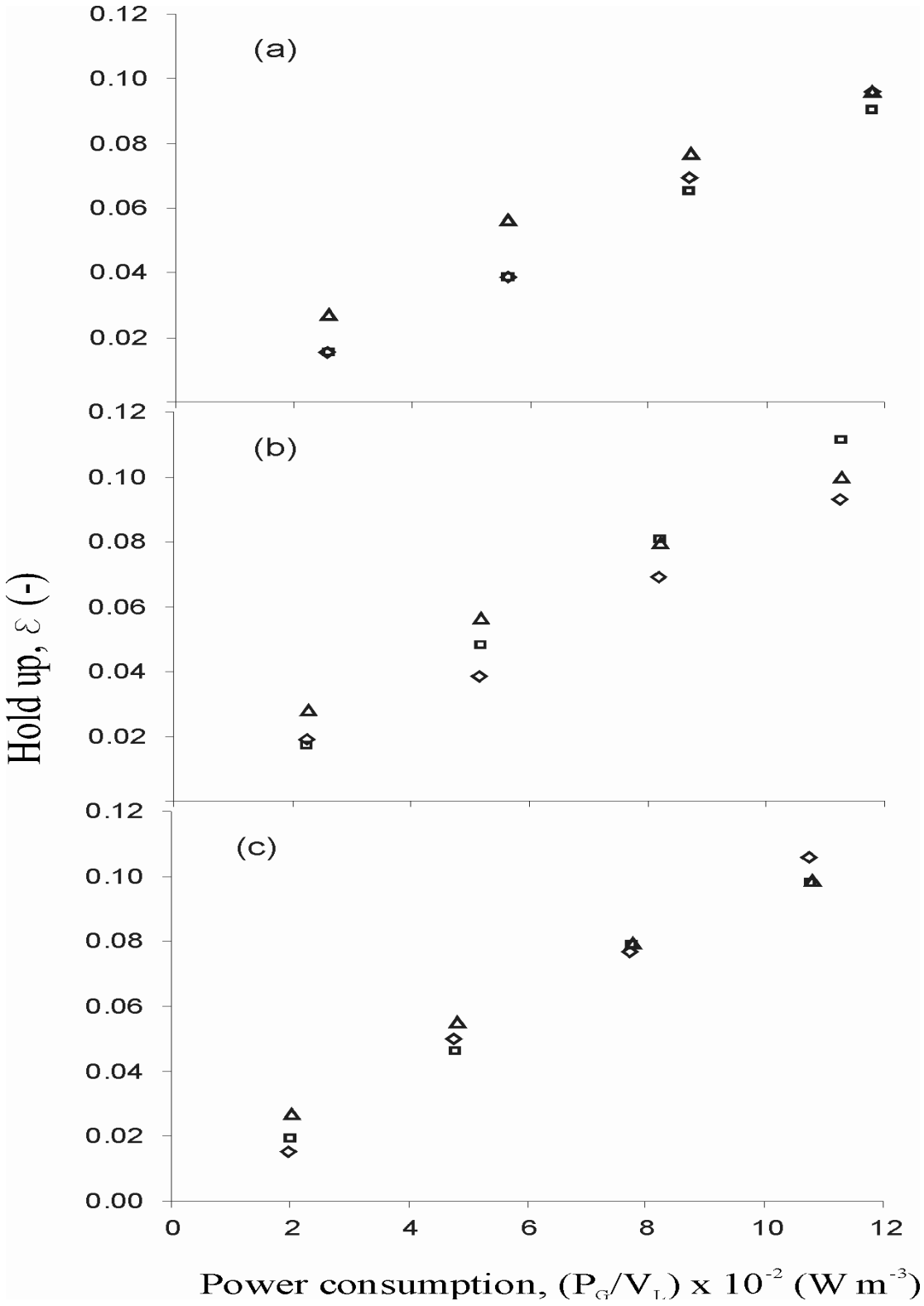

Figure 7.7. Hold up vs. power consumption in the column (air-water) at different column ratios (H_L/D_r): 11.4 (◊), 7.4 (□) and 3.7 (Δ) at three different temperatures 15°C (a), 25°C (b) and 30°C (c).

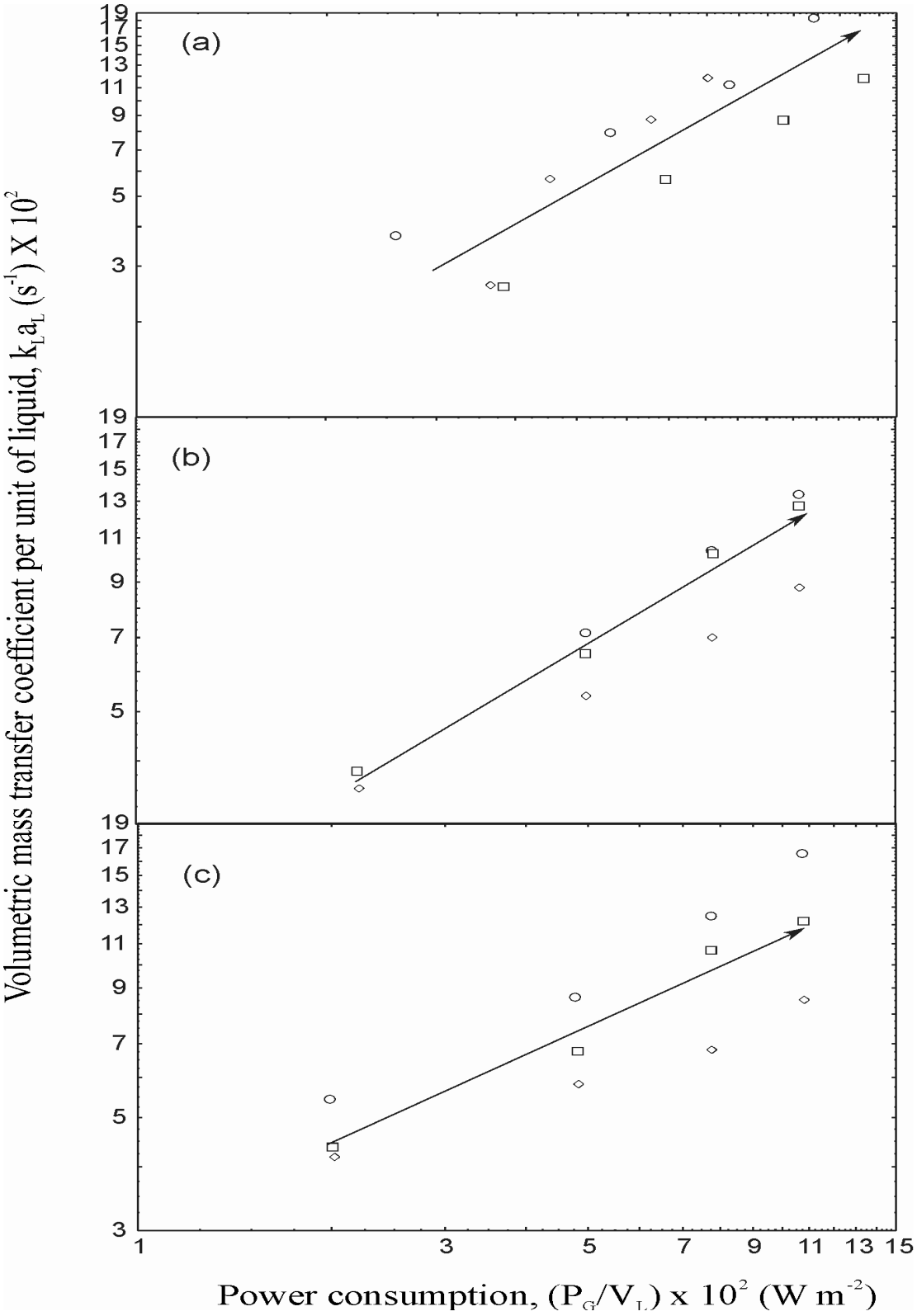

Figure 7.8. Mass transfer coefficient vs. power consumption in the column (air-water) at different column ratios (H_L/D_r): 11.4 (◊), 7.4 (□) and 3.7 (Δ) at three different temperatures 15°C (a), 25°C (b) and 30°C (c). The arrow represents the slope of the tendencies.

There are a large number of correlations for predicting the overall mass transfer coefficient k_La in bubble columns and in columns which have internal devices, such as draft tubes, to enhance liquid circulation. However, as far as we are aware no correlation has been proposed in relation to the H_L/D_r ratio. In the bubble columns, the key parameters are the superficial gas velocity *U* and the liquid properties that can be evaluated by the power consumption (P_G/P_V). The general model reported in the literature is presented here for the superficial gas velocity and power consumption (P_G/P_V) (after Chsti, 1989; Van't Riet, 1979).

$$k_L a_L = \alpha(U)^{\beta} \qquad \text{(Eq.7.5)}$$

$$k_L a_L = \alpha\left(\frac{P_G}{V_L}\right)^{\beta} \qquad \text{(Eq.7.6)}$$

The correlation of the model was good, however, a slight difference was produced at high superficial gas velocities. Figure 7.9 shows this and fit and the points that are above or below the diagonal line where under prediction was achieved. Thus several violations of the model were found in the analysis at high superficial gas velocities.

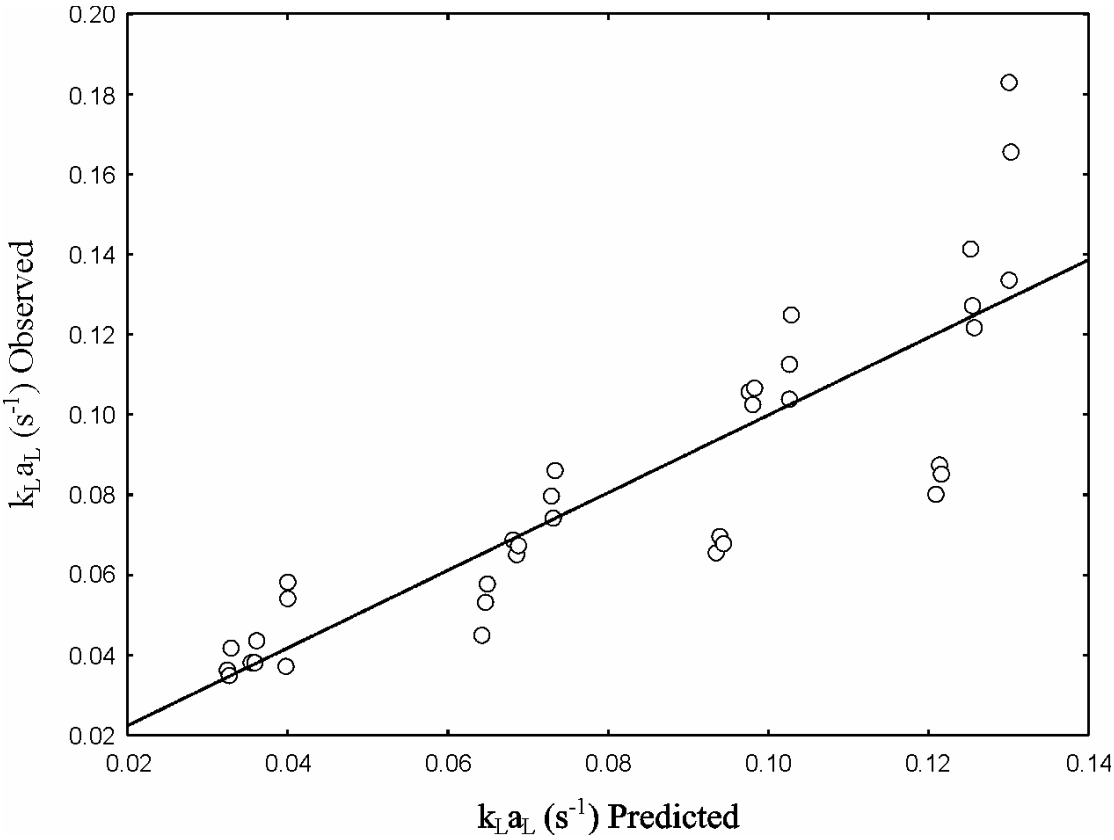

Figure 7.9. Residual diagnostics of the k_La_L surface generated by the model Eq. 6 (Van't Riet, 1979) of the predicted vs. observed.

