INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

THE EFFECTS OF SURFACTANTS ON THE SOLID SUBSTRATE FERMENTATION OF POTATO STARCH

by

Ana Paula Góes

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Department of Agricultural and Biosystems Engineering
McGill University
Macdonald Campus
Ste-Anne-de-Bellevue, H9X 3V9
Quebec, Canada

March, 1999

To my mother,

for giving me so much love and providing me support,

and most of all for being so present while so far in distance

THE EFFECTS OF SURFACTANTS ON THE SOLID SUBSTRATE FERMENTATION OF POTATO STARCH

ABSTRACT

The potential of surfactants for improving the yields of α-amylase during the solid substrate fermentation (SSF) of potato starch using pure and mixed cultures was examined. The microorganisms used in this study were *Aspergillus oryzae* ATCC 1011, *Bacillus subtilis* ATCC 21556 and *Bacillus subtilis* ATCC 21332. The surfactants tested were Tween 20, Tween 80, SDS and surfactin. The fermentations were carried out in perforated trays after the addition of 10% (v/w) inoculum and with temperature and humidity controlled at 30°C and 90% RH respectively. Samples were taken and analyzed quantitatively for the production of α-amylase and biomass and qualitatively by scanning electron microscopy (SEM) using a JSM-840 A scanning microscope at 10 kV accelerating voltage.

It was possible to increase fungal α-amylase production by as much as 6 fold in the process with the addition of either synthetic surfactants or the biosurfactant surfactin. The bacterial α-amylase yields increased up to 11.5 fold in with the addition and/or the co-culture production of surfactants. The highest enzyme activity was found in the fermentation of a mixed culture of the two *Bacillus* strains with the addition of Tween 80. During the SSF with *B. subtilis* ATCC 21332 and ATCC 21556 as a mixed bacterial culture, there was also the production of surfactin in yields comparable to those obtained in a submerged fermentation. The biofilm formation as observed by SEM appeared to be associated with the presence of surfactants in the process and was not formed when no surfactants were present. The biofilm was observed as an entrapment of the bacteria in the substrate, resulting in improved access to the starch and higher production of α-amylase.

LES EFFECTS DES SURFACTANTS SUR LA FERMENTATION EN ÉTAT SOLIDE DE L'AMIDON DES POMMES DE TERRE

RÉSUMÉ

Le potentiel de surfactants pour augmenter le rendement de la production d'α-amylase par fermentation en état solide (FES) de l'amidon des pommes de terre a été étudié. Des cultures mixtes e/ou pures d'*Aspergillus oryzae* ATCC 1011, *Bacillus subtilis* ATCC 21556 and *Bacillus subtilis* ATCC 21332 ont été utilisées comme souches microbiennes. Tween 20, Tween 80, SDS et surfactin étaient les surfactants testés. Les fermentations ont été conduites sur des plateaux aprés l'addition de 10% (p/v) d'inocula avec une temperature (30°C) et une humidité relative (90% HR) contrôlées. Les échantillons ont été analysés pour les déterminations quantitatives de la production d'α-amylase et qualitatives par microscopies speectrale (MES) en utilisant un microscope JSM-840 A à 10 kV d'accélération de voltage.

Il était possible d'augmenter près de 6 fois la production de l'α-amylase par les champignons en presence des surfactants synthétiques ou du biosurfactant surfactin. Les rendements des α-amylases bactériennes ont augmenté jusqu'à 11.5 fois aprés l'addition des surfactants ou aprés addition d'une co-culture microbienne productrice de surfactant. L'activité enzymatique la plus élevée a été trouvée quand des cultures mixtes des deux *Bacillus*, avec l'addition de Tween 80, on été réalisées. En fermentation FES d'une culture mixte de *Bacillus subtilis* ATCC 21332 et *Bacillus subtilis* ATCC 21556, les rendements de la production de surfactin obtenus étaient comparables aux rendements obtenus en fermentation liquide. La formation d'un biofilm, observée en microscopie d'électron que était associée à la présence des surfactants dans les procédés. Le biofilm n'était pas observé quand es surfactant n'étaient pas présents. Le biofilm servait à trapper les bacteries dans les substrats, et par conséquent il favorisa l'accés à l'amidon et augmenta la production de l'α-amylase.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to express my sincere thanks to my supervisor, Dr. John Sheppard, who gave me this opportunity and for all his support throughout the course of this study. I have benefitted immensely under his guidance and most of all from his knowledge.

My sincere thanks to many individuals have contributed to the completion of this work. Mr. Ray Cassidy who helped me in the construction of some of the equipment, Mr. M. Pikarnegar and Mr. R. Smith for their technical support with the Chamber room used in this project, Mr. G. Rimmer for his assistance with the electron microscope located in the Plant Sciences Department, Dr. S. Sotocinal for his help in the preparation of samples for the SEM pictures. I also thank Ms. Jeannie Mui, Ms. Matilda Cheung and Ms. Helen Campbell from the Departments of Anatomy and Cell Biology and Mining and Metallurgical Engineering for their help in the SEM sample preparation. My sincere thanks to Dr. H. Vali, Director of the Electron Microscope Center for the use of his laboratories, for helping in the SEM samples preparation and for his comments and suggestions.

I would like to thank Ms. Helen Cohen-Rimmer for her suggestions, the work done in the printing of the SEM pictures and help with slides preparation. My thanks are also extended to Mr. Luciano Germani from ICC for all his help.

Many thanks to CAPES, NSERC and McGill University for supporting me in my Ph.D. studies. My sincere thanks are also extended to the Department of Agricultural and Biosystems Engineering including Dr. G.S.V. Raghavan, Dr. Suzelle Barrington, Ms. Sandra Nagy, Ms. Susan Gregus, Ms. Maria Gabriel, Ms. Sharon Laduke and many others

for providing the flexible environment in which to work.

I take this opportunity to thank my colleagues in the laboratory for their friendship and support. Many thanks to my friends Dr. D. Lyew and Meda Venkatesh for all their help, constant support and friendship. My sincere thanks to Carla and Marcelo Molento, Dr. Ana Maria da Silveira and all my friends who have helped me with their support and encouragement through these years. I wish also to thank Dr. Veronique Barthet for proof-reading the French abstract. My special thanks to Dr. M.A.F. Belem for the French translation of the abstract, his support and most of all for his friendship.

Last, I owe special thanks and appreciations to my family. My parents for their love, care and prayers but most of all, my mother and best friend for her love, for being an example to my life, for her understanding and support. My sister Alessandra, my brother-in-law David Scullion, my nephew and niece Lucas and Natalia Scullion for their support, understanding, care and love. I wouldn't be here today if were not for them. Thank you God so much for everything.

LIST OF FIGURES

LIST OF TABLES

No. Page

CHAPTER 1

INTRODUCTION

The economic value of microorganisms is often enhanced through fermentation technologies, that is, by growing microorganisms under controlled conditions to obtain many higher value substances. Most fermentations are carried out in a liquid medium where the substrate to be fermented is diluted in water and is then inoculated with the selected microorganism to start the process. However, some microorganisms, primarily fungi, are capable of growing with a minimum of free water. Bacteria grow best when in a liquid phase or, at least, when the nutrients are in free water because, unlike fungi, they are unable to penetrate deep into a solid tissue for complete utilization of the substrate. In this case most of the moisture needed for the existence of the microorganism is found in the solid matrix in an absorbed or complex form, with moisture ranging between 12 and 80% by weight, depending on the degree of absorbency of the substrate. A process which exploits the growth of microorganisms under these conditions is generally known as a "solid substrate fermentation" (SSF) (Carrizales and Jaffe, 1986).

SSF processes have been used by man for many years. The term "SSF" is any fermentation in which the substrate is not a free liquid (Aido *et al.*, 1982). In other words, in SSF the moist water-insoluble solid substrate is fermented by microorganisms in the absence or near-absence of free water resulting in a semi-solid or solid fermentation system. Free water is defined as the water found within the pores or intergranular spaces of the food. This water retains the usual physical properties of water and often acts as a solvent or

dispersing agent for crystalline or colloidal substances. As there is some confusion in the literature regarding what constitutes free water, SSF will be described as the process which utilizes water insoluble materials for microbial growth and metabolism, and it is usually carried out in solid or semi-solid systems with reduced water content compared with submerged fermentation (Zheng and Shetty, 1998).

Research interest in SSF processes has been increasing considerably due to the opportunities SSF presents for increased productivity and product yields, reduced water consumption compared to submerged fermentation, and the possibility of using a wide range of agri-industry waste products as substrates. Different products can be produced using SSF, such as enzymes, organic acids, alcohols, surface active agents and antibiotics. Also, as the microorganisms in SSF grow under conditions closer to their natural habitats, they may be capable of producing metabolites that may not be produced in a submerged fermentation.

Among the enzymes being produced in SSF, hydrolytic enzymes, such as amylases, hold particular promise for SSF because they can be produced on plant biomass with little or no supplementation; an amylase-producing organism can simultaneously provide its own carbon source from starchy materials while producing the amylase product (Silva and Yang, 1998). Among the potential starchy materials for SSF, potato wastes are of great interest due to their availability in large amounts throughout the world and to their high starch content. According to FAO, in 1996 the world production of potatoes was 306 million tonnes, in the USA, 22.6 million tonnes and in Canada, 4 million tonnes. Potatoes are one of the main sources of food in the world, second only to wheat. Starch corresponds to 75% of the potato dry weight.

Objectives

The overall objective of the research to be presented here was to investigate the potential of SSF for α-amylase production using potato peel as the substrate, *Bacillus subtilis* ATCC 21556, *B. subtilis* ATCC 21332 and *Aspergillus oryzae* ATCC 1011 as the microorganisms, with the addition of surfactants for process optimization.

The specific objectives of this work include:

(1) To quantify α-amylase yields using a pure culture of *B. subtilis* ATCC 21556 and a pure culture of *A. oryzae* ATCC 1011 as well as to estimate the growth of these microorganisms by indirect measurement of biomass.

(2) To evaluate the production of α-amylase using a mixed culture of two strains of *B. subtilis* (ATCC 21556 and ATCC 21332) and a mixed culture of *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21332.

(3) To optimize the production of α-amylase by adding the synthetic surfactants Tween 80, Tween 20 and SDS and the biosurfactant, surfactin, at different concentrations.

(4) To compare the production of the biosurfactant surfactin in a SSF process by growing *B. subtilis* ATCC 21332 as a pure culture and a mixed culture of the two *B. subtilis* strains.

(5) To study the biofilm formation during a SSF process under different conditions by using scanning electron microscopy (SEM) analysis.

(6) To propose the mechanism of action occurring in the SSF process by establishing the most significant interactions between starch/enzyme, starch/surfactant, enzyme/surfactant, enzyme/water, surfactant/water and starch/ water.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Solid Substrate Fermentation (SSF)

Historically, solid substrate fermentation started in the Far East. This process was used to improve the nutritional and organoleptic qualities of various agricultural products such as rice and soya bean (Gervais and Bensoussan, 1994).

The koji process may be considered as the prototype of solid substrate fermentations (Moo-Young *et al.*, 1983). Koji is basically an enzyme preparation produced by growing a mould, either *Aspergillus oryzae* or *Aspergillus soyae*, on steamed rice or other cereals (Cannel and Moo-Young, 1980). It is used as a starter in the soy sauce (shoyu) industry, fermentation of miso (a semi-solid cheese like food) and in the brewing of the Japanese rice wine (saké). Another oriental fermented product prepared by the koji process is tempeh, a popular Indonesian food. It is a food most commonly prepared from soya, however, this fermentation technique is extremely versatile and has been applied to a variety of other legumes (Paredez-Lopes and Harry, 1988; Pandey, 1992). In addition to these types of foods, such processes have been used successfully in recent years for the production of protein enriched feed, single cell protein (SCP), fungal metabolites, and bioconversion of plant, animal and domestic wastes into useful products such as enzymes, organic acids and antibiotics (Paredez-Lopes and Alpuche-Solis, 1991).

2.2 Substrate and Applications

The solid substrate used and its preparation can significantly affect the success of SSF. The ideal solid substrate is the one that provides all the nutrients necessary for the optimum growth of the microorganisms. In SSF process, the substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells.

The properties of the solid substrate can be modified by pretreatment to improve the performance of the process. The steps involved in substrate preparation and pretreatment are: (1) size reduction by grinding or chopping, (2) chemical or enzymatic hydrolysis of polymers, (3) supplementation with nutrients if necessary, and (4) autoclaving.

A great variety of raw materials are currently available as a substrate for SSF processes. The spectrum, apart from traditional carbon and nitrogenous substrates, includes various agricultural and industrial by-products and waste materials. These agricultural feedstocks and their wastes have the advantages of being available in surplus, about 13×10^9 tonnes of wastes per year and of being produced in regions with temperate-to-tropical climates (Paredes-López and Alpuche-Solís, 1991; Makkar and Cameotra, 1997).

Starchy materials are some of the most significant substrates for SSF due to the large amount available and rate at which they can be fermented by a great number of fast growing microorganisms (Yang and Ling, 1989). Starch is a carbohydrate occurring in granular form in the organs of plants. It can be extracted commercially from many raw materials including corn, cassava, potatoes and wheat (Lee, 1991). The large amount of waste generated by the starch industry has the potential of being converted by microorganisms into useful products.

For example, sweet potato residue has been used for the production of the antibiotic tetracycline. This substrate was chosen for saving energy in antibiotic production, reducing the amount of agricultural waste and because it can be easily converted to biomass (Yang and Ling, 1989).