Based on this we propose a modification of Eq. 7.5 and Eq. 7.6 to include the bubble column ratio (H_L/D_r). The equation is valid in the range of 3.7- 1.4 (H_L/D_r) ratios (R^2=0.951 and R_{adj}^2=0.904)

$$k_L a = 1.34(U)^{0.74}\left(\frac{H_L}{D_r}\right)^{-0.43} \quad \text{(Eq. 7.7)}$$

Figure 7.10 shows the surface generated with the model. This shows the importance of the effect of the superficial gas velocity and how the H_L/H_D ratio becomes important at high *U*. Another parameter often used to forecast the volumetric mass transfer coefficient is the power consumption (P_G/P_V) and by adding the term of H_L/D_r ratio to the model we obtained a good fit (R^2=0.950 and R_{adj}^2=0.893)

$$k_L a = 1.6x10^{-3}\left(\frac{P_G}{V_L}\right)^{0.74}\left(\frac{H_L}{D_r}\right)^{-0.43} \quad \text{(Eq. 7.8)}$$

The surface generated with the model is shown in Figure 7.11. It shows the important effect of the power consumption and how the H_L/D_r ratio becomes important at high (P_G/V_L).

Subsequently we examined the observed versus predicted k_La_L generated with Eq.7.7 (Figure 7.12a). This shows slight violations of the model mainly at high superficial gas velocities, but generally a good fit of the model with the experimental data. Based on Eq. 7.8 the surface generated by the model is shown in Figure 7.12b. There was a good adjustment of the model with the experimental data.

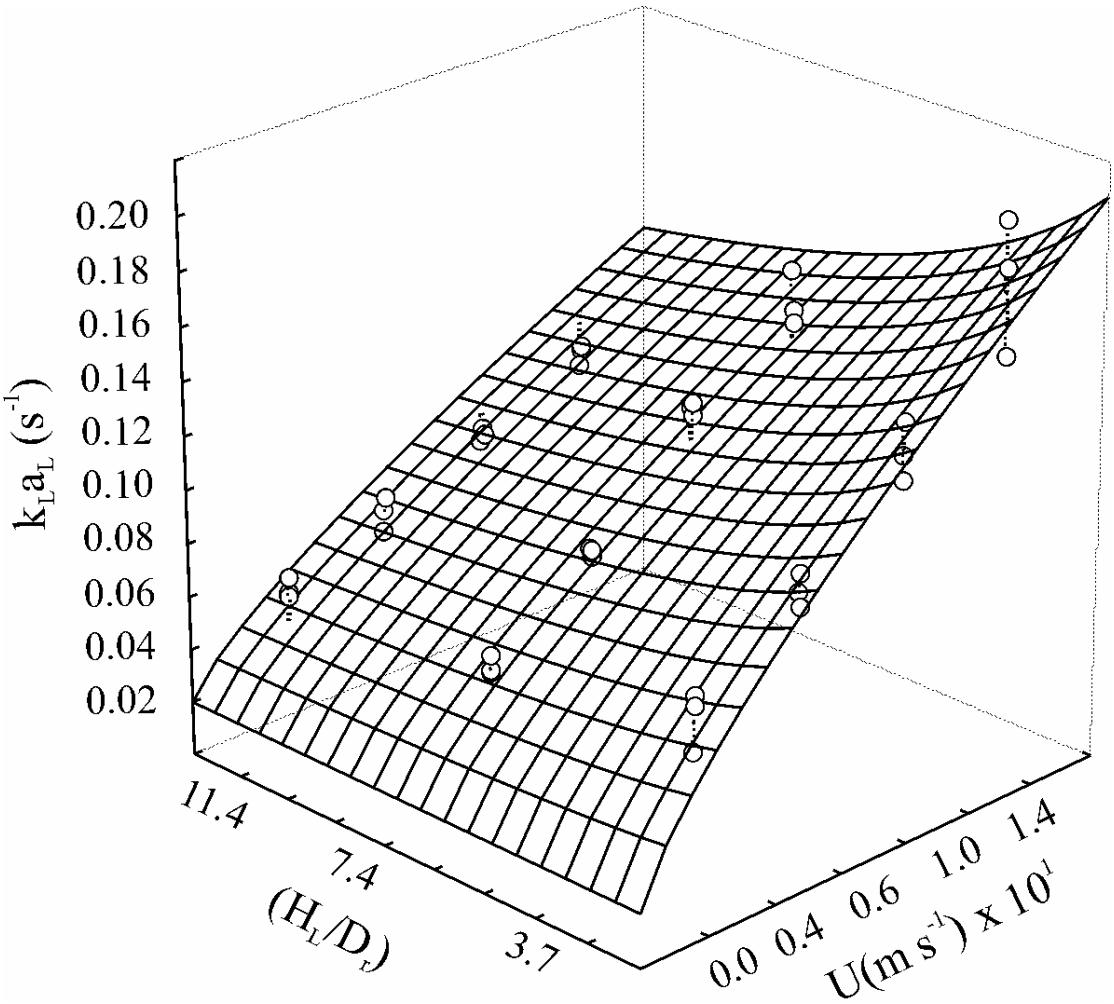

Figure 7.10. Volumetric mass transfer coefficient k_La_L surface (Eq. 7.8) in function of the superficial gas velocity (U) and the ratio (H_L/D_r). The experimental data is presented as open circles (○)

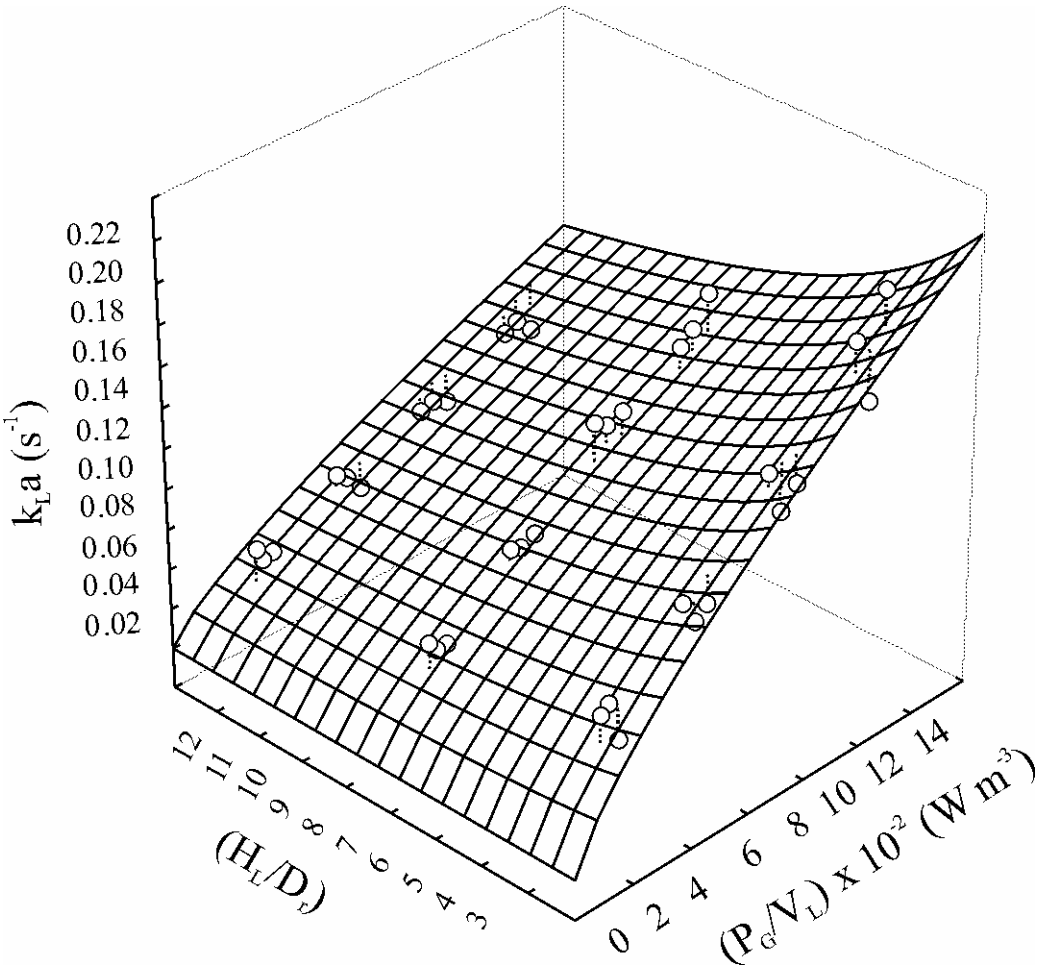

Figure 7.11. Volumetric mass transfer coefficient k_La_L surface (Eq. 7.9) in function of the power consumption (P_G/V_L) and the ratio (H_L/D_r). The experimental data is presented as open circles (○)

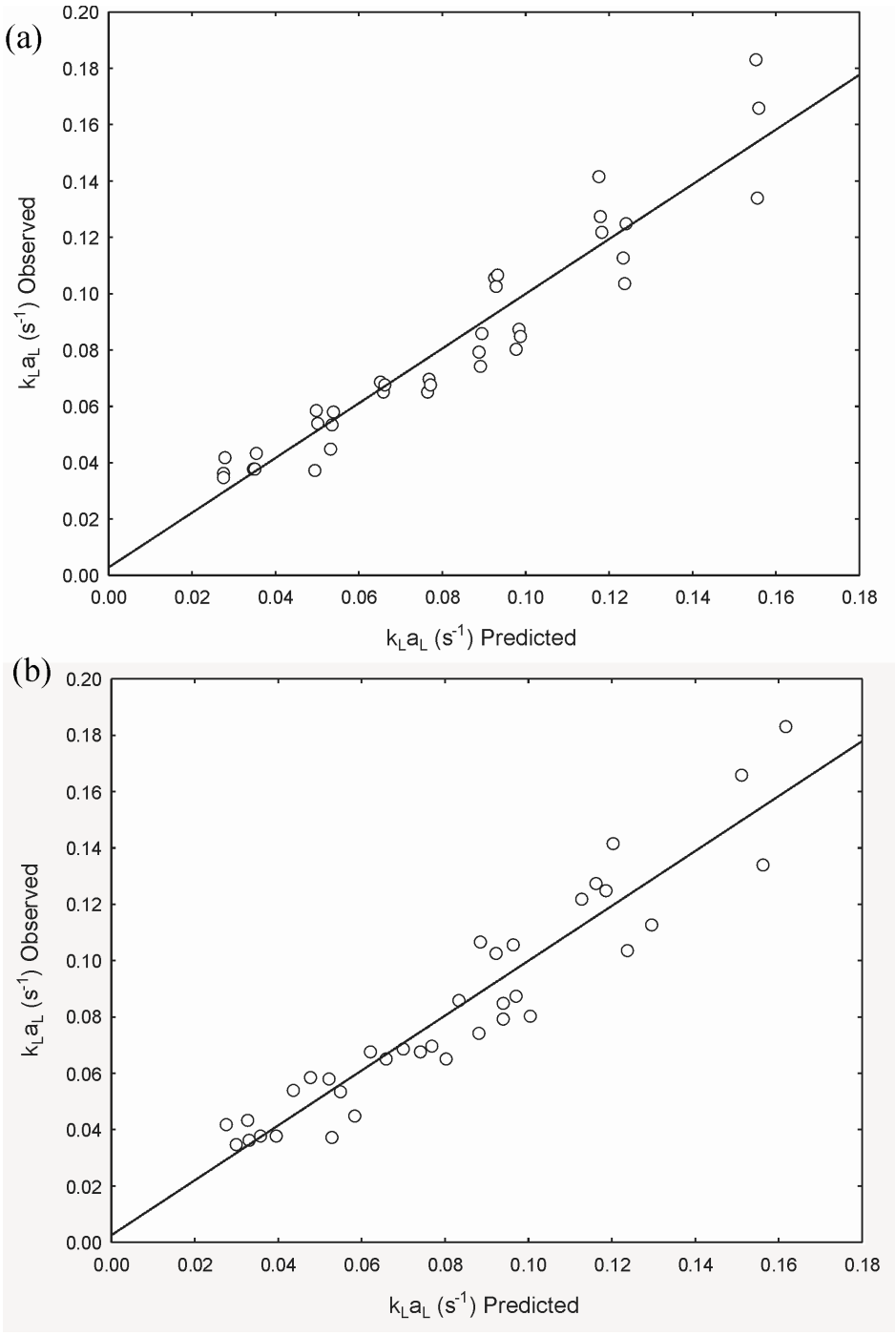

Figure 7.12. Residual diagnostics of the k_La_L surface generated by the model Eq. 7.8 (a) and Eq. 7.9 (b) of the predicted vs. observed

In order to establish the robustness of the experimental data a comparison with theoretical models was performed. The equation of mass transfer purely theoretically derived from mean bubble size (d_B) in dispersion and the overall gas hold up (ε) was:

$$\ln k_L a_L = \ln\left(6\frac{k_L}{d_B}\right) + \ln\frac{\varepsilon}{(1-\varepsilon)} \qquad \text{(Eq.7.9)}$$

In theory a log-log plot k_La_L against $\varepsilon/(1-\varepsilon)$ should have a slope = 1 for a perfect match of the experimental values and that derived from theory. The predicted k_La_L agreed remarkably well with the experimental values. In the experiment the average slope of all the data under the different operating conditions was 1.28. Chisti (1989) reported from a 200 point data set under different bioreactor/ fluid combinations a slope of 1.18. This result demonstrates that the gas hold up and the mass transfer measurements were quite reliable.

Figure 7.13 shows the growth kinetics of the *Phoma* sp. and the production of squalestatin S1 in the bubble column bioreactor using a H_L/D_r ratio of 3.7. Interstingly, no lag and stationary growth phases were observed. This absence of a stationary phase could be due to prevention of restricted oxygen conditions because of the high mass transfer coefficients of the geometrical ratio H_L/D_r. However, the excessive growth could also limit the total production of such secondary metabolites. Interactions between cells produce chemical signals which can control the production of secondary metabolites.

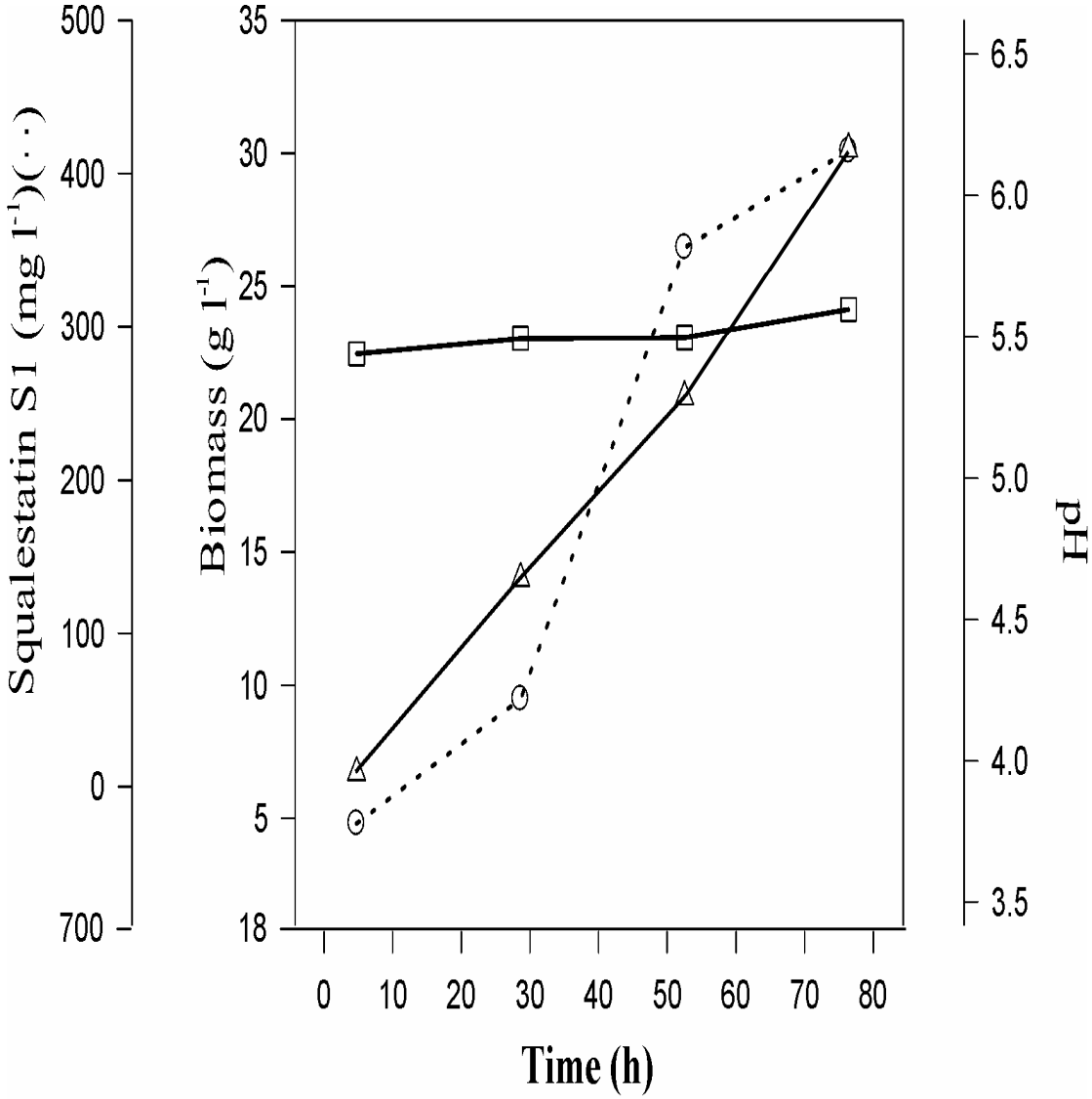

Figure 7.13. Squalestatin S1 fermentation kinetics at 25°C in the bubble column bioreactor (BC) ratio (H_L/D_r = 3.7, *Fg* =1.3 x 10^{-4} m^3 s^{-1}). Figures represent: pH (□), biomass (○) and squalestatin S1 concentration (Δ).

7.4 CONCLUSIONS

The following major conclusions can be drawn from this work.

1. For all the systems studied the gas hold up ε and in the volumetric mass transfer coefficient k_La were affected by the H_L/D_r ratio at high superficial gas velocities, i.e., U>0.08 m s^{-1} at 25°C.
2. The k_La/ε value tend to a constant which differs depending on the ratio H_L/D_r. Low ratios produced higher constant k_La/ε .
3. Regardless of the temperature and superficial gas velocities the maximum k_La values were obtained with the a H_L/D_r ratio of 3.7.
4. Levels of power consumption (P_G/V_L) <1.2 kW m^{-3} were found to be similar to other systems such as the stirred tank bioreactor. However, the bubble column produced almost double the mass transfer coefficient.
5. Two new models are proposed by adding the effect of the liquid level in the geometry of the fermentor to the widely applied correlations of superficial gas velocity and power consumption.
6. Successful scaled up produced similar concentrations of squalestatin S1 (420 mg l^{-1}) in the bioreactor when compared to shake flasks (434 mg l^{-1}) (Parra *et al*., 2004[c]). However the biomass produced was twice as much, the excessive growth could also limit the total production of secondary metabolite.
7. This evidence strongly suggests that evaluation of different superficial gas velocities is more suitable for secondary metabolites produced by the *Phoma* sp.
8. Further studies are needed to determine the effect of different superficial gas velocities in order to limit the biomass production which can restrict oxygen supply or limit growth by immobilization.

CHAPTER VIII

EXPLORATION OF NOVEL IMMOBILIZATION SYSTEMS IN BUBBLE COLUMN BIOREACTOR FOR THE PRODUCTION OF SQUALESTATIN S1 BY *A Phoma* SPECIES AND COMPARISON WITH A FREE CELL CULTURE STIRRED TANK REACTOR

8.1 INTRODUCTION

Squalestatins are a family of fungal secondary metabolites. These potent inhibitors of squalene synthase have the potential for development as cholesterol lowering agents and/or as antifungal agents (Bergstrom *et al*., 1995). Among the various species that produce squalestatins, a *Phoma* sp. is particularly important because of its potential concentration of squalestatins (Baxter *et al*., 1998; Dawson *et al*., 1991).

In 1969, the first industrial application of immobilization was applied. Since then, numerous improvements have been made in the immobilization techniques. A large number of supports for immobilization have been used (Emregul *et al*., 1995; Ohmori and Kurokawa, 1994; Sato *et al*., 1994; Escamilla *et al*., 2000) and the total number of their applications have also risen, particularly in the fields of environmental engineering, food processing and medicine (Gmeiner, 1992).

Gels are some of the most widely used supports; they can either be natural, derived from polysaccharides such as agar (Hayashi *et al*., 1993), alginate (Wu *et al*., 1994), k-carrageenan (Sakiyama *et al*., 1993), pectate (Escamilla *et al*., 2000) or synthetic, such as that derived from acrylamide (Axelsson *et al*., 1994). The immobilization of microbial cells in different carriers leads to changes in their microenvironment (Shreve and Vogel, 1993). Because of these changes, immobilized cells show various modifications in physiology and biochemical composition when compared to suspended cells (Hilg-Rotmann and Rehm, 1990).

In the past two decades advanced use of polysaccharides for gel-entrapment or encapsulation of cells and technique optimization has become a challenging area for the biotechnologist. The immobilization procedure can be carried out in a single step process under very mild conditions and is therefore compatible with most viable cells. The first step in fungal production is inoculation of vegetative cultures and subsequent inoculum development. Inoculum quality and quantity strikingly affect the overall production (Gancheva and Dimova, 1984; Brückner and Blechschmidt, 1991; Mussenden, 1993).

The optimization of immobilization parameters could permit development of an immobilized cell fermentation which improves S1 production in a bubble column bioreactor. However, the classical strategy of changing one variable at a time would be very time-consuming. Optimization approaches can be performed using a Placket-Burman experimental design and contour surfaces facilitating evaluation of interacting factors for optimising S1 production. While immobilization can be carried out using a mixture of polymers (alginate and pectate) and concentration of mixtures, the changes in water availability have never been considered and measured to evaluate the impact that such stress may have on the physiology of the microorganism and secondary metabolite production.

The objectives of this work were to evaluate the influence of immobilization design parameters and their interactions on S1 production and compare this with free stirred bioreactors. The factors considered were: polymer and copolymer (alginate and pectate) immobilization under different mixture concentrations, inoculum level, gel inducer (calcium chloride), reinforcing agent (chitosan) and air flow in the bubble column bioreactor. This was compared with S1 production in a free cell fermentation using a standard stirred tank bioreactor (STR).

8.2 MATERIALS AND METHODS

8.2.1 Fungal strains

See section 6.2.1

8.2.2 Activation and inoculation

See section 6.2.2

8.2.3 Immobilization

Growing mycelia of *Phoma* sp. were used for immobilization. In aseptic conditions the seeding fungus were immobilized and co-immobilized in Ca-pectate, Ca-alginate and mixtures of both using a modified version of that previously reported by Escamilla *et al*. (2000). The mycelial pellets were homogenised in a blender (50 seconds) and filtered through a sinter glass funnel to remove all the liquid. The biomass was recovered on Whatman paper (No. 2). The inoculum was adjusted in a

volumetric cylinder to either 10, 20 and 30 % wet mycelium inoculum:polymer ratio by immobilization in polygalactguronic or alginic acid or a mixture (alginate-poygalacturonate 50-50 (v/v)). The polymer concentration was 3 and 5 % w/v for alginate, 6 and 8 % w/v for polygalacturonate and 3.5% alginate and 3.5 % polygalacturonate for the 50-50 (v/v) mixture. The polymer-inoculum mixture a_w was modified with glycerol and forced through a multi-needle template (18 gauge for 5 mm beads) with a peristaltic pump (Watson Marlon) flowing at 10 ml min^{-1} and the droplets collected in sterile gel-inducer solutions of 3, 4 or 5 % (w/v) $CaCl_2$ with different concentrations of chitosan (0, 0.75 and 1.5 g l^{-1}). After soaking for 3 h the liquid was decanted and the spherical beads was washed with sterile RO water and stored at 4ºC for 24 hours. The water availability of the decanted beads were measured with a Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland).

8.2.4 Media preparation and incubation

See section 6.2.3

8.2.5 Bioreactors

A set of bubble column bioreactors (BCR) (Soham Scientific,United Kingdom) with the geometric ratio H_L/D_r= 3.7 equivalent to 1 l fermentation medium loaded into a BC. For aeration, a sintered glass plate (30 μm porosity, 10 mm thick) was used. From the optimal conditions for k_La found in previous work the fermentations were performed at different superficial gas velocities (U = 0.057, 0.089 and 0.120 m s^{-1}) and process temperature (T = 25ºC).

Parallel experiments with free cell fermentation were performed in a standard 5 l stirred tank reactor (STR) (Electochem Ltd, United Kingdom). The diameter of STR was D = 0.25 m (liquid height H_L=0.102 m) and the diameter of a Rushton radial impeller and axial turbine were D_R = 0.08 m. All experiments were performed at a superficial gas velocity of 0.024 m s^{-1} and the process set at T = 25ºC and 150 rpm.

All reactors were equipped with sterilizable pH (PHT20**)** (Thermo Russell) and oxygen (Bioengineering, Switzerland) probes, connected to a central controller (Bioengineering, Switzerland) for on-line measurements. Oxygen partial pressure measurements were measured by a polarographic electrode. Samples to determine

biomass and squalestatins were taken from a sterile suction port. Biomass was determined by filtering 4 ml through filter paper (Whaltman no. 2) and drying in an oven at 90^{o}C.