Wheat bran is another important substrate used frequently in SSF process. This substrate has been used for the production of various important metabolic products such as proteinases, glucoamylase, gibberellic acid and α-amylase, a starch-saccharifying enzyme (Ramesh and Lonsane, 1989). Microbial proteinases constitute a large and complex group of enzymes. Among these, the fungal proteinase finds extensive applications in the dairy, food processing, protein modification, pharmaceutical, meat tenderization, brewing, baking and other industries in North America. These enzymes are currently being produced by SSF and with extremely high titres (59,100 units of an extracellular proteinase/g substrate) (Padmanabhan *et al.*, 1993). Fungal glucoamylase, another important enzyme for the food industry, has also been produced by SSF. The culture used in this process is *Aspergillus niger* and improvements in the process such as nutrient supplementation, addition of whole corn flour, better process controls and a novel bioreactor have shown an increase in productivity (Pandey, 1990; Pandey *et al.*, 1996). Gibberellic acid is an important plant growth regulator and was originally produced by submerged fermentation at very high costs. SSF process has shown to be a potential technique for its production (Bandelier *et al.*, 1997).

Cassava fibrous waste is being used as a substitute to wheat bran in SSF for the production of pectinase (Budiatman and Lonsane, 1987) and for protein enrichment (Daubresse *et al.*, 1987). This substrate presents the advantages of being less expensive than

wheat bran, presenting a high content of starch and providing similar yields of pectinase when used as a substitute for wheat bran.

Fruit wastes are also an important substrate used in SSF process. Citrus wastes have been used for the production of single cell protein (Barreto de Menezes *et al.*, 1989), in fungal pretreatment for improved productivity of biogas and methane (Srilatha *et al.*, 1995) and as an alternative substrate for pectinase production. Pectic enzymes are of major importance to the food industry in processes of maceration and liquefaction of fruits and vegetables and to facilitate juice extraction and clarification (Garzón and Hours, 1992). Banana wastes have been used for α-amylase production using *Bacillus subtilis* CBTK 106 isolated from the same waste (Krishna and Chandrasekaran, 1996). Cranberry processing waste can be used in SSF to be converted into various potential value-added products such as fungal inoculants (Zheng and Shetty, 1998).

Sugarcane has been tested for the production of lactic acid which is a product widely used in the food industry as preservative or taste-enhancing additive and in the pharmaceutical industry to form polylactic acid (PLA), a polymer used in the manufacture of new biodegradable plastics, by *Lactobacillus casei* subsp *casei* (Xavier and Lonsane, 1994), and for the production of tannase, an enzyme used in the clarification of beer and fruit juices and manufacturing of coffee flavored soft drinks (Lekha and Lonsane, 1994).

SSF processes have also been applied to the alcoholic fermentation of sugar-beet (Cochet *et al.*, 1988), production of pyrazine in soybeans inoculated with *Bacillus subtilis* IFO-3013, a high-added-value compound used in the food industry as an additive for flavoring (Besson *et al.*, 1997), citric acid production (Lu *et al.*, 1998), extracellular lipase

from *Candida rugosa* (Benjamin and Pandey, 1997) and for the production of lipopetide antibiotics, iturin A and surfactin (Ohno *et al.*, 1995a).

2.3 SSF Process

According to Mitchell and Lonsane (1992) the steps involved in an SSF are: preparation of the solid substrate, sterilization, preparation of inoculum, incubation in appropriate fermentors, maintenance of optimal conditions, sampling and downstream processing.

2.3.1 Preparation of the solid substrate

The preparation of a solid substrate often involves a pretreatment to decrease the particle size or to increase the surface area and availability of nutrients to the microorganism.

Many substrates require particle size reduction to be suitable for SSF. Particle size is extremely important since it affects the surface area to volume ratio of the particle and the packing density within the substrate. Generally, smaller particle sizes provide a larger surface area for heat transfer and gas exchange, however, there must be a balance reached in the particle size. If the particles are too small they pack together too tightly and if the particles are too big then much of the substrate will remain inaccessible to the microorganism (Mitchell *et al.*, 1992 and Mudget, 1986). Particle size reduction includes milling, grinding and chopping. Despite the importance of particle size, only a few investigations have been done on this topic. Pandey (1991a) studied the effect of particle size of the substrate wheat bran

on glucoamylase production in an SSF showing that smaller particle substrate gave higher enzyme activity and Echevarria *et al.* (1991) have reported on the effect of particle size of sugar cane in an SSF for protein enrichment.

Nutrient supplementation is also another way of substrate pretreatment. It may be beneficial in nontraditional fermentations to provide supplemental media to initiate biomass production, induce enzyme synthesis, provide balanced growth conditions, or prolong secondary metabolite production (Mudget, 1986). The most frequent nutrients used are $(NH_4)_2HPO_4$, $CaCl_2.H_2O$, $MgSO_4.7H_2O$ and KCl. The addition of supplementary solid substrates along with the main substrate is also another way of nutrient supplementation.

2.3.2 Sterilization

Although SSF is often carried out non-asseptically, in particular cases it may be necessary to sterilize the substrate, the bioreactor, or the air used for forced aeration. This step is done in order to sterilize, or at least pasteurize the substrate, eliminating microorganisms that may spoil the end product, to destroy antinutritional factors that may be harmful and to allow absorption of water into the substrate particles (Mudget, 1986 and Paredes-López and Harry, 1988). Sometimes the solid medium requires cooking for physical modification of solid particles, softening and partial hydrolysis of starches and proteins. The cooking-sterilization temperature most frequently used is 121°C and the time varies between 15 and 60 minutes.

2.3.3 Inoculum

The preparation of a suitable inoculum, either by traditional techniques or pure culture technique, is necessary. For many SSF processes, the inoculum can be prepared and used in the same way as in liquid media. The even distribution and the density of the inoculum are also important (Mudget, 1986 and Mitchell *et al.*, 1992). Too low a density may give insufficient biomass and permit the growth of undesirable organisms; too high a density may produce too much biomass and deplete the substrate of nutrients necessary for product formation.

2.3.4 Fermentors

In a fermentation process, the bioreactor provides the environment for the growth and activity of the microorganisms which carry out the biological reactions. During the period of fermentation, the bioreactor should be capable of preventing the release of internal biomass/media into the environment as well as preventing foreign substances into the reaction media (Pandey, 1991b). An ideal fermentor should have several characteristics: in particular the material of construction should be nontoxic and able to withstand pressure (generally pressurized steam for sterilization).

The design of SSF systems has been for the most part highly empirical in nature. According to Mitchell *et al.* (1992), for the pilot-plant and large-scale fermentation of moist solid substrate, some factors need to receive special consideration for the construction or selection of the fermentation equipment: (1) inoculation, sampling and transfer techniques must be simple; (2) homogeneity of culture medium is important to prevent particle

agglomeration and settling of the substrate during fermentation; (3) appropriate aeration rate and prevention of heat build-up; (4) equipment sterilization; (5) handling must be simple; (6) materials used for construction of the fermentation vessel and (7) low capital costs and operating expenses. There are different types of reactors presently used for SSF: tray, packed bed, rotating drum, stirred bioreactor and others.

Tradionally, most SSF processes are conducted in shallow trays where a relatively thin layer is spread over a large horizontal area to avoid over heating and to maintain aerobic conditions. In these fermentors there is usually no mixing and no forced aeration although the base of the tray may be perforated and air may be gently circulated around the trays (Mitchell *et al.*, 1992 and Duran *et al.*, 1996). The fermentor is usually humidified by humidifiers and the temperature is controlled by placing these trays in a controlled cabinet or room. Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial and commercial scale (Daubresse *et al.*, 1987; Ghildyal *et al.*, 1992; Raghava Rao *et al.*, 1993).

Packed bed bioreactors are characterized by having a static substrate supported on a perforated base plate through which forced aeration is applied. The temperature regulation is carried out by using a jacket with water thermostatically regulated. They can be advantageous for some studies such as screening of strains and they are relatively simple allowing for better process control. Consequently, many workers have used a packed bed bioreactor, such as Laukevics *et al.* (1984), Sato *et al.*, (1985); Gowthaman *et al.*, (1995). Disadvantages of this bioreactor include difficulties with the emptying of final product, non-uniform growth, poor heat removal and problems with scale-up.

In the rotating drum bioreactor the design is based on the use of a drum-shaped container mounted on a system of rollers which act both as support and as rotating devices (Lonsane *et al.*, 1985). To ensure proper aeration, the air inlet and outlet should be situated at opposite ends of the bottle. Microbial growth in these fermentors appears prolific and uniform. Operation is simple and cleaning is fast. They present some problems such as microbial contamination, medium aggregation and heat build-up. Kargi and Curme (1985) have studied the influence of rotational speed of a rotating drum fermentor on the rate of ethanol fermentation and Reu *et al.* (1993) have studied temperature control in these reactors.

Stirred bioreactors have been used in SSF to convert wheat straw into protein-enriched feed (Viesturs *et al.*, 1981). They are of two main types depending on whether the axis of the bioreactor is horizontal or vertical. Horizontal stirred bioreactors are quite similar to rotating drum bioreactors except that the mixing is provided by an internal scraper or paddles, rather than by rotation of the body of the bioreactor. Vertical stirred bioreactors are often subjected to forced aeration. They differ from packed bed bioreactors by the fact they are agitated, either continuously or intermittently.

Once the substrate is placed in the appropriate fermentor and inoculated, the selection of temperature and time for incubation will be guided by the characteristics of the culture and the kinetics of product formation. The incubation time for enzyme production can vary between 30 hours and eight days but in most cases, the time employed varies between 30 and 72 hours.

2.3.5 Optimal Conditions

The maintenance of optimal conditions in SSF is not easy because the process involves many parameters, such as type of microorganism, substrate, type of reactor and environmental conditions.

Accurate measurement of variables related to biological activity in SSF is difficult but most important. There is a lack of good methods for these measurements and this is a disadvantage in research on SSF compared to that on submerged fermentation (Smits *et al.* 1996).

Only a little work has been done to optimize SSF processes. Abullah *et al.* (1985) have reported on the optimization of the conditions for SSF of wheat straw with *Chaetomium cellulolyticum*. They established the best pretreatment for the substrate and fermentation conditions such as moisture content, incubation temperature and time and thickness of the substrate.

2.3.6 Downstream Processing

The downstream processing is necessary in order to isolate the product and to convert the product to a form which is acceptable to market. The unit operations involved are separation of cell or suspended solids, product isolation, product purification, formulation of the product and effluent disposal. For SSF there is an extra unit operation necessary (when compared to submerged fermentation) which is the leaching of the product from the solids. The crude extract obtained from the leaching process may contain various soluble solids in addition to the product of interest. It may contain secondary metabolites, soluble constituents

of the medium and a variety of suspended solids such as finer particles, microbial cells and spores.

Enzyme recovery from SSF can be done by direct use of the product as source of enzyme, by drying, leaching where the enzyme can be leached from wet or dried solids yielding a crude extract, by precipitation and partial purification (Lonsane and Kriahnarah, 1992).

2.3.7 Comparison with submerged fermentation

It is important to compare the advantages and disadvantages of SSF with respect to the conventional submerged fermentation. Several advantages of SSF over submerged fermentation have been claimed by various workers (Cannel and Moo-Young, 1980 and Mitchell and Lonsane, 1992):

(A) The medium is relatively simple, consisting of an unrefined agricultural product which may contain all the nutrients necessary for microbial growth, so the substrate may require less pretreatment in SSF than in submerged fermentation;

(B) Limited amounts of liquid waste to deal with, consequently reduced cost of waste treatment;

(C) The restricted availability of water may help to select against contaminants, especially bacteria and yeasts, although contamination by fungi may be a problem. The low moisture level may favor the production of specific compounds which may not be produced or may be produced in low concentration in submerged fermentation;

(D) Aeration is easily achieved as there are airspaces between substrate particles;

(E) The concentrated nature of the substrate means that smaller reactors can be used in SSF compared to liquid fermentation to hold the same amount of substrate. Smaller reactor volumes result in lower capital and operating costs.

But according to Mitchell and Lonsane (1992) and Sargantis *et al.* (1993), SSF also present some disadvantages:

(A) SSF is restricted to microorganisms which can grow at reduced moisture level and, therefore, the range of possible processes and products is limited compared to submerged fermentation;

(B) The solid nature of the substrate can cause problems in monitoring process parameters. In addition, it is very difficult to ensure even distribution of any substances added during the process, so effective control of parameters such as pH, moisture content, biomass and substrate concentration is difficult.

(C) Many important basic scientific and engineering aspects in SSF are as yet poorly characterized, growth kinetics are poorly characterized and there is insufficient quantitative information to establish criteria for reactor design, scale-up and process optimization.

2.4 Environmental Parameters for Microbial Growth

Environmental conditions such as temperature, pH, water activity, aeration, concentration of nutrients and products significantly affect microbial growth and product formation. In submerged cultures environmental control is comparatively simple because of

the homogeneity of the suspension of microbial cells and of the solution of nutrients and products in the liquid phase.

2.4.1 Moisture

One of the differences between SSF and submerged fermentation is that in the former the moisture content of the substrate is low, resulting in a potential limitation to growth and metabolism of the microorganism (Xavier and Karanth, 1992). The concept of water availability in the substrate is thus very important in SSF. The water availability is quantified by the term 'water activity' (a_w) which represents the amount of unbound water available in the immediate surroundings of the microorganism (Ramana Murthy *et al.*, 1993). A_w is defined as relative humidity of the gaseous atmosphere in equilibrium with the substrate. It is an important parameter because it influences microbial growth, enzymatic and biochemical processes.