8.2.6 Determination of total nitrogen and carbon

All the samples were pre-filtered through a 0.2 ㎜ (Whatman) filter. Dilutions were preformed where nitrogen was > 10 mg l^{-1}. The total nitrogen was determined by on-line oxidation with persulphate in alkaline solution to nitrate in a segmented flow ion analyser (Burkard Scientific SFA-2000). The resultant nitrite reacts with sulphanilamide and N-1-napthylethylenediamine to form a red azo dye measured at 520 nm (Franso, 1995). The total dissolved carbon determination was carried out with a segmented flow ion analyser (Burkard Scientific SFA-2000). The sample was first acidified and sparged with carbon dioxide free air to remove inorganic carbon dioxide (based on a method supplied by Burkard Scientific SFA-2000). The sample was then mixed with acidified potassium persulphate and irradiated with UV light to convert organic carbon to dioxide. The carbon dioxide permeates through a gas diffusion membrane into a buffered phenolphthalein solution and the change in colour monitored at 550 nm.

8.2.7 Squalestatin analysis

See section 6.2.4

8.2.8 Experimental design and data treatment

The aim of this work was to investigate the relationship between the immobilization components and their concentration to maximize the production of squalestatin S1 by *Phoma* sp. in a bubble column bioreactor with a screening Placket Burman design and response surface methodology. The optimal immobilized cell fermentation was compared with free cell fermentation in a STB.

The immobilized design composition was selected according to the experimental design. The combinations of polymer type, polymer concentration, bead water activity, inoculum size, gel-inducer ($CaCl_2$), chitosan concentration and air flow rate were selected using the experimental design module of Statistica 6.0 (SoftStat, Inc. 1984-2001, USA). A screening experimental system of the Plakett-Burman (2^{4-7})

design with a resolution R=III and seven independent factors and four replicates at the centre was used. The levels of each factor were allocated into three categories: (-1) low, (0) medium and (1) high. For the analysis, the factors were given coded values between -1 (lowest concentration of substrate) and 1 (highest concentration of substrate). For the response surface methodology and the polynomial regression the coded values and the vector squalestatin production were loaded into the statistical analysis under the experimental design module of Statistica 6.0 (SoftStat, Inc. 1984-2001, USA). The experiment results were statistically evaluated.

A set of factors identified in the literature as potentially important in the design of immobilized cell fermentations were evaluated. These factors, along with low and high concentration of each level are shown in Table 8.1. The polymer type and concentration were chosen so that some immobilizations were pure polymer (alginate or pectate) and mixtures of both. When chitosan was evaluated the lower level indicates the absence of this compound in the immobilization. The inoculum treatment levels chosen were to enable differences to stand out, regardless of the organism variability.

This strategy was designed to balance the medium experimentation between the known factors that affect the immobilization and new sources that could represent an improvement in the immobilization technique evaluated under extreme hydrodynamic conditions generated in the bioreactor derived from the air flows. The 12 run Plackett-Burman design with four centre points were use to screen the effect of the immobilized design on S1 production.

8.3 RESULTS

The analysis of the data from the Plackett-Burman experiments involved fitting a first order (main effects) model together with the term for curvature. The low and high factor levels were coded as -1 and +1, respectively for the analysis of the data. The different conditions and the resulting titres forecasted by the model after 96 hours fermentation are shown in Table 8.2.

The theoretical model accompanying the analysis of the Plackett-Burman results is given by

Squalestatin S1 (mg l^{-1}) = $a_0+b_1X_1+b_2X_2+...+b_7X_7+c_0$CP+error

Where a_0 represent the intercept, b_i is the main effect or slope associated with the *i*th component of the immobilized design. The factors accompanying each main effect are represented by X for each immobilization components, while the CP indicates whether the experiment was a centre point (0) or a non-centre point (+1). The c_0 term is the gross curvature effect and indicates the degree to which the centre points differ from the non-centre points. A negative c_0 indicates the response at the centre exceeds the average response at the extremes in the design space. Otherwise, the higher responses occur at or beyond the extremes, which could prompt a shift in the factor levels and the design space.

The R^2 value was 0.998 and the model explained over 99.0% of the total variability in the data. The parameter estimate of the immobilized design are given in descending order in Table 8.3 and are interpreted as slopes for the change in the response for every unit change in the coded factor level. The immobilization factors that had the most significant effect (*P*-value < 0.001) were air flow, gel-inducer (calcium chloride, $CaCl_2$), polymer concentration and inoculum. Of secondary importance were inoculum level (*P*-value < 0.005) and water activity (*P*-value < 0.01), while polymer type and chitosan had marginal significance (*P*-value < 0.02). The curvature term was very significant, indicating that optimal conditions probably occur inside the experimental region.

One way to check the appropriateness of the selected model is to investigate the observed, predicted and residual values, which are the deviations between the actual titres and those predicted by the model. Patterns in the residuals or large residuals usually indicate inadequacies in the model or possible suspect data. Figure 8.1a shows the observed vs predicted concentrations of S1 with the Plackett-Burman model. Points above or below the diagonal line represent areas of over or under prediction. No significant violations of the model occurred in the analysis and a good fit of the model with the experimental data was achieved. Figure 8.1b shows the studentized (t) residual against the raw residuals. Good agreement with a Gaussian distribution of the residuals was found.

Table 8.1. Factor and levels evaluated in the Plackett-Burman screening design.

Factor	Levels		
	-1	0	1
Polymer (%)	100 % Alginate 0 % Pectate	50 % Alginate 50 % Pectate	0 % Alginate 100 % Pectate
Alginate (CAG) (g l^{-1})	3	3.5	5
Pectate (CPG) (g l^{-1})	4	6.0	8
Water activity (a_w)	0.98	0.96	0.94
Inoculum (v/v)	10	20	30
Gel-inducer ($CaCl_2$) (g l^{-1})	3	4	5
Chitosan (g l^{-1})	0	0.75	1.5
Air flow (l min^{-1})	4	6	8

Table 8.2. Plackett-Burman experimental and predicted sqaulestatine S1 production under the different immobilized cell fermentations.

Polymer		a_w	Inoculum	$CaCl_2$	Chitosan	Air flow	Squalestatin S1 (mg l^{-1})		Residual
Type	Conc.						Observed	Predicted	
-1	-1	-1	1	1	1	-1	473	473	0
1	-1	-1	-1	-1	1	1	51	51	0
-1	1	-1	-1	1	-1	1	85	85	0
1	1	-1	1	-1	-1	-1	4	4	0
-1	-1	1	1	-1	-1	1	0	0	0
1	-1	1	-1	1	-1	-1	883	883	0
-1	1	1	-1	-1	1	-1	124	124	0
1	1	1	1	1	1	1	42	42	0
0	0	0	0	0	0	0	49	28	21
0	0	0	0	0	0	0	27	28	-2
0	0	0	0	0	0	0	0	28	-28
0	0	0	0	0	0	0	38	28	10

Table 8.3. Parameter estimates of first order model, including curvature, from Plackett-Burman experiment

Term	Estimate	S.E.	*P*-value
Intercept	207.84	7.44	0.0001
Curvature	-179.41	12.88	0.0008
Air flow (l/min)	-163.43	7.44	0.0002
$CaCl_2$	162.99	7.44	0.0002
Polymer concentration	-143.84	7.44	0.0003
Inoculum	-78.06	7.44	0.0018
Water activity (a_w)	54.56	7.44	0.0052
Polymer	37.17	7.44	0.0154
Chitosan	-35.40	7.44	0.0176

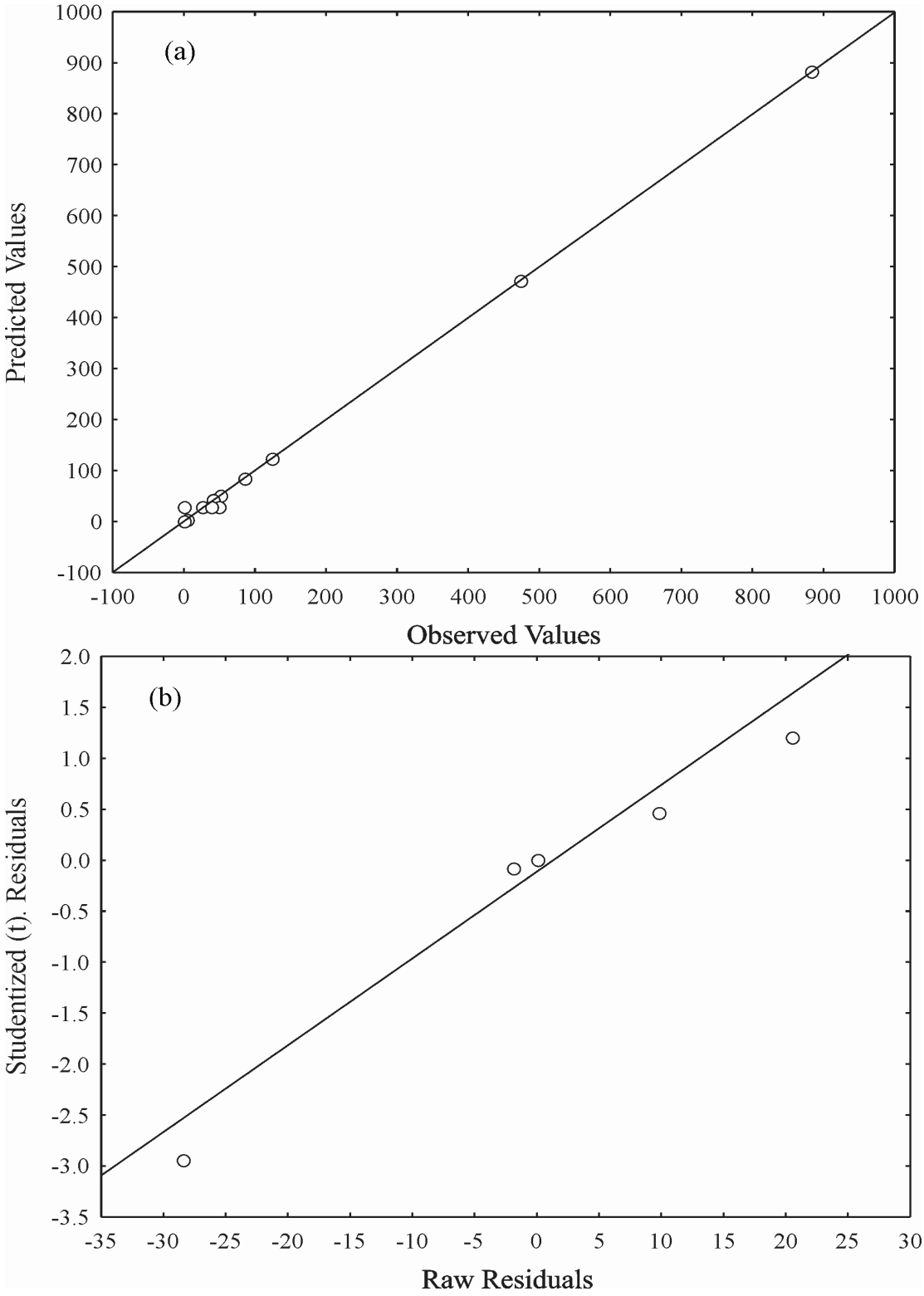

Figure 8.1. Residual diagnostics of the linear Plackett-Burman model of the (a) experimental observed (○) against the predicted (—) production of S1 (mg l^{-1}) and (b) experimental raw residuals (○) against the studentized (t) residuals (—)

8.3.1 Evaluation of optimization immobilization conditions

Confirmation of the optimal conditions was subsequently evaluated in different potential optimum regions. This gives a factor which predicts the maximum titre at the optimal distance between the factor levels. The experiments were grouped by factor and levels to determine the effect of changing factor levels on the production of squalestatin S1.

Figure 8.2 provides an illustration of the experimental strategy for the low and high levels arranging factors in a two by two format with the results for S1 production. For example Figure 8.2 (a) shows the effect of polymer concentration and polymer type. A high S1 titre (314-388 mg l^{-1}) was achieved at low polymer concentration in comparison with much lower levels at higher concentrations. Figure 8.2 (b) demonstrates the effect of inoculum size and water activity. Optimal conditions were at a low inoculum level and low water activity (340 mg l^{-1}). Optimal conditions were also achieved with a high gel-inducer concentration (CaCl2) and no added chistosan (406 mg l^{-1}; Figure 8.2c). For aw and polymer concentration low levels of water activity and polymer were optimum (406 mg l^{-1}; Figure 8.2d). For $CaCl_2$ and inoculum size, optimal conditions were at low inoculum levels and high gel-inducer ($CaCl_2$) (449 mg l^{-1}; Figure 8.2e). Figure 8.2 (f) shows that a low rate of air flow and absence of chitosan also produced high S1 concentrations (406 mg l^{-1}).