The influence of water activity in some SSF processes is well documented. Pandey *et al.* (1994) have reported on how a_w of the substrate affects the growth and activity of *Aspergillus niger* for glucoamylase production. Grajek and Gervais (1987) and Gervais *et al.* (1988a) studied the influence of water activity of sugar-beet pulp on the growth of *Trichoderma viride* TS and the relationship between the mycelial growth and water activity of substrates. Gervais *et al.* (1988b) have studied the effects of water content and water activity of cellulose substrate on the growth of a filamentous fungus *Penicillium roqueforti* and Gervais and Sarrette (1990) have reported on the influence of water activity of the medium on aroma production by *Trichoderma viride* grown on solid substrate.

The optimum moisture content for the cultivation of microorganisms in SSF processes is highly dependent upon the water binding properties of the substrate (Prior *et al.*, 1992). Bacteria mainly grow at higher a_w values (about 0.9) while the filamentous fungi and yeasts can grow at lower a_w values (0.6-0.7). In general, the optimum moisture content for growth and substrate utilization is between 40-70%, but this depends upon the microorganism and substrate used for cultivation.

2.4.2 pH

The hydrogen ion concentration is a very important factor in SSF. It is well known that pH affects the physiology of fungi, thereby strongly influencing the growth of microorganisms. The control of pH in SSF is very difficult because of the problems of achieving an equal distribution of acid or base solution throughout the solid medium. Therefore, it is desirable that the microorganism used should have a broad pH range for growth. Bacteria in general have a pH optimum between 6.5 and 7.5 but can grow between pH 4.0 and 9.0. Most fungi are able to grow in a wide pH range of between 2.0-8.0, with optimum pH in the region between 3.8 and 6.0.

2.4.3 Temperature

Temperature regulation is directly related to water activity and aeration. The optimum temperature for SSF processes depends on the type of microorganism utilized. For example, fungi usually grow better in a temperature range of 25-35°C, with optimum

enzyme production at 30-35°C. Bacteria normally grow better at higher temperatures, 30-45°C and enzyme production is optimum at 35-37°C.

Temperature variations during SSF are correlated to the metabolic activities of the microorganism and strongly determine the performance of fermentation (Auria *et al.*, 1993). High temperatures can cause moisture loss, reduce or even stop vegetative growth and product formation and induce nonproductive sporulation. Low temperatures are not generally favorable for growth of the microorganisms and can cause deactivation of biochemical reactions (Moo-Young *et al.*, 1983).

A limitation of SSF is the difficulty of removing heat, due to the low thermal conductivity of the solid substrate (Gervais and Bensossan, 1994). Control of temperature has been achieved by evaporative cooling, changing the relative humidity and other methods.

2.4.4 Aeration

Aeration in SSF process is needed for three main functions: (1) to maintain aerobic conditions, because the partial pressures of O_2 and CO_2 in the gas environment of SSF are critical factors for growth and product formation; (2) to control substrate temperature because the low moisture environment in SSF creates difficult conditions for heat transfer and temperature regulation and (3) to regulate water content/a_w of the substrate, CO_2 and volatile metabolites produced during metabolism

Ghildyal *et al.* (1992) and Gowthaman *et al.* (1993) have investigated the gaseous concentration in solid state fermentors as well as enzyme activity and their relationship. The required aeration rate depends on the microorganism used, the particular oxygen requirement

for growth and product synthesis on the nutrients supplied, the amount of metabolic heat to be dissipated, the degree to which CO_2 and other volatile metabolites are to be eliminated, the thickness of the substrate and its bulk density and moisture content (Prior *et al.*, 1992). Therefore, aeration must be optimized for each specific substrate, microorganism and process.

2.5 Substrate - Potato Peel

The potato of commerce belongs to a single species, *Solanum tuberosum* L. (Hawkes, 1992). After maize, the potato is the most widely distributed crop in the world. It is grown in about 140 countries and most of the production is concentrated in the temperate regions of the industrialized countries (Beukema and Van der Zaag, 1990).

The potato is the most important vegetable crop in Canada. Canadian production is concentrated in P.E.I. (34%), Manitoba (17%), Québec (16%) and New Brunswick (13%). Varieties of potatoes vary from province to province with Superior as one of the main chipping and table varieties. In 1997/1998 Canadian potato production was 4,169,000 tonnes (Agriculture Canada). By the year 2000, Canada's potato production is expected to be 4.5 million tonnes. About 50% of all potatoes grown in Canada are processed. During the processing about 10-20% of the potato is discarded during peeling, resulting in a substantial waste disposal problem (Arora *et al.*, 1993).

Potato is one of the world's three most important sources of starch, the others being maize and wheat (Christensen and Madsen, 1996). Consequently, potato processing wastes

are also composed of starch, containing pieces of potato material that are rejected during processing (Polman *et al.*, 1995).

Various studies have been conducted in order to transform potato waste into useful products for the food industry. This by-product has been tested as a potential source of dietary fiber in bread (Camire *et al.*, 1997) and phenolic compounds with antioxidant activity (Rodriguez de Sotillo *et al.*, 1994). Because of the low price of this waste and the high starch content, it could also be used as a source of biomass-derived fermentable sugar for industrial production of chemicals, such as glycerol and biosurfactants (Polman *et al.*, 1995) and in this particular study, for the production of α-amylase.

Starch is a glucose polymer and is one of the most widely available polysaccharides. Of all the polysaccharides, starch is the only one universally produced in small individual packets called granules. Potato has the largest starch granules with a diameter ranging from 15-100 nm (Whistler and Daniel, 1985). Starch comprises 65 to 80%, typically about 75%, of the dry weight of the potato. The chemical composition of the starch granules is shown in Table 2.1 (Mitch, 1984). Normal starches contain roughly 25% amylose. Amylose chains of potato starch are long and are composed of glucose subunits. The other fraction of starch is amylopectin, which is a ramified structure containing a substantial amount of α-1,6 glycosidic linkages (Lee, 1991 and Lisinska and Leszczynski, 1989).

Table 2.1 Chemical Composition of Potato

	Range (% by weight)
Water	63.2-86.9
Total solids	13.1-36.8
Proteins	0.7-4.6
Fat	0.02-0.96
Carbohydrate	13.3-30.53
Ash	0.44-1.9

2.6 Mixed Culture

The modern fermentation industry has been dominated by the pure culture approach. However, recent years the properties of mixed cultures have attracted increasing attention. Among the reasons for this interest is the exploitation of defined mixed cultures for a range of biotechnological purposes, including single cell protein production and bioconversion (Bull, 1985).

A defined mixed culture involves the inoculation of the substrate with more than one pure culture, so that both microorganisms grow simultaneously (Mitchell, 1992). It seems that there are many synergistic combinations of microorganisms that can synthesize substances not produced by pure culture or that can operate faster or more efficiently than do pure cultures (Haas *et al.*, 1980). Some examples of processes employing defined mixed

culture in submerged fermentation are production of ethanol (Abate *et al.*, 1996), Swiss cheese (Reinbold and Takemoto, 1988), lysine (Fields *et al.*, 1988), biosurfactants from molasses (Ghurye *et al.*, 1994) and many others.

There is a great potential for the use of mixed cultures in SSF for enhancing the productivity and the rate of bioreactions (Mitchell and Lonsane, 1992). A mixed culture fermentation using bacterial and fungal cultures or two different species of *Bacillus* may result in improved enzyme production (Lonsane and Ramesh, 1990).

SSF has been used to produce ethanol by using sweet sorghum carbohydrates and a mixed microbial culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae* (Mamma *et al.*, 1996), xylanase by co-culturing *Trichoderma reesei* with either *Aspergillus niger* or *Aspergillus phoenicis* in a SSF on sugar cane bagasse (Gutierrez-Correa and Tengerdy, 1998) and to produce cellulolytic enzyme on sweet sorghum silage inoculated with *Trichoderma reesei* LM-1 (a Peruvian mutant) and *Aspergillus niger* ATCC 10684 (Castillo *et al.*, 1994) and on sugar cane bagasse by a mixed culture of *Trichoderma reesei* LM-UC4 with *Aspergillus phoenicis* QM 329 (Gutierrez-Correa and Tengerdy, 1997).

The literature shows that by using a co-culture of two or more fungal strains or a co-culture of yeast and fungi in SSF, there was an increase in the productivity of one enzyme or a combination of enzymes. But, there was no evidence for the previous use of a co-culture of two bacteria strains or a co-culture of a bacteria and a fungus for enzyme production. Thus, in this work the potential of using a mixed culture in SSF for the production of α-amylase is a novel approach.

2.7 Alpha-amylase

Alpha-amylases (1,4-α-D-glucan-4-glucano-hydrolase, E.C. 3.2.1.1) are endoglucanases widely distributed among animals, plants, fungi and bacteria. Amylases have been well characterized through the study of various microorganisms. They hydrolyze the polysaccharide chains consisting of α-1,4-linked glucose residues, producing initially oligosaccharides of various lengths and, in some cases, maltose and glucose as final products (Virolle *et al.*, 1990; Selvakumar *et al.*, 1996).

The worldwide market for industrial enzymes in 1990 had an estimated value of $625 million, with the U.S. as the largest sector (Guzman-Maldonado and Paredes-Lopez, 1995). About 62% of the enzymes produced are applied in the food industry and amylolytic enzymes are among the industrially most important enzymes, being used in such processes as starch hydrolysis for sugar syrups, baking and brewing and are obtained from *Aspergillus* and *Bacillus* species (Nigam and Singh, 1995). These enzymes have been produced throughout the world by submerged fermentation, however, this process is expensive due to the presence of the product in low concentration and the consequent handling and disposal of a large volume of water during down-stream processing.

Interest in α-amylase has been increasing due to the fact that in the starch processing industry, acid-catalyzed methods are now being replaced by enzymatic processes (Siqueira *et al.*, 1997). Consequently, a search for the most cost-effective fermentation strategy for the production of α-amylase is desirable. SSF could help overcome these problems as the yield

of the product can be many times higher than that in submerged fermentation (Ramesh and Lonsane, 1987a).

2.7.1 Bacterial origin

Bacterial strains, especially those belonging to the genus *Bacillus*, have been used throughout the world for the production of enzymes in submerged fermentation (Stephenson and Harwood, 1998). Bacterial cultures reported for the production of α-amylase by SSF are limited to the genus *Bacillus* (Babu and Satyanarayana, 1995). These enzymes are used in the saccharification of starch. They randomly attack α-1,4-glucosidic linkages in starch thereby leading to the production of limit dextrins. The uses of these amylases extend to a variety of food, textile manufacturing, brewing, distilling, paper, adhesive and sugar industries (Fogarty *et al.*, 1974). It represents about 12% of the total sales of world enzymes.

Bacterial α-amylases produced by *Bacillus* species are thermostable even up to 110°C. The commercially produced enzymes have pH optima for enzyme activity at neutral or slightly acidic pH ranges. These enzymes are widely accepted by the starch processing industry for the hydrolysis of starch, however, this is one of the most expensive unit operations in the overall saccharification process due to the cost of the enzyme (Ramesh, 1988). The potential for the production of bacterial α-amylase by SSF has been demonstrated by many reports. Ramesh and Lonsane (1989,1990, 1991) and Padmanabhan *et al.* (1992) have reported the characteristics and novel features of thermostable α-amylase produced by *Bacillus licheniformis* in SSF. The substrate utilized for α-amylase production by *B. licheniformis* was wheat bran due to the high availability of nutrients present in this substrate.

Also reported was the critical moisture content of the medium and its control during fermentation, and the pH optima for the enzyme production at 6.0-7.0 with temperature optimum at 30-45°C. Also, α-amylase has been produced by *Bacillus megaterium* (Ramesh and Lonsane, 1987; Ramesh, 1988) using wheat bran as the substrate. The levels of amylase production varied between species of *Bacillus* over the range of 9.2 to 33,000 units/g dry substrate (Lonsane and Ramesh, 1990). The use of different substrates also resulted in variations in the enzyme yields.

2.7.2 Fungal origin

Filamentous fungi have a number of properties which make them important both scientifically and industrially. Industrially they are important in a range of applications, such as for the production of organic acids, antibiotics and different types of enzymes (Carlsen *et al.*, 1996; Eriksen *et al.*, 1998). Amylolytic enzymes derived from filamentous fungi have been extensively investigated because of their importance during the starch saccharifying process at lower temperatures. These enzymes have been found in several genera of fungi, but *Aspergillus* species have been recognized as an important source for industrial production (Alazard and Baldensperger, 1982).

The action of fungal α-amylases is to break down α-1,4 bonds of starch molecules into water soluble chains of maltose, oligomers such as dextrins and a small quantity of glucose (Meyrath, 1966 and Lonsane and Ghildyal, 1992). They have lower thermostability, usually up to 50-60°C, as compared to 90-105°C for bacterial α-amylases. Among the amylases, α-amylase has reached particular importance since it is now extensively used in baking,

brewing, the distillery and pharmaceutical industries. The production of this enzyme through SSF has been reported to present enormous potential and economic viability (Ahmed *et al.*, 1987).

Aspergillus oryzae is the species most frequently used for α-amylase production in submerged fermentation (Lachmund *et al.*, 1993; Spohr *et al.*, 1998) and in SSF. Chou and Rwan (1995) have studied the production of α-amylase on various rice extrudades and steamed rice. Maximum α-amylase activity was 69 units/g dry matter after 72-84 hours of cultivation. Other reports include the production of α-amylase by *A. oryzae* grown on polyurethane foams (Torrado *et al.*, 1998; Murado *et al.*, 1997), rice (Silva and Yang, 1998; Narahara *et al.*, 1982) and wheat bran (Nakadai and Nasuno, 1988).

There are also some other *Aspergillus* species that produce α-amylase. Sudo *et al.* (1994), studied the production of α-amylase by *A. kawachii* on rice and compared with submerged fermentation. Michelena and Castillo (1984) have reported on the production of amylase by *A. foetidus* using rice flour as a substrate.