Figure 8.3 illustrates the effects when a third factor interacting factor is considered to enable the effect of three conditions at two levels to be examined. We can summarise the optimum conditions:

(a) polymer concentration = -1 (3 - 4 % w/v), polymer = +1 (100% CAG) and water activity = +1 (0.94) with a level of 443 mg l^{-1} S1 concentration.
(b) inoculum = -1 (10 v/v), water activity = +1 (0.94) and $CaCl_2$ = +1 (5 w/v) produced 503 mg l^{-1} S1 concentration
(c) water activity = +1 (0.94), polymer concentration = -1 (3 - 4 % w/v) and inoculum = -1 (10 v/v) produced 484 mg l^{-1} S1 concentration
(d) $CaCl_2$ = +1 (5 w/v), inoculum -1 (10 v/v) and chitosan +1 (1.5 g) produced 484 mg l^{-1} S1 concentration

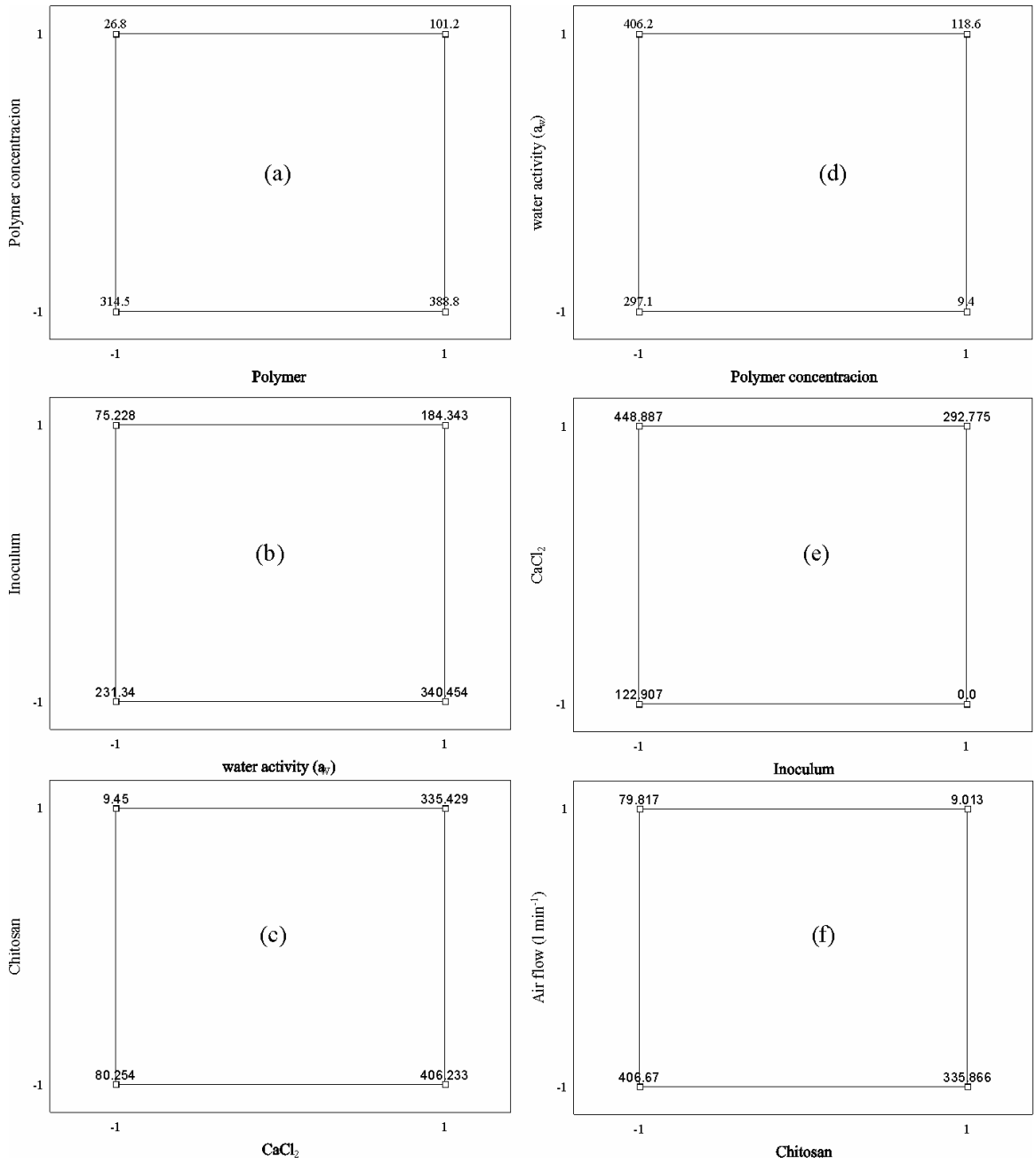

Figure 8.2. Illustration of squalestatin S1 concentration produced under different factor and levels combinations on the experimental design: (a) polymer concentration (-1 = 3 - 4 % w/v, +1 = 5 - 8 w/v) vs polymer (-1= 100% CPG, +1 = 100% CAG); (b) inoculum (-1 = 10 v/v, +1 = 30 v/v) vs water activity (-1 = 0.98, +1 = 0.94); (c) chitosan (-1 = 0 g, +1 = 1.5 g) vs $CaCl_2$ (-1 = 3 w/v, +1 = 5 w/v); water activity vs polymer concentration; (e) $CaCl_2$ vs inoculum; (f) air flow (-1 = 4 l min^{-1}, +1 = 8 l min^{-1}) vs chitosan. Internal boxes vertex represent concentration and external boxes coded factor levels.

Overall the combined conditions in (a) to (d) is shown in (e):

(e) chitosan = -1 (0 g), $CaCl_2$ = +1 (5 w/v) and air flow =-1 (4 l min^{-1}) produced 569 mg l^{-1} S1 concentration

which represents the highest S1 concentrations.

Figure 8.4 shows the plot of the Plackett-Burman model for polymer with all the factors at level 0 and the uncoded concentration of the binary polymer concentration. Uncoding the polymer concentration it was observed that the two polymers had opposite effects. Increasing the calcium alginate gel (CAG) concentration increased the S1 concentrations. In contrast, an increase in the concentration of calcium pectate gel (CPG) led to a reduction in S1 production. The centre point for both polymers are considerably different. For CAG the range is between 800-880 mg l^{-1} S1 and for CPG between 600-675 mg l^{-1} S1. The optimal composition of polymer is with pure calcium alginate gel (CAG) (coded = +1).

Figure 8.5a shows the forecasted S1 concentrations as a function of inoculum size. The effect of increasing the inoculum size was to reduce the total S1 produced. Optimum conditions for the production of S1 was obtained with 10 % v/v wet inoculum. Increasing the gel-inducer ($CaCl_2$) level resulted in a linear increase in S1 production with optimum at +1 (5 % w/v; Figure 8.5b).

For the reinforcing agent chitosan, an inverse relationship between concentration of chitosan and S1 was observed (Figure 8.6a). The optimum level was -1 (0 g l^{-1}) when no chitosan was added. Figure 6b shows that an inverse relationship between air flow ratio and S1 production occurred. The optimum level was -1 (4 l min^{-1}). After analysis of all the factors and levels, the optimal factor conditions of immobilization on the production of squalestatin S1 is summarised in Table 8.4.

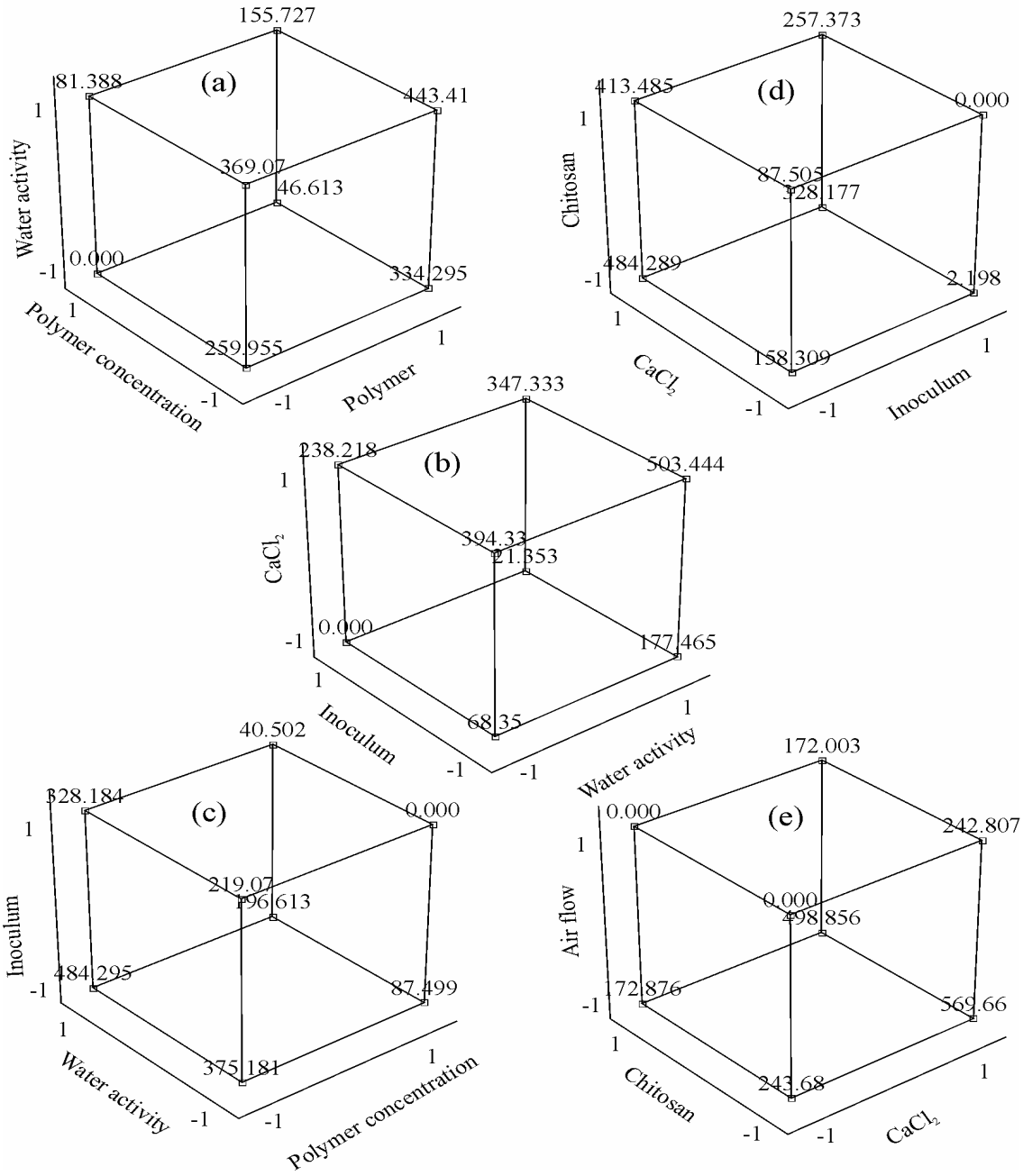

Figure 8.3. Illustration of squalestatin S1 concentration produced under three different factors and two levels combinations on the experimental design: (a) polymer concentration (-1 = 3 - 4 % w/v, +1 = 5 - 8 w/v), polymer (-1= 100% CPG, +1 = 100% CAG) and water activity (-1 = 0.98, +1 = 0.94); (b) inoculum (-1 = 10 v/v, +1 = 30 v/v), water activity and $CaCl_2$ (-1 = 3 w/v, +1 = 5 w/v); (c) water activity, polymer concentration and inoculum; (d) $CaCl_2$, inoculum and chitosan (e) chitosan, $CaCl_2$ and air flow (-1 = 4 l min^{-1}, +1 = 8 l min^{-1}).