2.8 Surfactants

Surfactants or surface active agents are substances which alter the conditions prevailing at interfaces. In other words, surfactants are amphiphilic molecules that tend to partition preferentially at the interfaces between fluid phases of different degrees of polarity and hydrogen bonding, such as oil/water or air/water interfaces (Lin, 1996). The hydrophobic group, usually a hydrocarbon chain, tends to be repelled by the water, while the hydrophilic

group tends to remain in water (Rosenberg, 1986 and Parkinson, 1985). Because of these properties, surfactants find applications in an extremely wide variety of industrial processes including emulsification for emulsion polymerization, foaming for food processing, detergency, wetting and phase dispersion for cosmetics and textiles, dispersing and solubilization of agrochemicals (Lin, 1996 and Desai and Banat, 1997). Surfactants are one of the most versatile process chemicals.

The surfactant market is around 10 billion dollars per year and their demand is expected to increase at a rate of 35% per year toward the end of the century. The majority of synthetic surfactants are chemically derived from petroleum feedstocks. However, interest in microbial produced surfactants has increased due to their potential as lytic agents in biological systems, diversity, environmentally friendly nature, and their potential applications in the environmental protection, crude oil recovery, health care and food processing industries.

Biosurfactants are produced as metabolic by-products by bacteria, yeasts and fungi. They are not only potentially as effective but offer some distinct advantages over the more common synthetic surfactants. Microbial surfactants present remarkable specificity and are consequently suited to new applications, they have effective physicochemical properties (low interfacial tensions and critical micelle concentrations), can be less sensitive to extremes of temperature, pH, or salinity, generally have lower toxicity, higher biodegradability and the ability to be synthesized from renewable feedstocks (Cooper, 1986, Parkinson, 1985, Mulligan and Gibbs, 1993, Desai and Banat, 1997).

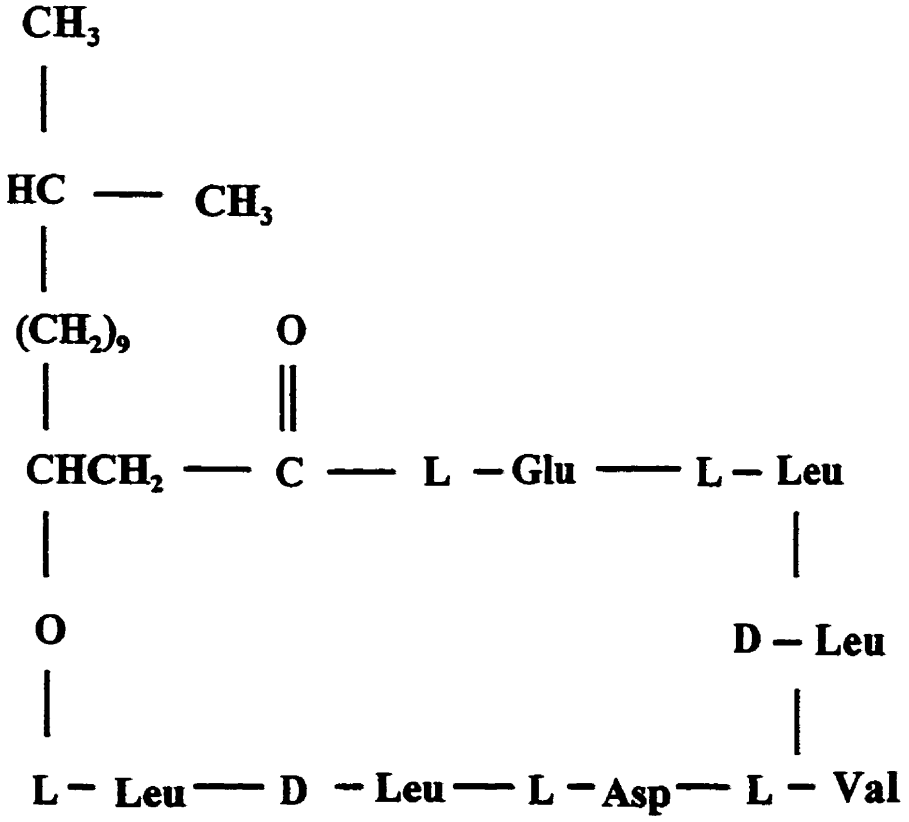
In this research the biosurfactant of interest is surfactin, a lipopeptide produced by

Bacillus subtilis ATCC 21332 by submerged fermentation in a glucose and mineral salts medium. It contains seven amino acids bonded to the carboxyl and hydroxyl groups of the 14 carbon acid (Figure 2.1). It is known to be one of the most powerful biosurfactants, capable of lowering the surface tension of water bellow 30 mN/m (Cooper *et al.*, 1981, Sheppard and Cooper, 1991) and acting as an antibiotic by solubilizing major components of microbial cell membranes. Sheppard *et al.* (1991) have shown how surfactin produces ion-selective channels in black lipid membranes. More recently, surfactin has been also produced by SSF using okara as substrate and by recombinant *Bacillus subtilis* (Ohno *et al.*, 1995a, 1995b).

Synthetic surfactants have been used in submerged fermentation and SSF to increase enzyme activity. The addition of nonionic surfactants resulted in a marked increase in yields of the enzymes cellulase, amylase, sucrase, β-1-3-glucanase, xylanase, β-glucosidase, purine nucleosidase, benzoyl esterase and ligninases (Reese and Maguire, 1969; Sukan *et al.*, 1989; Gashe, 1992; Pushalkar *et al.*, 1995; Pardo, 1996; Jäguer *et al.*, 1985). Other enzymes that have their productivities improved by the addition of surfactants are phytase, which has been produced in canola meal during the SSF process using *Aspergillus ficuum* (Ebune *et al.*, 1995) and, at low concentrations, surfactants enhance lipoxygenase activity (Sunivasulu and Rao, 1993).

Enzymatic hydrolysis of bagasse was accelerated by pretreatment with 3.33% wt nonionic surfactant. The enzymatic hydrolysis rate increased because of an increase in the surface area of cellulose accessible to the enzyme compared to those pretreated with water.

Figure 2.1 Structure of the lipopetide surfactin from *Bacillus subtilis* ATCC 21332.



SURFACTIN

This effect is based on the fact that surfactant makes hydrophobic degradation products extractable by water (Kurakake *et al.*, 1994). Effects of surfactants on enzymatic saccharification of cellulose have also been studied by Ooshima *et al.*, 1986. From this work it was concluded that nonionic, amphoteric and cationic surfactants enhanced the saccharification process. Helle *et al.*, 1993, have also reported on the effect of surfactants on cellulose hydrolysis, showing that Tween 80 and the biosurfactant sophorolipid increased the rate of cellulose hydrolysis by as much as seven times.

2.9 Biofilm

According to Costerton *et al.* (1995), biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media. Biofilm consists of living cells and extracellular products, mainly composed of polysaccharides. The structure of the biofilm is held together by biopolymers synthesized by these microorganisms attached to the substratum. This material acts like a glue for the biofilm structure entrapping nutrients and protecting the cells from a hostile environment (Wirtanen and Mattila-Sandholm, 1993 and Zottolo and Sasahara, 1994).

Microbial biofilms, especially bacterial, accumulate as a consequence of the ability of the microbes to adsorb to a surface (termed as substratum), replicate, produce extracellular polymers, and metabolize nutrients and substrates dissolved in the surrounding fluid phase (Bryers, 1994). Biofilm formation is usually considered to be detrimental, but, it can also

serve beneficial purposes in natural and in some modulated or engineered biological systems (Brading *et al.*, 1995, Characklis and Marshall, 1989).

Some examples of microbial processes that have provided economic or process advantage by being carried out in natural biofilm include, biofilm reactor used in fermentation process for acid acetic production; in wastewater treatment for degradation of soluble organic or nitrogenous waste; in the microbial decomposition of cellulose fibers; in ethanol production; and in polysaccharide production, which is critical to the formation of the gel matrix surrounding cells in biofilms.

On the contrary, biofilm formation can also cause problems, such as: formation of undesirable deposits on industrial equipment thereby reducing the rate of heat transfer; increased fluid frictional resistance; plugging; corrosion; and other types of deterioration.

The evaluation of biofilm formation has been performed according to the substratum, liquid medium, carbon source, pH and hydrodynamic parameters, including flow rate (Little *et al.*, 1991). Many of the conclusions about biofilm development, composition, distribution and relationship to substratum have been derived from scanning electron microscopy (SEM). According to Ladd and Costerton (1990) SEM can reveal some details about the mechanism(s) of attachment such as individual cells, micro colonies, fibrillar strands, and condensed polymeric material coating the cells. Lindsay and Holy (1997) have used SEM to evaluate laboratory-grown bacterial biofilms. Another technique used for studying biofilm, is epifluorescence image analysis, described in detail by Wirtanen *et al.*, 1996 and Wirtanen and Mattila-Sandholm, 1993. In this work biofilm formation on the solid substrate was studied by SEM.

CHAPTER 3

MATERIAL AND METHODS

3.1 Quantitative Experimental Methods

3.1.1 SSF on ground potato peel

3.1.1.1 Microorganisms

Aspergillus oyrzae ATCC 1011, *Aspergillus foetidus* ATCC 10254, *Bacillus subtilis* ATCC 21556 and *Bacillus subtilis* ATCC 21332 were obtained from the American Type Culture Collection (Rockville, Maryland), stored on slants of agar in a refrigerator at 4°C and subcultured every 30 days.

3.1.1.2 Inoculum preparation

Aspergillus oryzae ATCC 1011 was grown on slants of malt agar (DIFCO, Detroit). The culture was kept in a refrigerator at 4°C and it was subcultured every 30 days. The liquid medium of *Aspergillus oryzae* for SSF was prepared using 1.5% (w/v) malt extract (DIFCO, Detroit), sterilized and inoculated with the culture. The flasks were incubated in a shaker (G-25 Shaker Incubator, New Brunswick Scientific, N.J., USA) at 30°C for 48 hours and 200 rpm, prior to the use in SSF process. *Aspergillus foetidus* ATCC 10254 was also grown on slants of malt agar. It was then transferred to a sterilized liquid medium with malt

extract, 20 g/l; glucose, 20 g/l and peptone, 1.0 g/l and grown in a shaker at 30°C for 48 hours at 200 rpm.

Bacillus subtilis ATCC 21556 was transferred from a nutrient agar slant and grown for 72 hours at 30°C in a sterile nutrient medium containing: beef extract, 3.0 g/l; peptone, 5.0 g/l and 1.0% potato starch. *Bacillus subtilis* ATCC 21332 was grown for 72 hours at 30°C in a glucose mineral salts medium (Cooper *et al.* 1981). The glucose was sterilized separately and mixed with the mineral salts just before inoculation with cells grown on agar plates. For both *Bacillus* strains the 500 mL flasks containing 250 mL of media were placed in a shaker incubator at 200 rpm (G-25 Shaker Incubator, New Brunswick Scientific, N.J., USA).

3.1.1.3 Substrate preparation

Potato peel was used as source of starch. Potatoes from a variety called *Superior* were bought from a local market. They were washed, peeled by hand and the peels ground into small particles with a blender (Proctor-Silex 10 Speed Blend Master) for 20 seconds, to about 3 mm diameter. The moisture content of this substrate was in the range of 78-80% on a wet basis.

Barley was obtained from a local market. As barley is a substrate with low moisture content (11%), it was necessary to soak this product in distilled water in a proportion of 5 volumes of water (based on the weight of barley). The time of soaking was 3 hours, after which the moisture content of the product was between 40% to 45% on a wet basis. Orange peel was also tested as a solid substrate for the process. The oranges were washed, peeled

by hand and the peels ground into small particles with a blender to about 2 mm diameter. The moisture content of this substrate was in the range of 75-80% on a wet basis.

3.1.1.4 Fermentation process

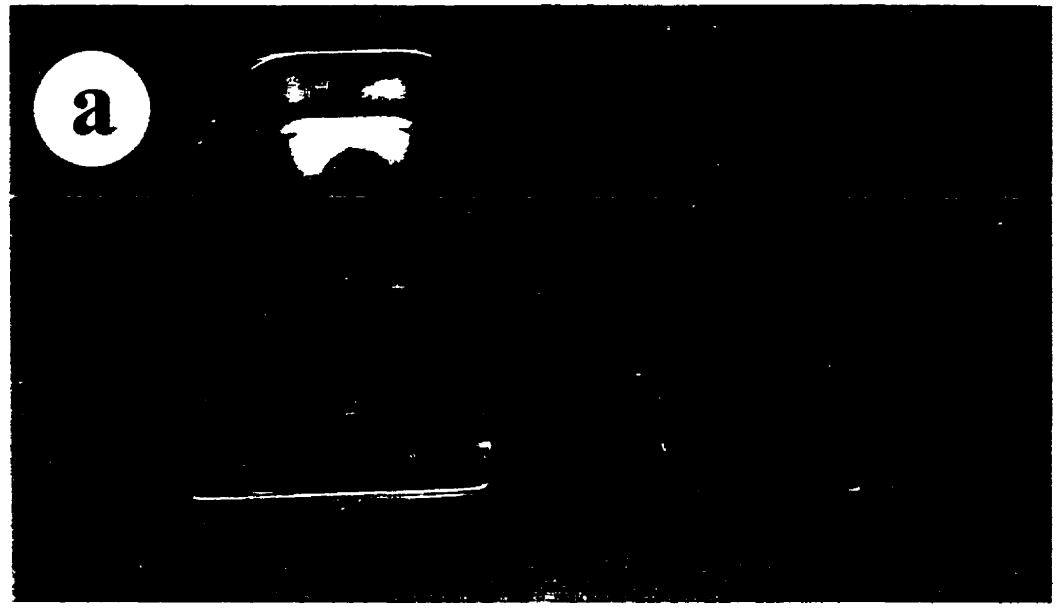
The fermentation process was carried out in perforated stainless steel trays 32.2 cm in length, 17.8 cm in width and 6.4 cm in depth. In the bottom of these trays there were three holes, 4.1 cm in diameter, spaced 5 cm apart to allow for air circulation. Placed on the bottom of each tray was a perforated stainless steel mesh, with 1.6 mm hole size. A 250 g quantity of the substrate was spread on this mesh and the tray covered with aluminum foil. Refer to Figure 3.1.