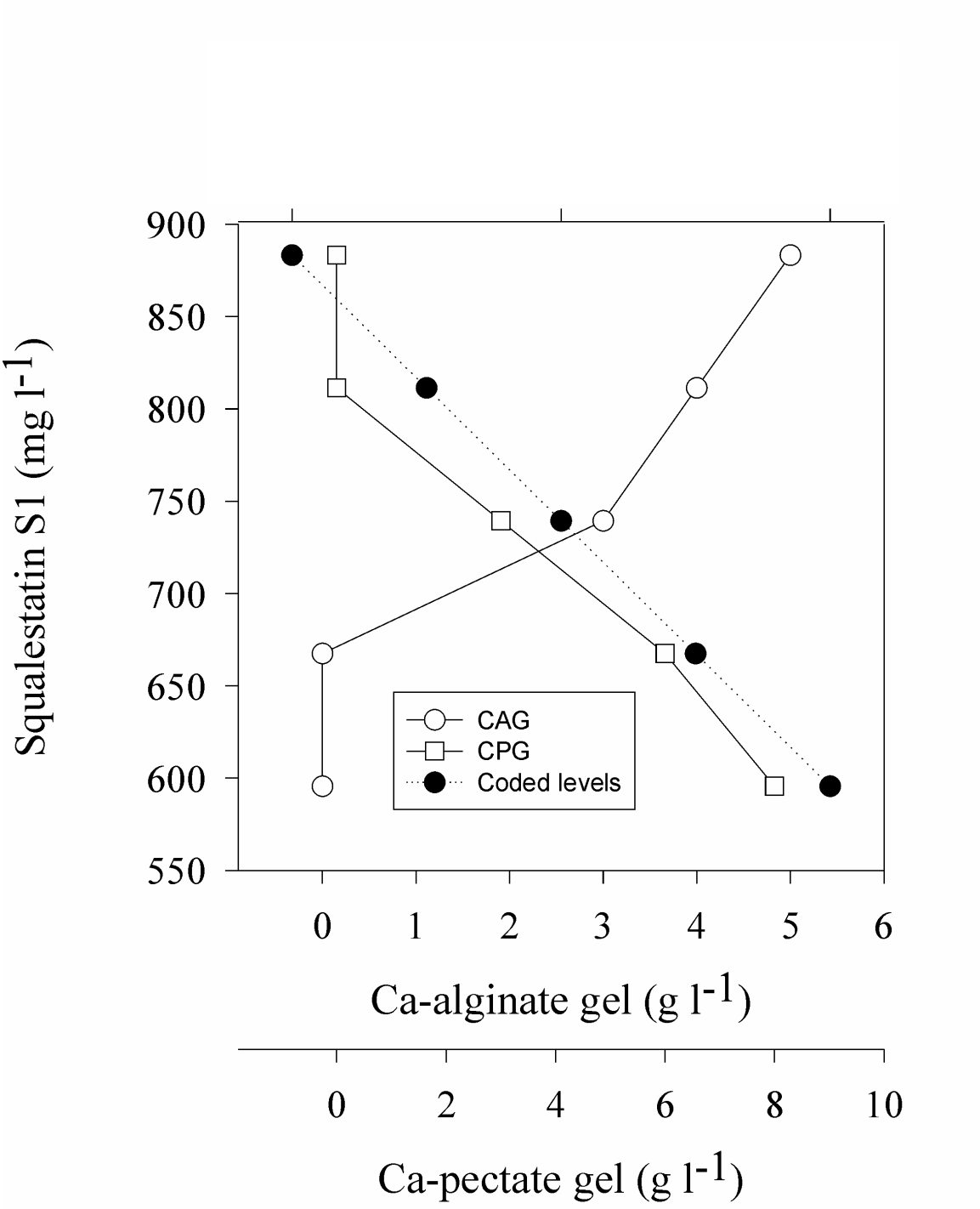

Figure 8.4. Coded experimental (●) and predicted (···) production of squalestatin S1 and the uncoded levels for calcium alginate gel CAG (○) and calcium pectate gel CPG (□) gel levels of squalestatin S1 production at 72 hours of fermentation.

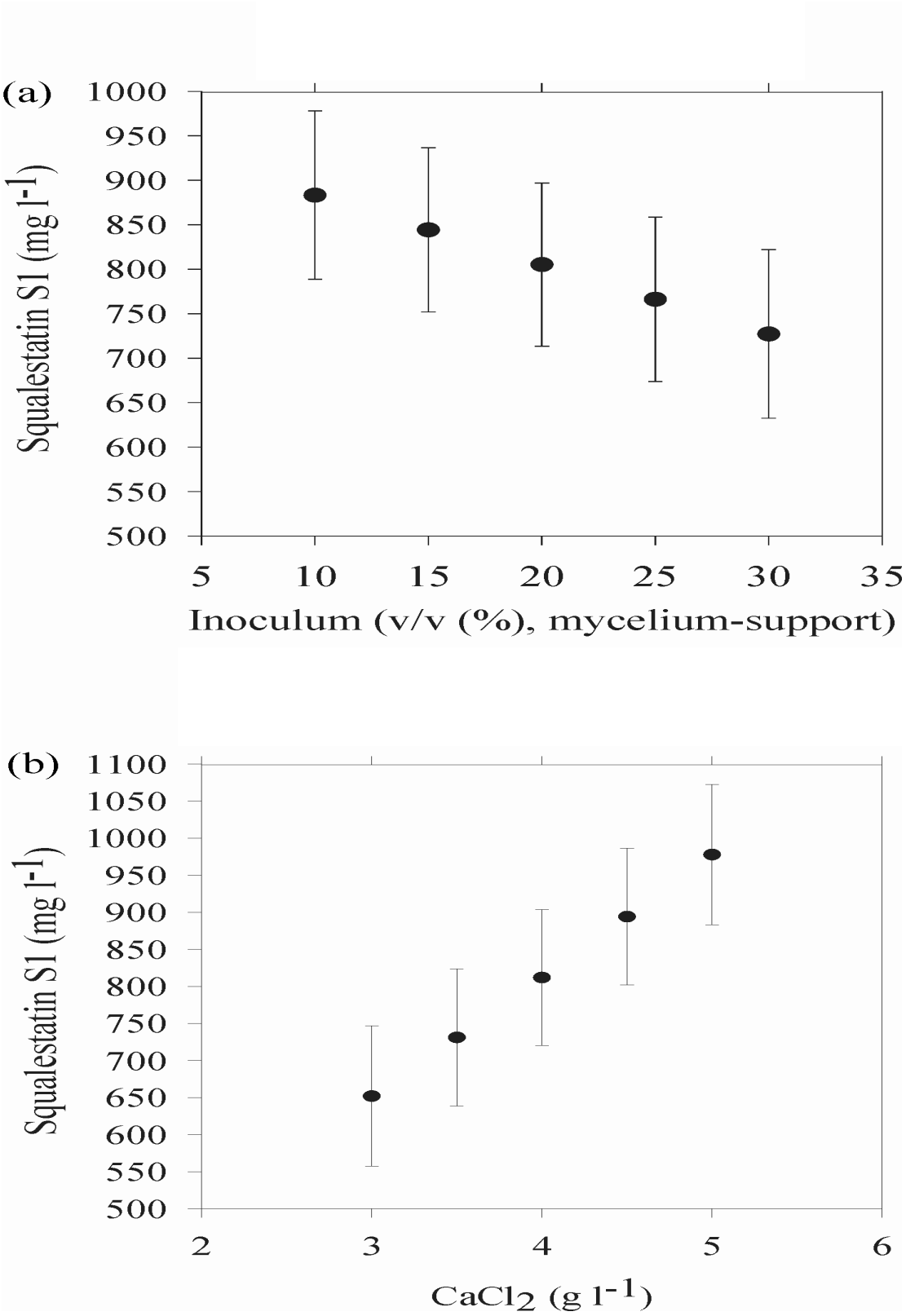

Figure 8.5. Squalestatin S1 produced under different inoculum sizes (a) and gel-inducer (CaCl2) concentration (b). The experimental (○) and forecasted (●) squalestatin concentrations at 72 hours of fermentation, the bars represent the 95% of confidence.

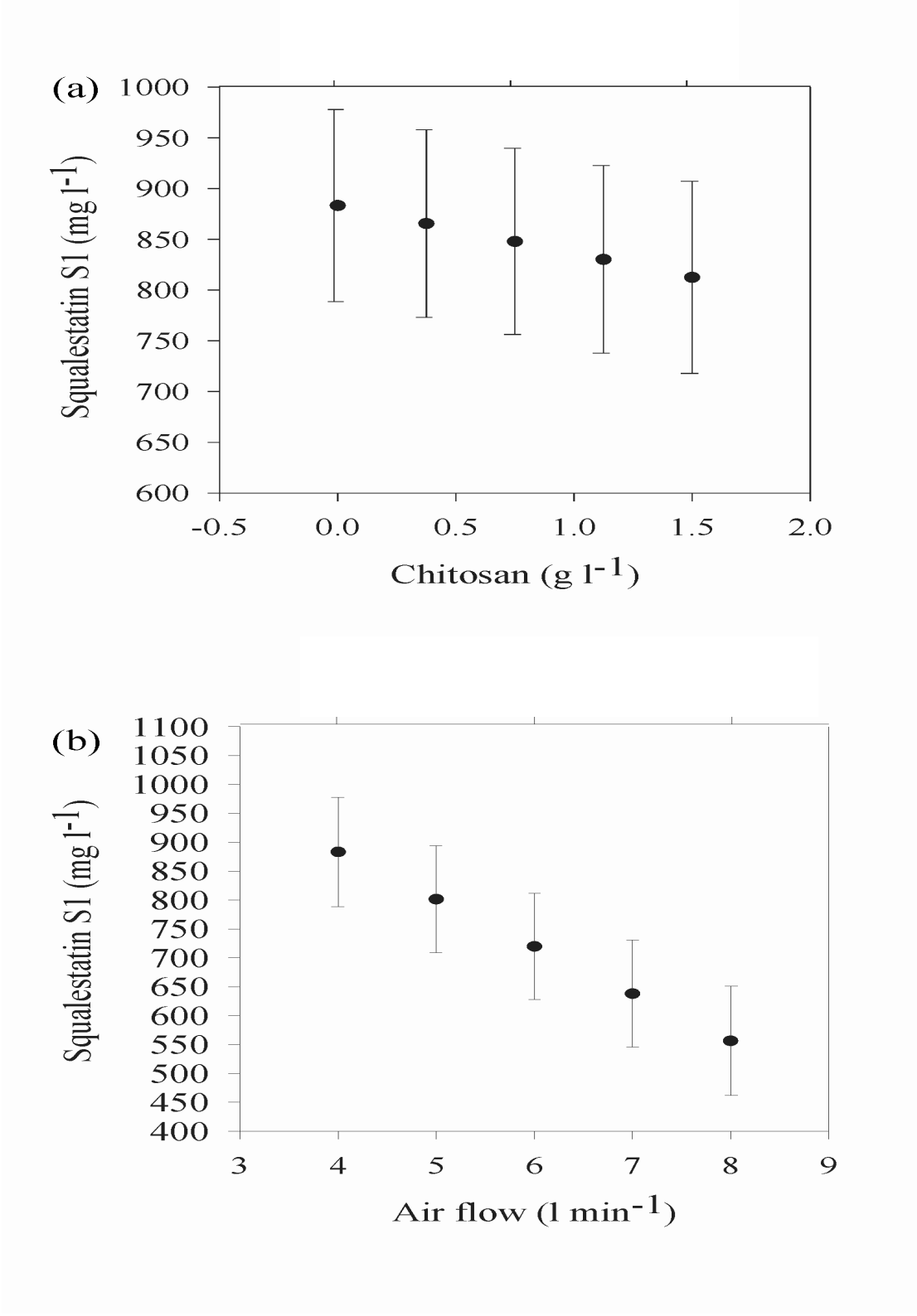

Figure 8.6. Squalestatin S1 produced under different gel- reinforce agent (chitosan) concentration (a) and air flow ratos (b). The experimental (○) and forecasted (●) squalestatin concentrations at 72 hours of fermentation, the bars represent the 95% of confidence.

Table 8.4. Optimal conditions for Phoma sp. immobilization for the production of squalestatin S1 evaluated in the Plackett-Burman screening design.

Factor	Value
Polymer (%)	0 % Alginate 100 % Pectate
Alginate (CAG) (g l^{-1})	3
Pectate (CPG) (g l^{-1})	8
Water activity (a_w)	0.94
Inoculum (v/v)	10
Gel-inducer ($CaCl_2$) (g l^{-1})	5
Chitosan (g l^{-1})	0
Air flow (l min^{-1})	4
Predicted	883.28

Figure 8.7 shows the optimal immobilized design for the production of squalestatin S1. Figure 8.7(a) presents the kinetics of S1 production together with the biomass and nitrogen changes. A very high S1 production of 883 mg l^{-1} was achieved with the optimal design levels. The sample was taken from the medium secondary metabolite production was only initiated when a certain nitrogen concentration was reached (1.02 g l^{-1}). The low biomass in the medium during the whole fermentation period suggests that the cell entrapment was very effective. Figure 8.7(b) shows the carbon and nitrogen ratio, carbon consumption and changes in pH. The pH remained constant for 80 hours and only then increased to 6.0. The carbon uptake ratio fell sharply after 20 hours, then continued decreasing more slowly until the 80^{th} hour. In the middle of the fermentation period (60 hrs) when a peak production of squalestatin S1 was observed. A linear increase in C:N ratio was due to nitrogen uptake associated with the growth within the beads. The maximal production of squalestatin S1 was achieved with C:N ratios of between 39-42. After 80 hours a change of the C:N ratio (C/N) was observed.