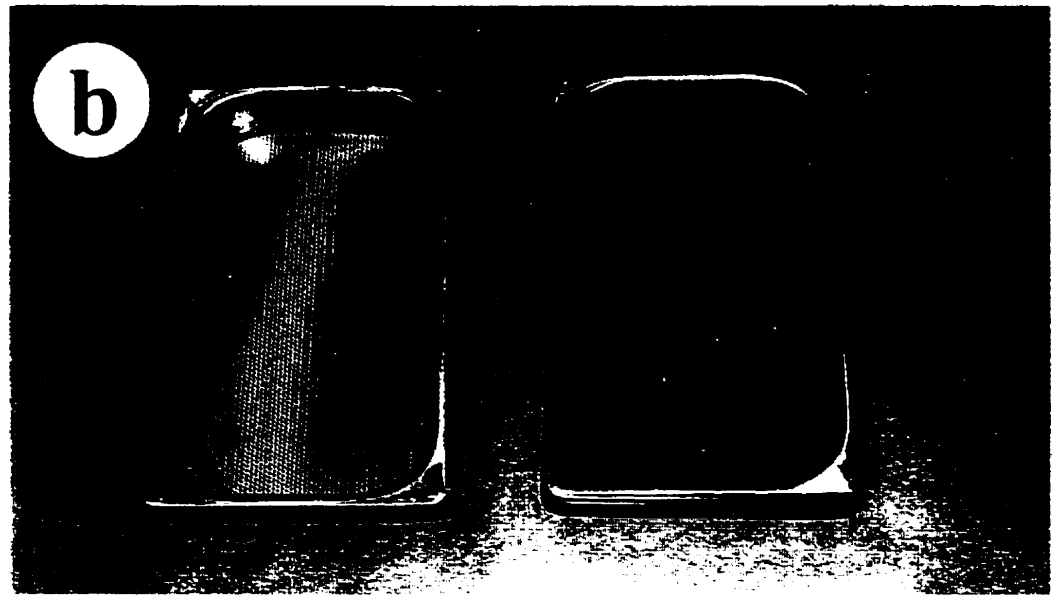
The trays were sterilized in an American Sterilizer Company (U.S.A) autoclave at 121°C and 15 psi for 60 min, cooled, inoculated with a 10% (v/w) preparation of a pure culture or, when a mixed culture was used, 10% (v/w) of each culture. Also added was the synthetic surfactants, Tween 80, Tween 20 and SDS, or surfactin, the biosurfactant produced by *Bacillus subtilis* ATCC 21332.

Tween 80 and Tween 20 are two nonionic surfactants also known as polyoxyethylenesorbitan monooleate and polyoxyethylenesorbitan monolaurate sorbitan respectively. Lauryl sulfate sodium salt (SDS) is an anionic surfactant.

The trays were incubated in closed chambers (Conviron CMP 3244, Winnipeg) with automatic control of temperature (30°C), relative humidity (90%) and with uniform upward air flow. Samples were taken every 12 hours (about 5 g).

Figure 3.1 Tray fermentor showing (a) the tray with three holes and the perforated mesh; (b) mesh placed on the tray with and without the ground potato peels.


a


b

3.1.2 Growth on agar plates

The growth on agar plate was used to estimate the rate of growth of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 by themselves, with or without the addition of Tween 80, and of a mixed culture of these two *B. subtilis* strains in the presence or absence of Tween 80.

Plates containing either potato dextrose agar (PDA) or plate count agar (PCA) (DIFCO, Detroit) were inoculated with 0.2 mL of each culture (pure or mixed), spread with a glass rod over the PDA and PCA plates. The plates were incubated at 30°C for 72 hours. Samples were taken every 12 hours and the biomass was washed from the agar plates with distilled water and poured into pre-weighed aluminum dishes. The dishes were dried in an oven at 104°C for 24 hours and the biomass dry weight calculated in g.

3.2 Analytical Methods

3.2.1 pH

The pH was measured after suspending 1 g of wet fermented solid in 9 mL of distilled water. This dilution was then mixed and the pH measurement obtained with a combination pH electrode and a pH/ion meter Accumet Model 25 (Fisher Scientific, U.S.A.).

3.2.2 Moisture Content

Every 12 hours, 1 g of fermented solid was sampled and dried in an oven (Isotemp Oven, Fisher Scientific Co.) at 104°C for 24 hours, and its weight was measured for the calculation of moisture content. Moisture content was calculated based on weight loss using the following formula:

$$MC(\%\ \text{wet basis}) = ((W_1-W_2)/W_1) \times 100$$

where,

W_1: is the weight of the substrate before drying (g);

W_2: is the weight of the substrate after drying (g).

3.2.3 Enzyme extraction

Alpha-amylase was extracted by using a leaching process. The leaching process was established after studying the effect of three different solvents on enzyme recovery. The solvents included two concentrations of NaCl (0.5% (w/v) and 1.0% (w/v)) and a phosphate buffer (10 mM). One gram of fermented substrate was washed with 9 mL of each solvent for three hours at room temperature. The mixture was filtered in a wet cheese cloth and the liquid centrifuged in a table top centrifuge (International Equipment Company, model HN-S, U.S.A.) at 3000xg for 20 minutes. The clear supernatant contained the enzyme extract which was then analyzed for enzyme activity.

3.2.4 Enzyme activity

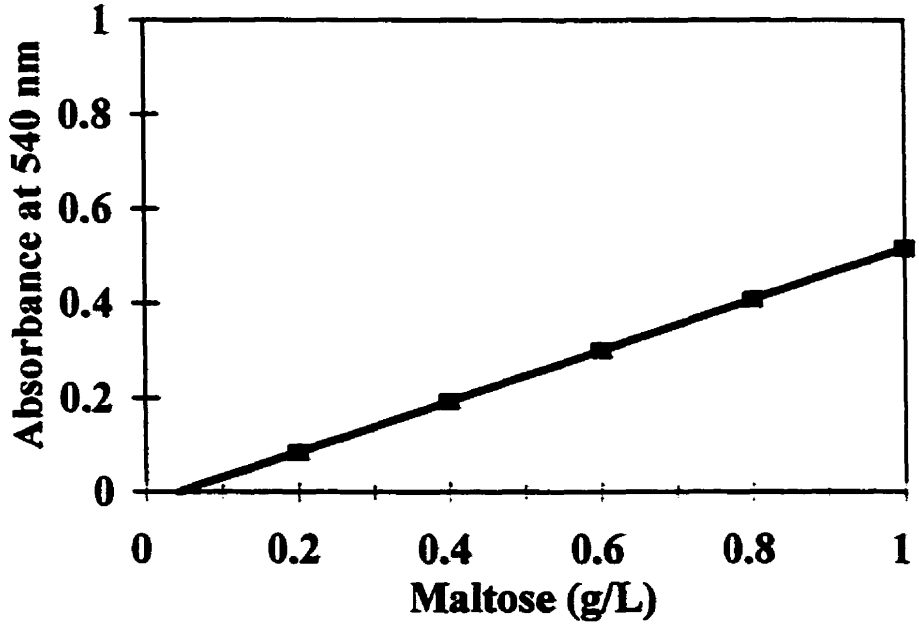
Alpha-amylase is typically quantified by measuring the degradation of starch either via the amount of reducing sugars produced or from the amount of starch which is hydrolyzed. Different methods to determine α-amylase activity have been reported. Carlsen *et al.*, 1994 described a flow-injection analysis (FIA) system for measuring α-amylase. Virolle *et al.*, 1990 reported a method for α-amylase assay which relies on the reduction of turbidity that occurs upon digestion of a starch suspension.

The methodology used in this work was that of Bernfeld (1951), a colorimetric method based on saccharifying activity. In this method starch was hydrolyzed to maltose and the reaction was stopped by adding 3,5-dinitrosalicylic acid. The complete methodology including the list of reagents is described in the enzymatic assay of α-amylase (EC 3.2.1.1) from Sigma Chemical Co. (St. Louis). The concentration of maltose was determined from a standard curve based on absorbance at 540 nm obtained using a spectrophotometer (NOVASPEC II, Cambridge, England) (Figure 3.2). One unit of enzyme is defined as that amount required to liberate 1.0 mg of maltose from starch in three minutes at pH 6.9 and 20°C. Enzyme activity was obtained and expressed in units/g solid.

3.2.5 Biomass estimation

Biomass is a fundamental parameter in the characterization of microbial growth. However, direct determination of biomass in SSF is very difficult due to problems with the separation of the microorganism from the substrate (Mitchell *et al.*, 1992).

Figure 3.2 Standard curve of maltose based on absorbance at 540 nm used for determination of α-amylase activity.


Absorbance at 540 nm
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
Maltose (g/L)

3.2.5.1 Fungal Biomass Estimation

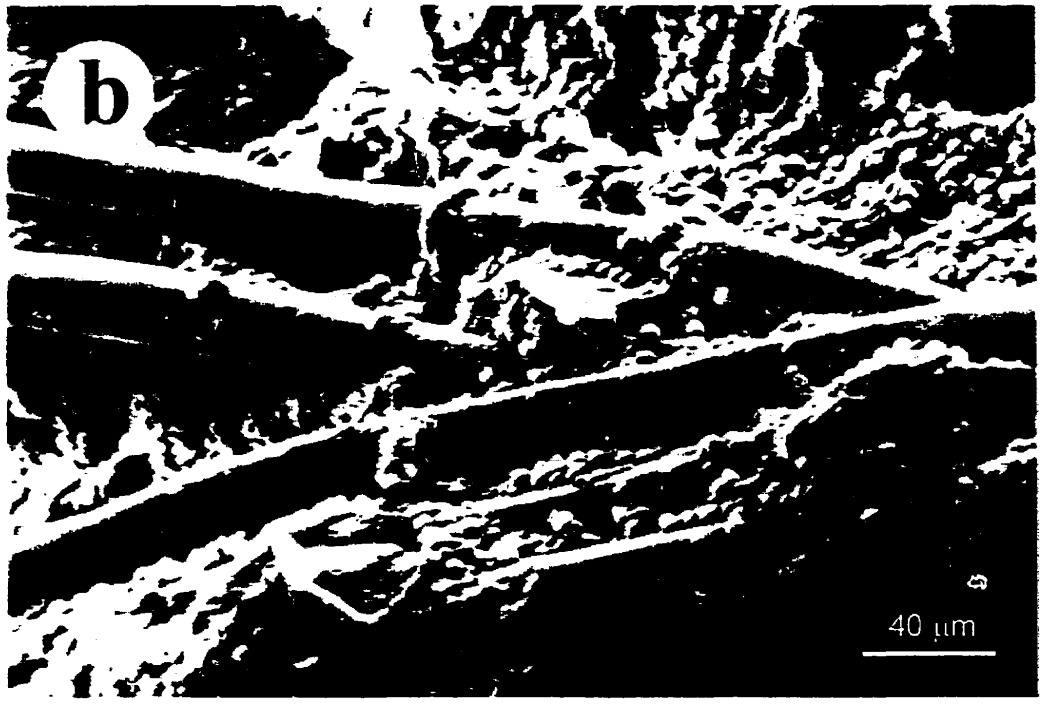
Fungi penetrate deep into the intra and intercellular spaces in the substrate by mechanical and enzymatic means and firmly anchor themselves to the substrate for optimum attack (Figure 3.3). This makes the separation of the fungal mycelia from the residual solid substrate difficult, and consequently direct estimation of biomass is not feasible (Ramana Murthy *et al.*, 1993b). Because of the difficulties associated with direct measurement of biomass in SSF systems, most of the methods reported in the literature are indirect ones. Thus can be based on glucosamine content (Ride and Drysdale, 1972, Aido *et al.*, 1981, Matcham *et al.*, 1985), ergosterol content (Desgranges *et al.*, 1991a) or protein content (Raimbault and Alazard, 1980). On-line monitoring methods have also been reported, based on carbon dioxide evolution rate and infrared, (Desgranges *et al.*, 1991b) or visible light reflectance (Ramana Murthy *et al.*, 1993b).

In this work, the methodology chosen for determination of fungal growth was the glucosamine (chitin) content. Chitin is a poly-N-acetylglucosamine with the monomers connected by α,1-4 links in a straight chain and is one of the most frequently occurring polymers in fungal walls.