For comparison, Figure 8.8a shows the production of squalestatin S1 in a standard stirred tank reactor (STR) with a Ruston turbine. Fig 8.8a shows the kinetics of S1 production, biomass and nitrogen uptake by free cells of *Phoma* sp. Concentration levels of 660 mg l^{-1} S1 were produced in the STR. The bubble column with the optimal immobilized cell fermentation produced 34% more than the STR. The secondary metabolite production began when a certain nitrogen concentration was reached. In this case the squalestatin S1 production started at 1.4 g l^{-1} of nitrogen. Figure 8.8b shows the C:N ratio, carbon consumption and pH. The pH remained constant at value of 5.4 during the whole fermentation time. Carbon uptake ratio increased rapidly after 20 hours and continued increasing during the fermentation. The effect of the change of the carbon uptake had an effect in the squalestatin production and the peak of S1 occurred between 50- 70 hrs. Possibly the production could be continued for more than 72 hrs but the medium became very viscous. A fast linear increase in the C:N ratio at the beginning of the fermentation was due to the nitrogen uptake associated with the growth. An average value of 29.5 was established for the rest of the fermentation.

8.4 DISCUSSION

As the kinematical phases of a bioprocess are dynamic and are the consequences of directed functioning of the micro-environmental generated by the immobilized cell fermentation interacting strongly with the bioreactor operation parameters. The influence of different immobilization systems was evaluated by the capability to produce squalestatin S1 by statistical tools and is presented next.

It was observed an inverse effect of polymer type (alginate and pectate) on the production of S1. The concentration of S1 produced increase as the alginate concentration increase and pectate decrease. In this work Ca-alginate gel (CAG) was shown to be a better polymer for cell immobilization than Ca-pectate gel (CPG) in the production of secondary metabolite S1. Although, Ca-pectate resulted in a superior polymer than alginate our previous work in the production of heterologous proteins lysozyme (Parra *et al*., 2004d). This results point out the importance of the particular selection of polymer according the strain and the product cause this influence the outcome of the fermentation. Ca-alginate has been considered to be the most important material for cell immobilization (Kumar and Lonsane, 1988; Vassileva *et al*., 1998; Vílchez *et al*., 2001).

A 2.1 times higher production of S1 was achieved by the optimal immobilized cell fermentation over our previous work with free cells fermentation in a fluidized bed bioreactor (Chapter VII). Ca-alginate has a higher intrinsic viscosity than Ca-pectate (Tomáška *et al*., 1995). Succinate has reported as precursors in the biosynthesis of squalestatins (Bergstrom *et al*., 1995). The beads microenvironment produces an hypoxic condition that may result in a higher expression of secondary metabolites metabolic pathways. It is been reported the production of antibiotics with immobilized *Streptomyces Lividans* was 2.4 times higher than with free cells in a fluidized bed bioreactor (Sarrà *et al*., 1997). Moreover, *Saccharomyces cereviciae* immobilized in ceramic had a productivity of ethanol 3.2 times that obtained with free cells (Kukaya *et al*., 1996). The production of penicillin in a fluidized bed bioreactor obtained three times higher production with immobilized *Penicillium chrysogenum* in a resin (ceolite) in comparison with free cells (Gbewonyo and Wang, 1983).

The addition of chitosan as part of design of the immobilized cell fermentation resulted in a reduction in the concentration of squalestatin produced. The addition of chitosan into gelling agent it was reported to improve the mechanical strength and thermal stability of the hydrogel was greatly improved (Wang and Qian, 1999). The addition of chitosan into the new immobilized cell fermentation may represent a reduction of mass transfer due to the reinforcement of the gel network and lead to lower S1 concentrations. The opposite effect observed with the gel-inducer $CaCl_2$ may be attributed to the geometrical arrange of the mesh and the strength of the bead permit an easy interchange of mass (substrates and product) without releasing the fungi to the bulk. The volume of channels across the beads that allows the transport of product and substrates increases when the water activity increases. Reducing the immobilized inoculum load into the beads results in an increase in the S1 concentration produced. Mussenden *et al*. (1993) observed that a reduction of the immobilized viable spores load of *Penicillium chrysogenum* from 4×10^4 to 2×10^3 spores ml^{-1} produced an increase in the penicillin titer of 5 times.

The bubble column (BC) bioreactor produced 34% higher S1 production than the stirred tank reactor (STR). This could be attributed to the high mixing conditions and high mass transfer achieved in the BC in comparison to a standard STR (Chapter VII) It was observed that the low level of air flow (4 l min^{-1}) produced higher S1 concentrations than higher (8 l min^{-1}). This is attributed to the shift in the metabolic pathway due to hypoxic conditions inside the beads. A higher air flow leads to higher growth rates, as observed by the amount of nitrogen consumed. This was twice as much as under the lower air flow rate.

The statistical experimental design Plackett-Burman is a methodology suitable for immobilization design and optimization of multi-factor experiments. This work showed that the proper use of these tools can allow a scientist to reduce the complexity of a problem by identifying the most important components of the design of an optimal cell immobilization. The experimental optimal conditions resulted in much higher concentrations (34 %) of S1 compared to a free cell fermentation in a standard bioreactor with the same fermentation medium. Some immobilization conditions produced no squalestatin S1 and this methodology identifies which immobilization designs should be avoided.

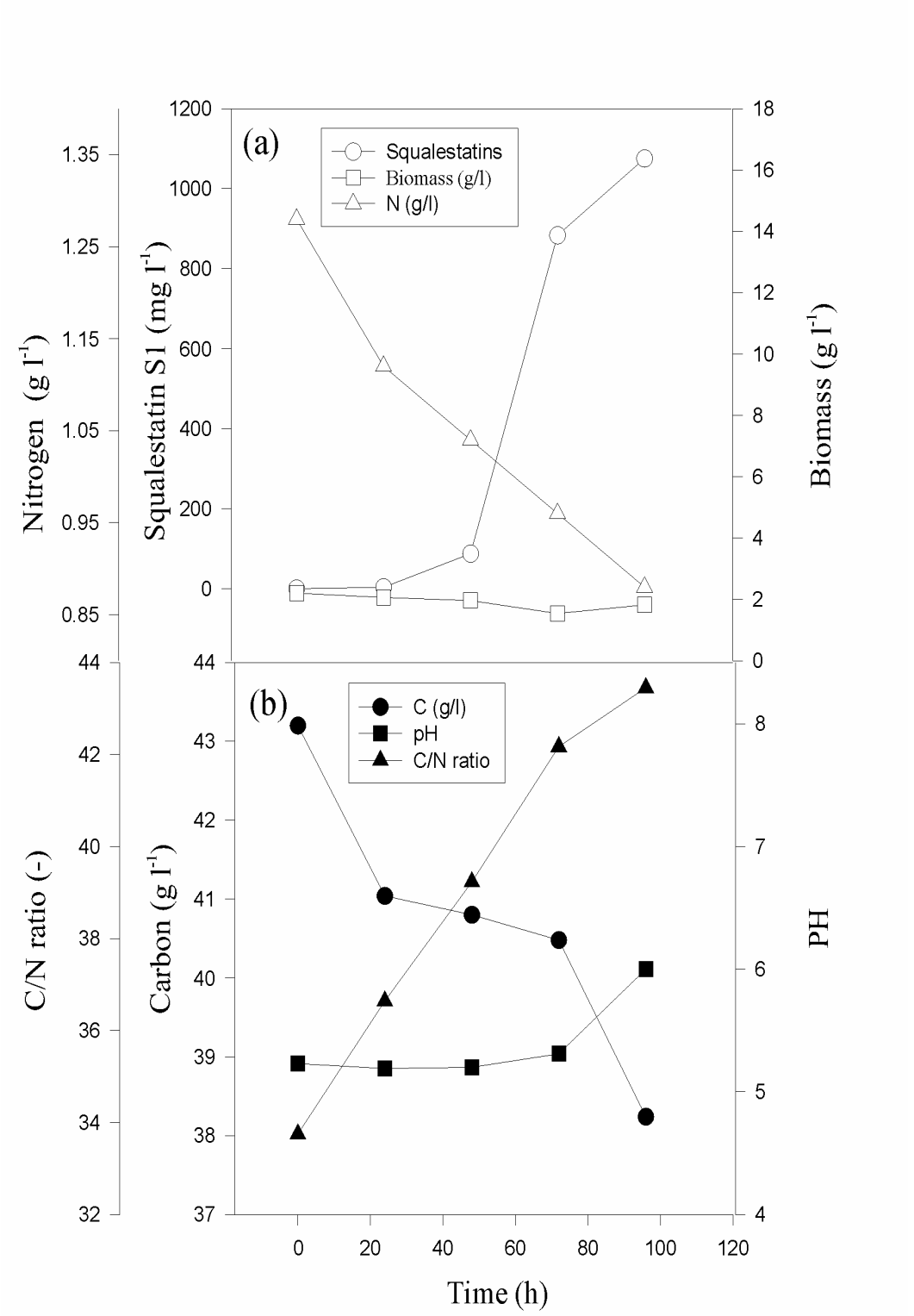

Figure 8.7. Kinetics of squalestatin S1 production in bubble column bioreactor inoculated with under optimal immobilized cell conditions. (a) S1 (mg l^{-1}) (○), biomass (g l^{-1}) (□),and Total nitrogen (g l^{-1}) (Δ). (b) Carbon (g l^{-1})(●), pH (■) and ratio C/N (▲)

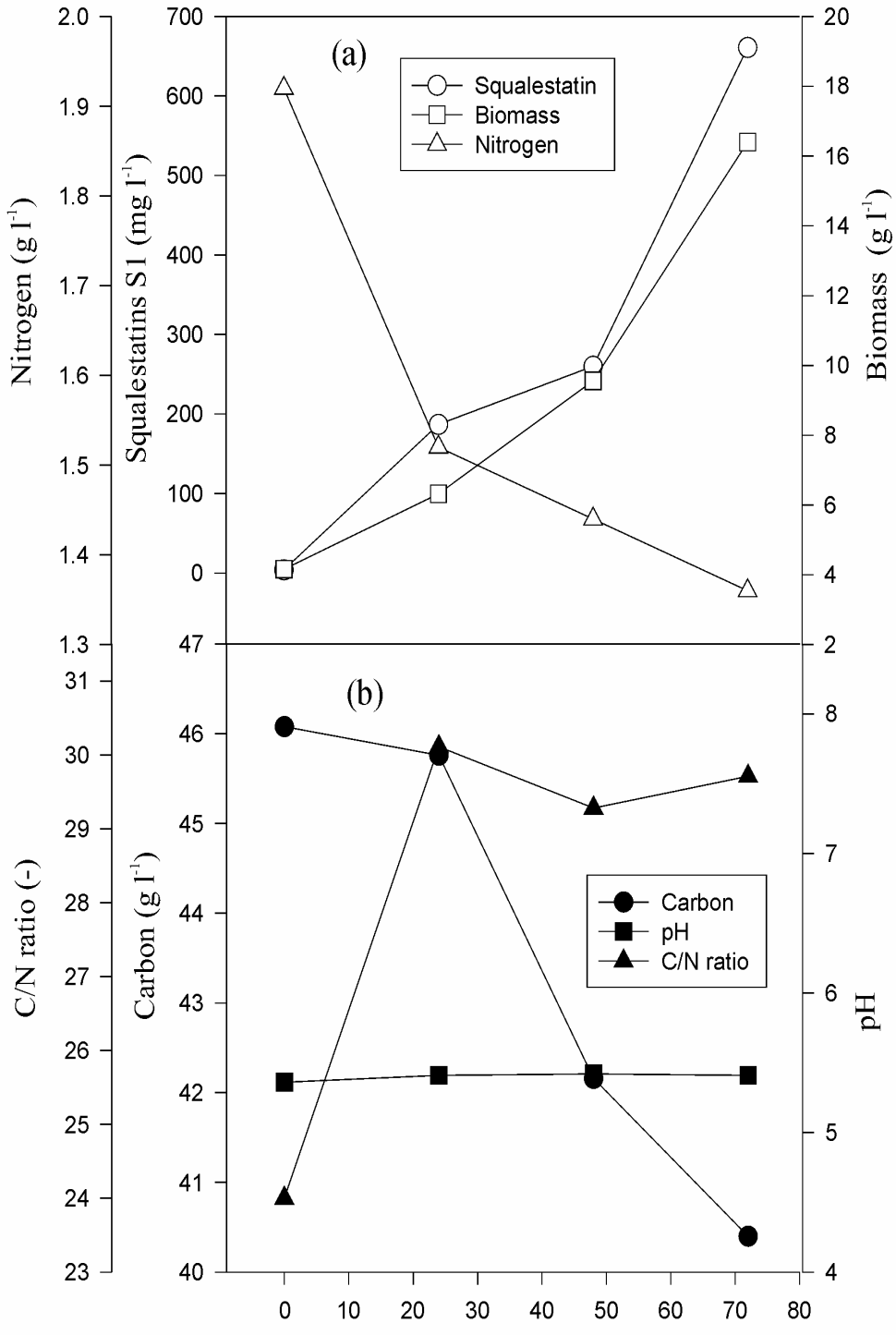

Figure 8.8. Kinetic of squalestatin S1 production in stirred tank bioreactor inoculated with free cells. (a) S1 (mg l^{-1}) (○), biomass (g l^{-1}) (□),and Total nitrogen (g l^{-1}) (Δ). (b) Carbon (g l^{-1})(●), pH (■) and ratio C/N (▲)

CHAPTER IX
CONCLUSIONS AND FURTHER WORK

9.1 CONCLUSIONS

Biotechnological processes have been flourishing over the last few years and their applications are increasing in number day by day. New metabolites are required for the production of new drugs for the treatment of diseases now common in the population, such as high blood pressure or heart disease.