The accuracy of chitin assay techniques for determination of fungal biomass in any tissue depends upon having a reliable conversion factor for relating glucosamine content to unit dry weight of mycelium. The quantitative liberation of glucosamine by hydrolysis from the mycelium is necessary prior to the estimation. There are different methods for conversion

Figure 3.3 Scanning electron micrograph of *Aspergillus* grown on ground potato peel: (a) x500 magnification, (b) x1000 magnification.


a
100 µm


b
40 µm

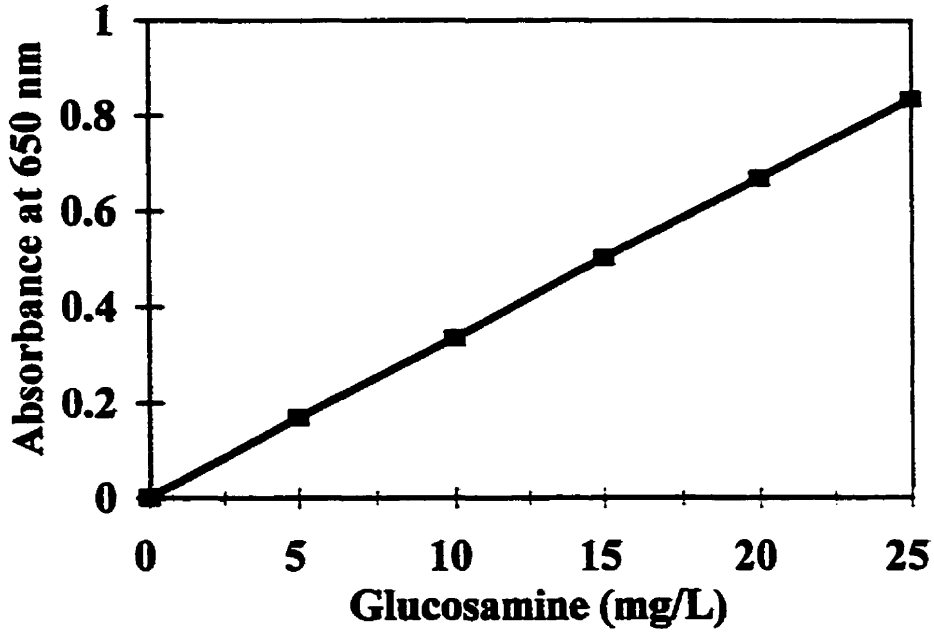
of chitin to glucosamine, each involving either an acid or alkaline hydrolysis. In this experiment, the Ride and Drysdale's method (1972) was used. This method is based on alkaline depolymerization and deacetylation of the chitin. The glucosamine is then estimated by an assay with 3 methyl-2-benzothiazole hydrazone. By using a spectrophotometer (NOVASPEC II) at 650 nm the optical density was determined and the glucosamine content converted to mg/g dry substrate with a standard curve constructed from a stock solution of glucosamine (Figure 3.4). This was then converted to biomass based on a standard curve obtained from a pure culture of *A. oryzae* ATCC 1011 or *A. foetidus* ATCC 10254 grown in a submerged fermentation.

3.2.5.2 Bacterial biomass

Measurement of bacterial biomass in SSF is important to be able to monitor the bacterial growth as well as to establish a correlation with α-amylase production.

The technique evaluated was the plate count method. A series of dilutions (up to 10^{-10}) were prepared from 1 g of fermented substrate in 9 mL of 0.1% (w/v) peptone solution. The tubes were vortexed for 5 seconds. Then, 0.1 mL were obtained with an eppendorf pipette (10μL-100μL) and spread with a glass rod over a 9 cm plate count agar petri dish. The plates were incubated at 30°C for 72 hours and the individual colonies were counted.

Figure 3.4 Standard curve of glucosamine based on absorbance at 650 nm used for estimation of fungal biomass.


Absorbance at 650 nm
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
Glucosamine (mg/L)

3.2.6 Recovery of surfactin

3.2.6.1 From submerged fermentation

Surfactin was obtained for addition to the various experiments as part of cell-free liquid of *B. subtilis* ATCC 21332 grown for 72 hours in a glucose and mineral salts medium.

3.2.6.2 From SSF

A quantity of 0.5, 1, 2, 5 and 10 g of solid fermented substrate were placed in tubes with 10 mL of distilled water and mixed in a vortex (Fisher Scientific Model K-550-G), and centrifuged (International Equipment Company, model HN-S, U.S.A.) at room temperature for 20 minutes at 3000xg. The supernatant was then filtered and the surface tension measured using a Fisher Tensiometer (Model 20).

3.2.6.3 Analysis of surfactin

According to Sheppard (1989) surfactin can be assayed indirectly by measuring surface tension of the samples. The theoretical critical micelle concentration (CMC) can be determined by plotting surface tension as a function of different proportions of the cell free broth obtained in submerged fermentation. Surface tension measurements were done using a surface tensiometer in dynes/cm (mN/m) with a 6 cm diameter platinum ring (Fisher Scientific, U.S.A., Model 20) employing the principle of Du Nuoy's ring method. The broth proportion at which the surface tension started rising abruptly was denoted CMC and corresponds to approximately 10 mg/L of surfactin (Sheppard, 1989). The dilution factor required to reach the CMC represents the quantity of surfactin present in the cell-free broth

and needs to be determined (CMC^{-1}) to convert the broth proportion into actual quantities of surfactin (w/w).

3.3 Qualitative Experimental Methods

These analyses were done in order to investigate the mechanism of action of surfactants in the SSF process and to help in understanding the interrelationship between surfactants, solid media and microbial cells.

3.3.1 Scanning electron microscopy (SEM)

The SEM analyses were done in an attempt to observe how the microorganism grew and its physical relationship with the substrate. The samples were from SSF of both ground and sliced potatoes. A SSF process on sliced potatoes was conducted just for these analyses.

Samples taken for SEM analysis were preserved overnight in a fixative solution of 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer. The fixative was removed and the samples were washed with 0.1 M sodium cacodylate buffer once and then preserved in this same buffer until the samples were prepared for SEM analyses as described below.

The preparation of the samples for SEM was done in four steps: washing, freezing in liquid nitrogen, freeze-drying and coating. The samples were first washed with 30% ethanol three times for 5 minutes each, followed by 2 washes with 50% ethanol and finally one washing with 70% ethanol. This was done in order to wash out the fixative from the samples. These samples were then placed in a thermanox slide with double sided tape. Using

tweezers, the samples were immersed on liquid nitrogen at -162°C for about 10 seconds, placed on disposable sterile cryogenic polypropylene vials and then immersed in liquid nitrogen at -180°C until the freeze drying step. Twenty four hours before the SEM analysis, the samples were freeze-dried in a floor model freeze-drier (Labcanco, Fisher Scientific).

The freeze-dried samples were mounted on stubs and placed in the Hummer VI Sputter Coater (ANATECH LTD) for Au/Pd coating. The coating of 6 samples took about 15 minutes. Once the samples were coated, they could then be examined in a JSM-840A Scanning Microscope (JEOL, USA) at 10 kV accelerating voltage. Pictures were taken using 500x, 1000x and 3500x magnification with a Polaroid camera.

3.3.2 Diffusivity tests

The following diffusivity tests were done to verify if the presence of surfactant was affecting the mobility of the microorganisms, nutrients or products in the SSF process.

3.3.2.1 Diffusivity in solution

This test was done in order to evaluate the effect of diffusivity of two different dyes in an aqueous solution in comparison to a surfactin solution with a surface tension of 30 mN/m. The dyes used were safranin and crystal violet.

The experiment was conducted in triplicate by adding either distilled water or surfactin solution to petri dishes, then adding a drop of one of the dyes to the center of the petri dishes and timing how long it took for the dye to diffuse throughout the different solutions.

3.3.2.2 Diffusivity in plate count agar (PCA)

Plates containing plate count agar were prepared with 0.3%, 0.5% and 1.0% (v/v) basis of Tween 80 and 0.5%, 1.0%, 2.0% and 5.0% (v/v) basis of surfactin solution. Two mL of crystal violet were added to the center of each plate and the diffusivity of the dye was observed for 24 hours and compared with plates without surfactant.

3.3.2.3 Diffusivity in potato slice

Slices of potatoes were prepared by adding surfactant on the surface and then by adding two mL crystal violet for evaluation of the diffusivity of this dye on a starch material in the presence and absence of surfactant. The amount of surfactant added was based on the weight of the potato slices.

3.3.3 Colony formation of *B. subtilis* ATCC 21556

Plate count agar (PCA) plates were prepared with different concentrations of surfactin solution and Tween 80 to evaluate the growth of *B. subtilis* ATCC 21556 when this strain was inoculated in the center of each PCA plate. The control plates did not contain any surfactant.

Tween 80 was added during the PCA preparation at 0.1%, 0.2%, 0.3% (v/v) basis. Surfactin was added at 0.5%, 1.0%, 2.0.% and 5.0% (v/v) basis. The broth was sterilized and the plates were prepared in triplicate.

Bacillus subtilis ATCC 21556 previously grown in a liquid nutrient broth medium for 72 hours at 30°C was used to inoculate the plates. The plates were kept at 30°C for 96

hours and the growth shape was evaluated every 24 hours. The same experiment was conducted with the plates placed in a shaker at 200 rpm.

3.3.4 Starch film formation

The study of starch film formation was done to evaluate the different interactions that could be occurring in the SSF between starch/enzyme, starch/surfactant, enzyme/surfactant, enzyme/water, surfactant/water or starch/water.

Starch film was formed by using two different types of starch and at two temperatures (30°C and 100°C). In the studies of starch film formation at 30°C, 1.0% and 10% soluble potato starch (Sigma Chemical Co., St. Louis) and purified potato starch powder were stirred for 60 minutes and allowed to settle for 30 minutes before film preparation. The solutions were also heated up to 100°C and then cooled to 30°C with stirring and settled for 30 minutes.

Different combinations of surfactants and amylase enzyme were used for this study. Tween 80 was added on a vol/vol basis at 0.1%, 0.5%, 1.0% and 2.0%. Surfactin solution was added on a broth vol/vol basis at 1.0%, 2.0%, 5.0%, 10% and 20%. Amylase (Sigma Chemical Co., St. Louis) was added at either 10 μL or 20 μL, corresponding to 178 to 357 units. One unit of enzyme is defined as that amount required to liberate 1.0 mg of maltose from starch in three minutes at pH 6.9 and 20°C.

The starch films were prepared by adding 10 μL of each solution to the top of the slide. The slides were air dried for 24 hours and then observed under a light microscope using phase contrast objectives at a total magnification of x300 Diastar (Reichert-Jung).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preliminary studies

The primary objective of the preliminary studies was to evaluate the potential of three different substrates, potato peel, barley and orange peel to be used in a SSF process for the production of the enzyme α-amylase. Secondly, a screening of five different microorganism was done in order to select two of them for further studies. Among these microorganisms there were three bacteria and two fungi. The strains of bacteria were *Bacillus licheniformis* ATCC 39326, *Bacillus subtilis* ATCC 21556 and *Pseudomonas saccharophila* ATCC 15946. The fungal strains were *Aspergillus foetidus* ATCC 10254 and *Aspergillus oryzae* ATCC 1011.

The choice of these microorganisms was based on the fact that they are all known to be α-amylase producers. Also, some of them had already been used in SSF for α-amylase production but in different substrates from the three mentioned above.

Potato peel and barley are substrates rich in starch and consequently have potential for α-amylase production. In addition, potato peel is one of the largest wastes from food processing being generated in Canada. According to Agriculture Canada, about 50% of all potatoes grown in Canada are processed. In 1997/98 the amount to be processed was 2.1 million tonnes and the wastes generated varied between 10-15% of this amount. Due to environmental concerns, the reuse of this waste for the production of a value-added product

for the food industry would be of great interest. Orange peels were tested due to the fact that there is a large amount of this waste in tropical countries. Only in the southeast of Brazil, 75 million tonnes of oranges are processed annually (Barreto de Menezes *et al.*, 1989). Orange peels are not a starchy substrate, they present low protein and high cellulose contents but they have been used in SSF for the production of other products such as biogas, methane and pectinase (Srilatha *et al.*, 1995, Garzón and Hours, 1992).

The preliminary studies conducted with orange peels were not successful. There was little or no α-amylase production. The trials with *Bacillus subtilis* ATCC 21556 resulted in the production of 6.0 units of α-amylase per g of solid after 72 hours of fermentation. When using *Aspergillus oryzae* ATCC 1011, the highest yield found was 16.2 units/ g of solid. *Aspergillus foetidus* and *Bacillus licheniformis* were not tested. With barley, even though some amylase was produced, the sample handling of this substrate became difficult and the drop in moisture content during the SSF process also became a drawback. Consequently, potato peel was chosen as the substrate for this research. The preliminary results with ground potato peel were on average 30 to 40 units of enzyme per g of solid when inoculated with *B. subtilis* ATCC 21556 or *A. oryzae* ATCC 1011.

The five microorganisms were bought from the American Type Culture Collection (ATCC). They were first grown in their respective agar medium which was specified by ATCC, then from the agar slants they were grown in the liquid medium in shake flasks.

Among the bacteria, *Pseudomonas saccharophila* was discarded in the beginning of the screening due to its slow growth, taking 5 days to produce 0.40 g/L of biomass in liquid medium. *B. licheniformis* produced 0.70 g/L of biomass in 72 hours. Whereas, *B. subtilis*

also reached its maximum biomass concentration in 72 hours, but was able to produce around 1.0 g/L. It was decided to pursue this study with *B. subtilis*, due to the large amount of work already done with *B. licheniformis* in SSF and to the challenges of using *B. subtilis* in SSF, a microorganism that has been extensively studied in submerged fermentation for the production of important enzymes (Fogarty *et al.*, 1974). Also, there was a better chance of success for co-culturing two *Bacillus* species.

Aspergillus oryzae and *A. foetidus* grew very well on the agar slants as well as in the liquid media. When in the liquid media these two *Aspergillus* species grow in a pellet format. Both fungi also grew very well when used for SSF and the choice of the species of fungi was done after some preliminary studies with both, as shown in the following section.