Biotechnology is facing this new challenge by developing an interdisciplinary approach, applying tools such as mathematical modelling and statistical strategies. This way the research into new processes, that in the past used to take several years, can now be optimized in a few months. The advantages include, apart from the time saved, economical and environmental benefits, as fewer resources are consumed.

The main findings of the individual phases of the process are presented below.

Conclusions summary

Chapter II

WATER ACTIVITY , SOLUTE AND TEMPERATURE MODIFY GROWTH AND SPORE PRODUCTION OF WILD TYPE AND GENETICALLY ENGINEERED *Aspergillus niger* STRAINS

- ✓ Maximum growth rates were achieved for both strains considered (L11 and B1) under moderate a_W levels.

- ✓ L11 showed a higher growth rate than B1. Fastest growth was achieved at 30^{o}C, using glycerol as solute, and 0.97 a_w.

- ✓ Optimal conditions for growth of strain L11 were estimated by means of contour plot surfaces and found to be 0.965 a_w with glycerol as solute at 35ºC.

- ✓ The predicted value of the optimum growth rate on agar was 10.5 mm day^{-1}. A value of 10.85 mm day^{-1} was obtained experimentally giving a good correlation between the estimated and the measured results.

- ✓ Sporulation was optimum for the wild type strain at 0.99-0.95 and 35°C. However, a significantly higher production of conidia was achieved by L11 at 0.97-0.93 a_w and at 0.97 a_w and 35°C for strain B1.

- ✓ Optimal conditions for spore production were different from those for growth for both genetically engineered strains and wild type.

- ✓ Under similar ecological conditions the wild type and both the genetically engineered strains had different growth and sporulation patterns.

Chapter III

MODELLING THE EFFECT OF TEMPERATURE AND WATER ACTIVITY ON GROWTH OF *Aspergillus niger* STRAINS AND APPLICATIONS FOR FOOD SPOILAGE MOULDS

- ✓ An extended combined model describing the growth of two strains of *A. niger,* as a function of temperature (25-30°C) and a_w (0.90-0.99) was developed for the first time.

- ✓ This extends the previous square root model of water activity showing the relationship between temperature and bacterial growth rate developed by Ratkowsky *et al*. (1983) and the parabolic relationship between the logarithm of the growth rate and a_w developed by Gibson *et al*. (1994).

- ✓ A good correlation between the experimental data and the model predictions was obtained, with regression coefficients (R^2) > 0.99.

- ✓ The estimation of the minimum a_w levels (a_{wmin}) was in accordance with data in the literature for similar and a range of other *Aspergilli* and related species, regardless of the solutes used for a_w modification.

- ✓ The estimation of the optimal a_w (a_{wopt}) and the optimal growth rate (μ_{opt}) were in good agreement with the experimental results and data from the literature.

- ✓ This approach enables accurate prediction of the combined effects of environmental factors on growth of spoilage fungi for rapid prediction of cardinal limits using surface response curves.

- ✓ This approach is a rapid method for predicting optimal and marginal conditions for growth of a wide range of spoilage microorganisms in relation to interacting environmental conditions and will have applications for improving the shelf-life of intermediate moisture foods.

Chapter IV

EFFECT OF WATER ACTIVITY, INOCULUM SIZE AND AUTOCLAVING PROCEDURE ON LYZOSYME PRODUCTION BY TWO GENETICALLY ENGINEERED *Aspergillus niger* STRAINS

- ✓ The most important factors were the autoclaving procedure and the interaction between the autoclaving procedure, a_w level and initial spore concentration.

- ✓ Although both strains of *A. niger* had similar lysozyme concentrations (15 mg l^{-1}), different production patterns were found under the experimental conditions.

- ✓ Strain B1 produced relatively higher amounts of lysozyme under water stress (0.96 a_w) with all the substrates autoclaved together. This represented an important process advantage in the scale-up process because autoclaving all

the medium substrates reduces the risk of media contamination and it's more economical

- ✓ The concentration achieved by *A. niger* B1 in the autoclaving treatment (A), low a_w (0.96) and high inoculum size ($2.7x10^5$ spores/ml) was twice that achieved with the same strain when using the two other autoclaving treatments (B; C).

- ✓ The optimal conditions found on the contour surfaces for lysozyme production with *A. niger* B1 was 0.96 a_w, $2.0x10^5$ initial inoculation concentration and autoclaving together all the components of the medium.

Chapter V

A NOVEL IMMOBILIZED DESIGN FOR THE PRODUCTION OF THE HETEROLOGOUS PROTEIN LYSOZYME BY GENETICALLY ENGINEERED *Aspergillus niger* STRAINS

- ✓ Overall, immobilization in Ca-pectate resulted in higher lysozyme production compared to immobilization in Ca-alginate. Similar effects were observed when the polymer concentration was reduced. Regardless of polymer type and polymer concentration, increasing the inoculum level increased lysozyme production.

- ✓ A 10 fold increase in lysozyme production was achieved with Ca-pectate in comparison with Ca-alginate (20-23 and 0.5-1.7 mg l^{-1} respectively).

- ✓ The highest lysozyme concentration achieved was 23.0 mg l^{-1} by immobilization in 2% (w/v) Ca-pectate and 33% (v/v) mycelium with 3.5% (w/v) of gel-inducing agent ($CaCl_2$).

- ✓ The optimal pH condition for the production of lysozyme was in the range of 4.5-5.0.

- ✓ Only 0.94 a_w produced significant lysozyme concentrations regardless of polymer type.

Chapter VI

MEDIUM OPTIMIZATION FOR THE PORODUCTION OF THE SECONDARY METABOLITE SQUALESTATIN S1 BY A *Phoma* sp COMBINING ORTHOGONAL DESIGN AND REPONSE SURFACE METHODOLOGY

- ✓ A combined statistical methodology of orthogonal design $L_{27}(3^{13})$ and response surface techniques succeeded in optimizing the composition and concentration of a liquid fermentation medium for the production of squalestatin S1 by a *Phoma* species.

- ✓ The sources of carbon, their concentration and interactions with oily precursors were statistically significant factors.

- ✓ The optimal medium composition produced average concentrations of 434 mg l^{-1} in a five day fermentation at 25^{o}C. This represented an improvement of 60% of the maximum concentration predicted, and a two-fold increased productivity in comparisons with reported productivities for S1 in liquid fermentations with different fungal species.

- ✓ This approach enables rapid identification of key control parameters for optimizing production systems.

Chapter VII

DESIGN OF A NOVEL BUBBLE COLUMN BIOREACTOR BASED ON THE VOLUMETRIC MASS TRANSFER COEFFICIENT AND SCALE UP OF SQUALESTATIN S1 PRODUCED BY *Phoma sp*.

- ✓ Two k_La models are proposed. One representing a function of the superficial gas velocity (m s^{-1}) and ratio (H_L/D_r) (R^2=0.951).

- ✓ and the other a function of the power consumption (P_G/V_L) and ratio (H_L/D_r) (R^2=0.950).

- ✓ Regardless of the temperature effect, high volumetric mass transfer coefficients (0.14-0.17 s^{-1}) were found under the lower ratio (H_L/D_r) at the highest superficial gas velocities (0.115 m s^{-1}).

- ✓ A free cell fermentation was performed in the bubble column with a ratio of (H_L/D_r)= 3.7 and superficial gas velocity of U= 0.120 m s^{-1}, at 25^{o}C. The production of the secondary metabolite squalestatin S1 reached 420 mg l^{-1}.

- ✓ The bioreactor scale up succeeded in maintaining the high S1 concentration obtained in flask culture (434 mg l^{-1}) but in a shorter time period.

Chapter VIII

EXPLORATION OF NOVEL IMMOBILIZATION SYSTEMS IN BUBBLE COLUMN BIOREACTOR ON THE PRODUCTION OF SQUALESTATIN S1 BY A *Phoma* SPECIES AND COMPARISON WITH A FREE STIRRED TANK REACTOR

- ✓ A linear model for S1 concentration gave a very good fit (R^2 = 0.99).

- ✓ The factors having the most significance (*P*-value < 0.001) were air flow, gel-inducer ($CaCl_2$), polymer concentration and inoculum level.

- ✓ Optimal conditions for production of S1 resulted in concentrations of 883 mg l^{-1} which represented a 34 % improvement over that achieved (660 mg l^{-1}) in a stirred tank bioreactor (STR) system.

Overall findings

This is the fist time that a series of optimization strategies have been applied in two different models, with a secondary metabolite and a heterologous protein. The results were more promising for the secondary metabolite S1, as the work achieved the highest reported production of S1 reported in the literature for liquid fermentation. Lysozyme production was strongly influenced by the immobilization of the engineered fungi.

The present study succeeded in designing and developing a novel biotechnological process for the production of two important compounds: the secondary metabolite squalestatin S1 and the heterologous protein lysozyme, as well as establishing the guidelines for the production of similar compounds produced by fungi. These guidelines were:

- The development of a mathematical model which describes the growth rate of fungi under various environmental conditions.
- An ecophysiological approach to enhance the production of enzymes.
- The use of statistical tools to optimize the medium composition to enhance secondary metabolite production.
- The design of a novel immobilization design succeeded in improving the production of secondary metabolites and enzymes as compared with free-cell systems.

The use of these guidelines resulted in a considerable saving of time and resources and the robustness of the process was demonstrated.

9.2 *FUTURE WORK*

Lysozyme produced by *Aspergillus niger* B1

- ✓ Evaluation of growth rate by modifying the water activity with a polar solute (NaCl)
- ✓ Evaluation of the effect of immobilized *Aspergillus niger* B1 in the bioreactors and comparison with free cell fermentations
- ✓ Evaluation of the bubble column operation parameters such as air flow, pH and temperature on the production of lysozyme
- ✓ Evaluation of the kinetic parameters of *Aspergillus niger*, and correlations of the production of the heterologous and homologous proteins

Squalestatin 1 produced by *Phoma sp.*

- ✓ Evaluation of the effect the dynamic k_La on S1 production
- ✓ Elucidation of the correlation between volatiles and biochemical stages on the S1 production
- ✓ Evaluation and modelling a non-invasive method to quantify biomass on immobilized cell fermentations by optical methods
- ✓ Developing techniques for assessing the rheological properties in the bioreactor

APPENDIX I REFEREED PAPERS IN THIS THESIS

Parra R., Magan N (2004) Modelling the effect of temperature and water activity on growth of *Aspergillus niger* strains and applications for food spoilage moulds. *Journal of Applied Microbiology* **35**:232-237.

Parra R., Aldred D., Archer D., Magan N (2004) Water activity, and temperature modify growth and spore production of wild type and genetically engineered *Aspergillus niger* strains. Enzyme and Microbial Technology. **97**:429-438. (Available on line at www.sciencedirect.com)

Parra R., Aldred, D., Archer D & Magan N (2004) Effect of Water Activity, Inoculum Size and Autoclaving Procedure on Lysozyme Production by Two Genetically Engineered *Aspergillus niger* Strains. *Biotechnological Progress. Submitted*.

Parra R., Aldred D., Archer D & Magan N (2004) A novel immobilized design for the production of the heterologous protein lysozyme by a genetically engineered *Aspergillus niger* strain. *Applied Microbiology and Biotechnology. In Press.*

Parra R., Aldred D., Magan N (2004) Medium optimization for the production of secondary metabolite squalestatin S1 by *Phoma* sp combining orthogonal design and response surface methodology. *Applied and Environmental Microbiology. Submitted.*

Parra R & Magan, N (2004) Design of a novel bubble column bioreactor based on the volumetric mass transfer coefficient and scale up of squalestatin S1 produced by *Phoma* sp. *Biotechnology and Bioengineering. In preparation*

Parra, R & Magan, N. 2004. Exploration of novel immobilization systems in bubble column bioreactor on the production of squalestatin S1 by *Phoma* sp and compared with free cell fermentations in a stirred tank reactor. *Journal of Industrial Microbiology and Biotechnology. In preparation.*

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