4.2 Pure cultures

4.2.1 *Aspergillus*

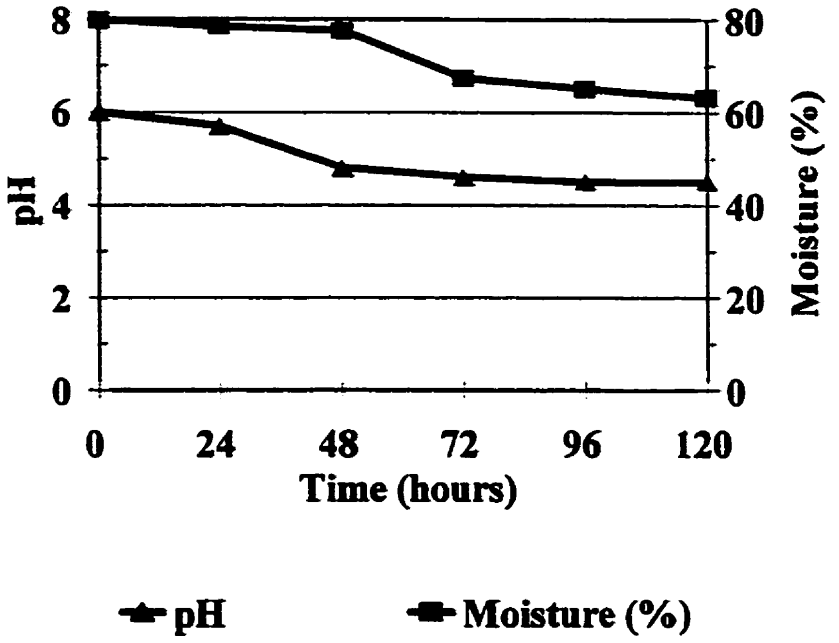
Aspergillus oryzae and *A. foetidus* have the potential of producing α-amylase in SSF using rice as a substrate, as already described in Section 2.7.2. SSF of the two *Aspergillus* strains was carried out in the tray fermentor with 250 g of ground potato peel, inoculated with 10% v/w medium previously grown for 48 hours in a liquid medium to a biomass content of 4.0 g/L for *A. foetidus* and 0.4 g/L for *A. oryzae*. The trays were placed in the incubator at 30°C and 90% RH. Samples of approximately 5 g were taken every 12-24 hours and then analyzed.

For pH and moisture content, the analyses were performed right after sampling. All the pH and moisture content analyses for both strains were done in duplicate and the results shown in Figure 4.1 are the mean values. The pH and moisture content analyses were done in order to monitor changes in the substrate due to metabolic activity. The initial pH of the potato peel after inoculation with *A. foetidus* as well as for *A. oryzae*, was 6.0. Over the course of the SSF the pH decreased to, reaching a final value of between 4.5 and 4.8. This change may have been caused by the secretion of metabolic by-products such as acetic or lactic acid.

The initial moisture content for the peel inoculated with *A. foetidus* was 79.9%, remaining relatively constant until 48 hours and then dropping to 67.4% after 72 hours and ending at 63.2% after 120 hours of fermentation. For the peel inoculated with *A. oryzae* the initial moisture content was 67.3%, and with only slight variations in this parameter during the 84 hours of fermentation.

Other analyses, such as biomass and α-amylase activity were done at the end of the fermentation after all the samples had been collected. In the first 48 hours of fermentation the colonies of both *Aspergillus* strains spread over the entire surface of the substrate. The colonies of *A. oryzae* were light yellow (Figure 4.2) and the ones from *A. foetidus* were black. Due to the difficulties of separating the biomass from the fermented substrate, a methodology to determine fungal biomass for the two strains was established. An analysis for the glucosamine content was chosen as a correlation for biomass due to the fact that it is a

Figure 4.1 The change in pH and moisture content during the SSF of *Aspergillus foetidus* (top) and *Aspergillus oryzae* (bottom) on potato peel.


8
6
4
2
0
pH
80
60
40
20
0
Moisture (%)
0
24
48
72
96
120
Time (hours)
pH
Moisture (%)

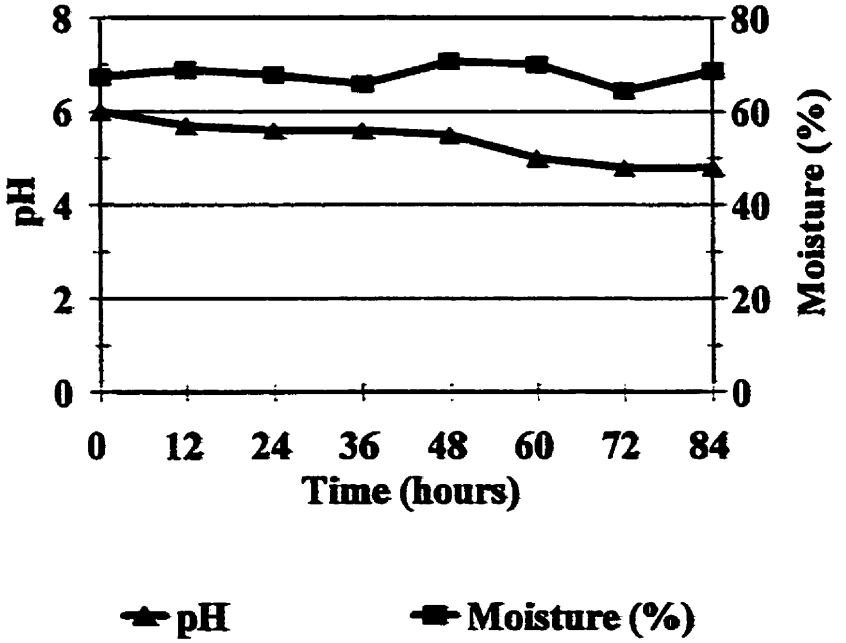
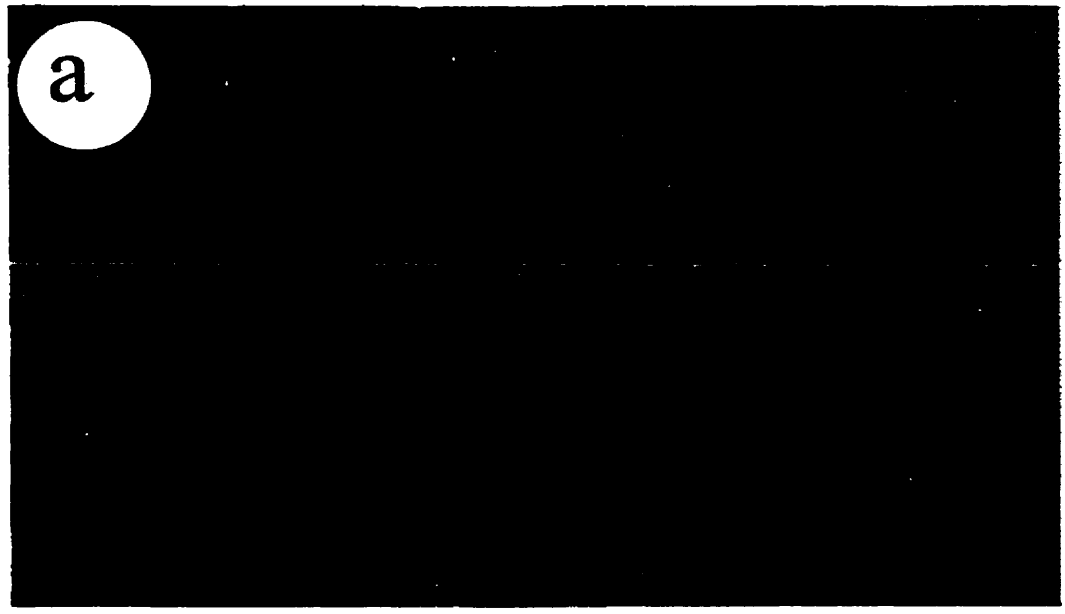
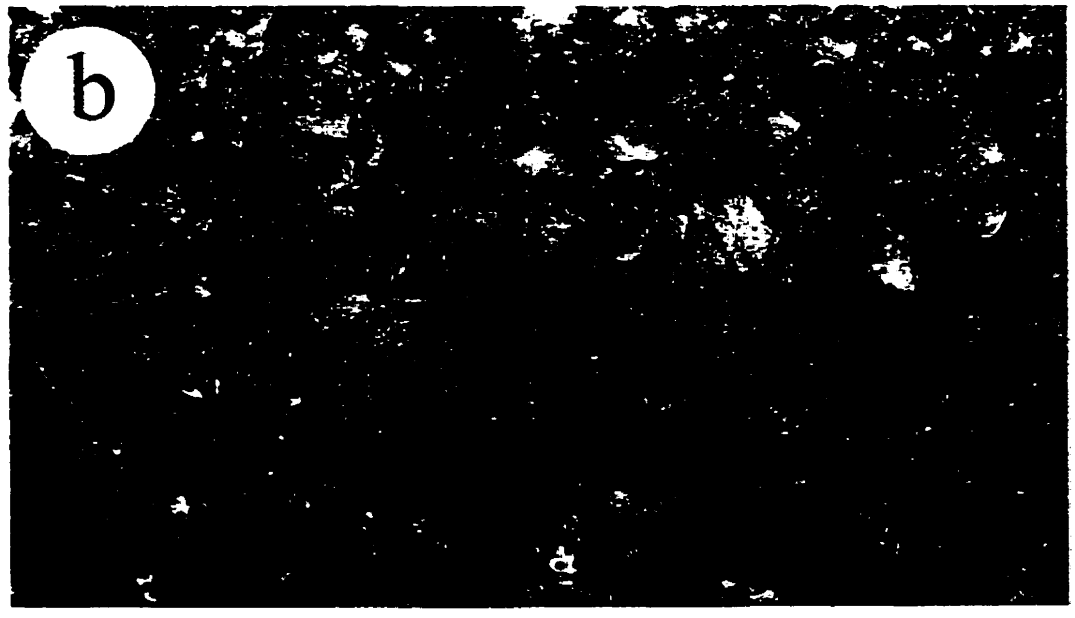

8
6
4
2
0
pH
80
60
40
20
0
Moisture (%)
0
12
24
36
48
60
72
84
Time (hours)
pH
Moisture (%)

Figure 4.2 Growth of *Aspergillus oryzae* on ground potato peel after 48 hours (top) and at zero hours (bottom).


a


b

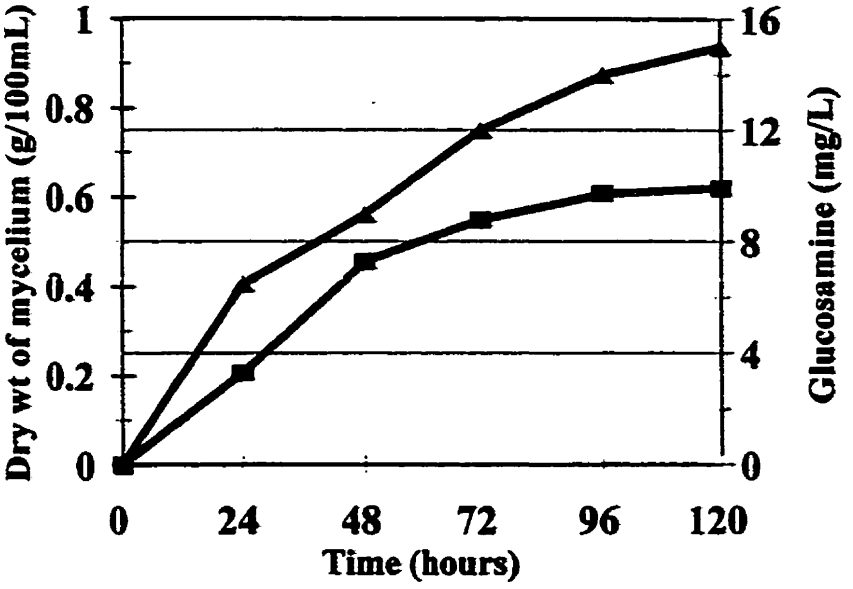
characteristic component of the fungal cell wall. In some cases, glucosamine may comprise up to 60% of the dry weight of the fungal cell wall but it is not found in measurable quantities in potato. To calculate the fungal biomass in an SSF process it was necessary to obtain a conversion factor between glucosamine and biomass dry weight for each one of the species. The conversion factor was calculated as the mean glucosamine content of the liquid cultured mycelium during the period of growth when a linear correlation existed between glucosamine and mycelium dry weight (from four samples obtained between 0 to 120 hours of fermentation). The amount of glucosamine was calculated according to the methodology already described in Section 3.2.5.1 and using the standard curve of glucosamine in Figure 3.4. The values obtained from submerged fermentations of *A. foetidus* and *A. oryzae* are shown in Figure 4.3. Using these values, the conversion factor calculated for *A. foetidus* was 0.0021 mg glucosamine/mg dry weight and for *A. oryzae* the conversion factor was 0.0195 mg glucosamine/mg dry weight. Using these conversion factors, it was possible to calculate the amount of biomass being produced during the SSF of both *Aspergillus* species when grown on ground potato peel. Figure 4.4 shows the increase in the amount of glucosamine content measured during the SSF process.

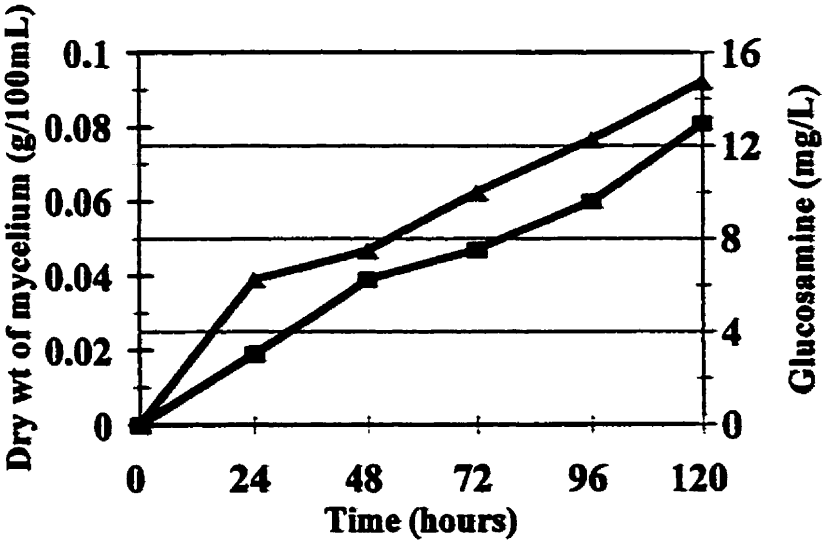
4.2.1.1 Enzyme recovery from an SSF with *Aspergillus oryzae* ATCC 1011

For the determination of α-amylase activity, three different solvents were studied for the extraction of this enzyme. The solvents used were NaCl at two different concentrations (0.5% and 1.0% (w/v)) and 10 mM phosphate buffer. These three solvents were chosen based on previous work where α-amylase was extracted from different substrates than potato

Figure 4.3 Correlation between dry weight of mycelium and glucosamine content in submerged fermentations of *A. foetidus* (top) and *A. oryzae* (bottom).

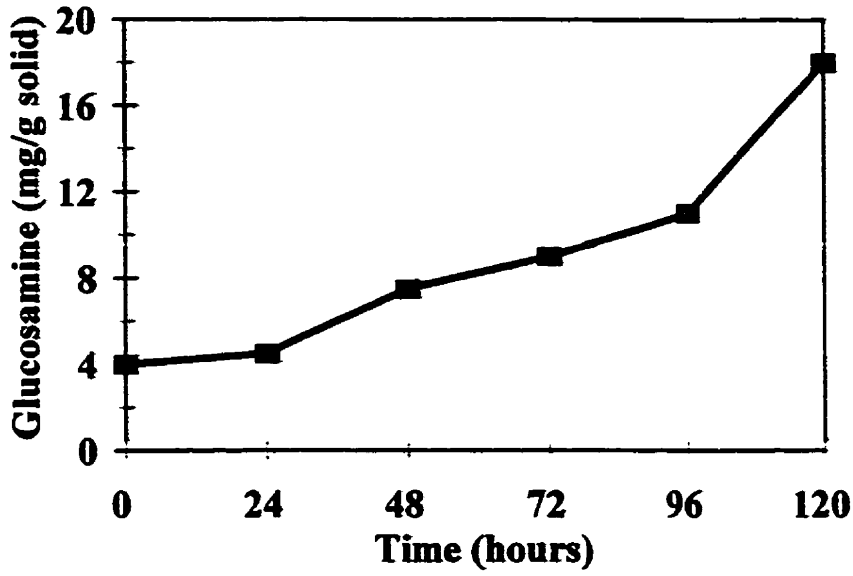
Figure 4.3 Correlation between dry weight of mycelium and glucosamine content in submerged fermentations of *A. foetidus* (top) and *A. oryzae* (bottom).

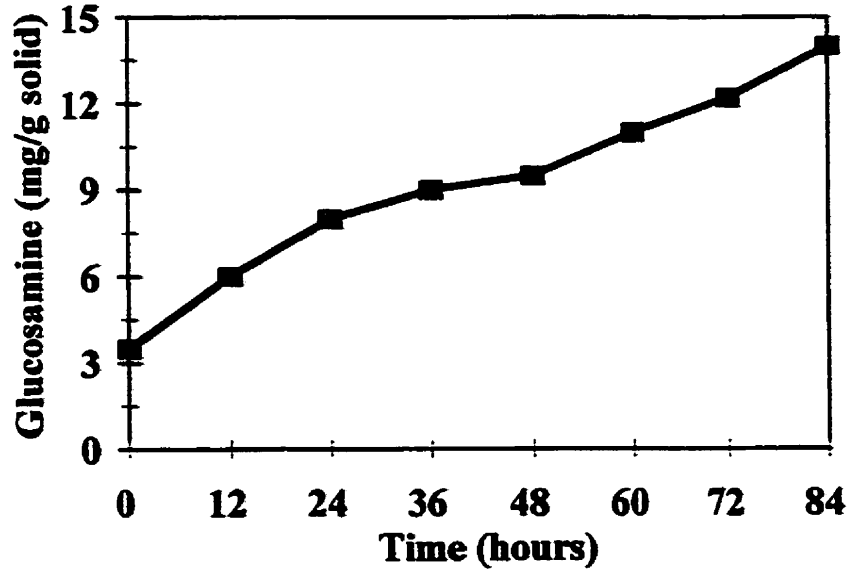




Dry wt glucosamine

Figure 4.4 Changes in glucosamine content during an SSF with *A. foetidus* (top) and *A. oryzae* (bottom).

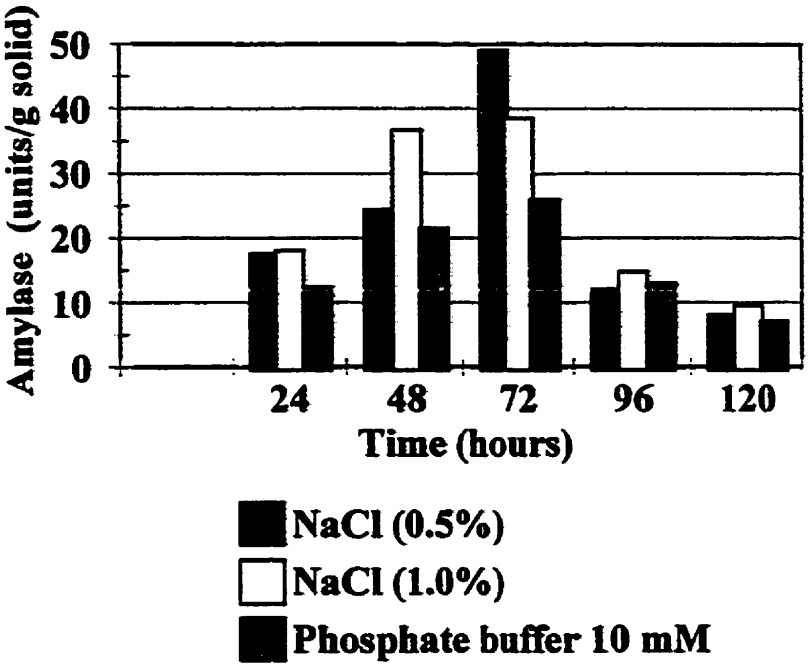

Glucosamine (mg/g solid)
20
16
12
8
4
0
0
24
48
72
96
120
Time (hours)

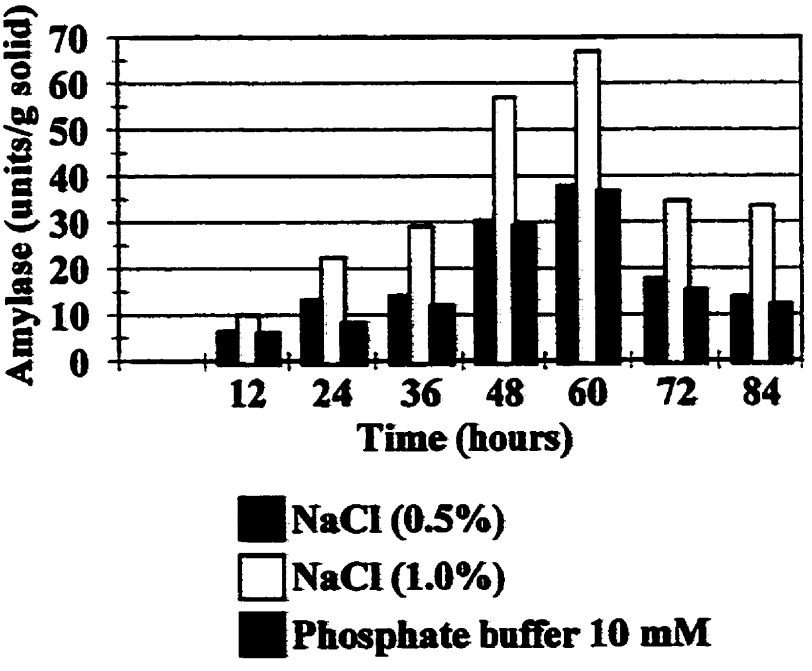

Glucosamine (mg/g solid)
15
12
9
6
3
0
0
12
24
36
48
60
72
84
Time (hours)

peel and from different sources of microorganisms. Sodium chloride had been tested for the extraction of this enzyme in a SSF of rice with *A. oryzae* (Chou and Rwan, 1995). According to Ramesh and Lonsane (1990), 10 mM phosphate buffer is an optimum solvent for α-amylase extraction from a SSF of wheat bran with *Bacillus* species. In Figure 4.5 the mean values are shown for the leaching of potato peel of *A. foetidus* and *A. oryzae* respectively. For both species the highest enzyme activity was obtained when NaCl was used as the solvent. The peak of enzyme activity obtained for *A. foetidus* was 49 units/g solid after 72 hours of fermentation. One unit of enzyme is that amount required to liberate 1.0 mg of maltose from starch in three minutes at pH 6.9 and 20°C. The best solvent was 0.5% NaCl, followed by 1.0% NaCl and phosphate buffer. *A. oryzae* produced maximum enzyme activity, 66.9 units/g solid after 60 hours of fermentation and through all the sampling, 1.0 % NaCl was the best solvent. There was little or no difference in enzyme activity when using either 0.5% NaCl or phosphate buffer.

At this point it was decided to work with only one of the *Aspergillus* strains. *Aspergillus oryzae* was chosen because it provided higher α-amylase activity and is also well known as one of the most important sources of α-amylase in submerged fermentation as well as in an SSF of rice and wheat bran. The work with *A. oryzae* will be described in detail later.

Figure 4.5 Enzyme yields resulting from leaching SSF samples with 0.5% (w/v) and 1.0% (w/v) NaCl and 10 mM phosphate buffer during an SSF with *A. foetidus* (top) and *A. oryzae* (bottom).


Amylase (units/g solid)
50
40
30
20
10
0
24
48
72
96
120
Time (hours)
NaCl (0.5%)
NaCl (1.0%)
Phosphate buffer 10 mM


Amylase (units/g solid)
70
60
50
40
30
20
10
0
12
24
36
48
60
72
84
Time (hours)
NaCl (0.5%)
NaCl (1.0%)
Phosphate buffer 10 mM

4.2.2 *Bacillus subtilis* ATCC 21556

Bacillus subtilis ATCC 21556 was the specific bacterial strain chosen for this work to evaluate the production of α-amylase in an SSF using potato peel as the substrate. This strain was previously grown in a liquid nutrient medium for 72 hours before utilization in the SSF process. The amount of biomass produced after 72 hours was between 0.9 to 1.0 g/L. The volume of inoculum added to the process was again 10% v/w and the SSF incubation was done at 30°C and 90% RH. In the beginning of this work *B. subtilis* and *Aspergillus* strains were incubated in the same chamber room but there was cross contamination between them. For this reason, the experiments were subsequently conducted at different times.

The pH and moisture content of the potato peel inoculated with *B. subtilis* ATCC 21556 was monitored over 84 hours of fermentation. Since bacterial growth was not as visible as the fungal growth, samples were collected every 12 hours so the process could be followed more closely. The pH of the substrate was essentially constant throughout the 84 hours of fermentation process, starting at a pH of 6.3, increasing up to 6.5 and at the end of the process, 6.2. The moisture content varied between 76% and 69%. Figure 4.6 shows the pH and moisture content of the substrate. By monitoring some of the environmental parameters of SSF, pH and moisture content, it was possible to verify the process was well controlled. The optimum pH for bacterial growth is between 6.5-7.5 which was the range of pH measured throughout the process for this pure culture. Moisture content is a key element for regulating and optimizing SSF. The optimum moisture content for the growth and substrate utilization is between 40-70% but depends upon the microorganism and substrate

used for cultivation (Prior *et al.* 1992). In this particular case, the moisture content was kept in this range by control of the relative humidity in the surrounding air.

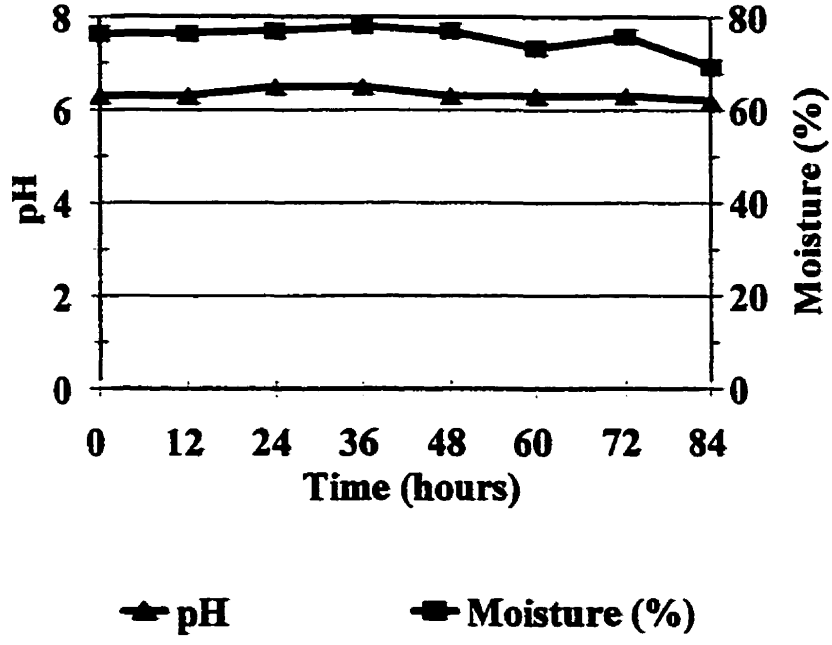
4.2.2.1 Enzyme recovery from an SSF with *Bacillus subtilis* ATCC 21556

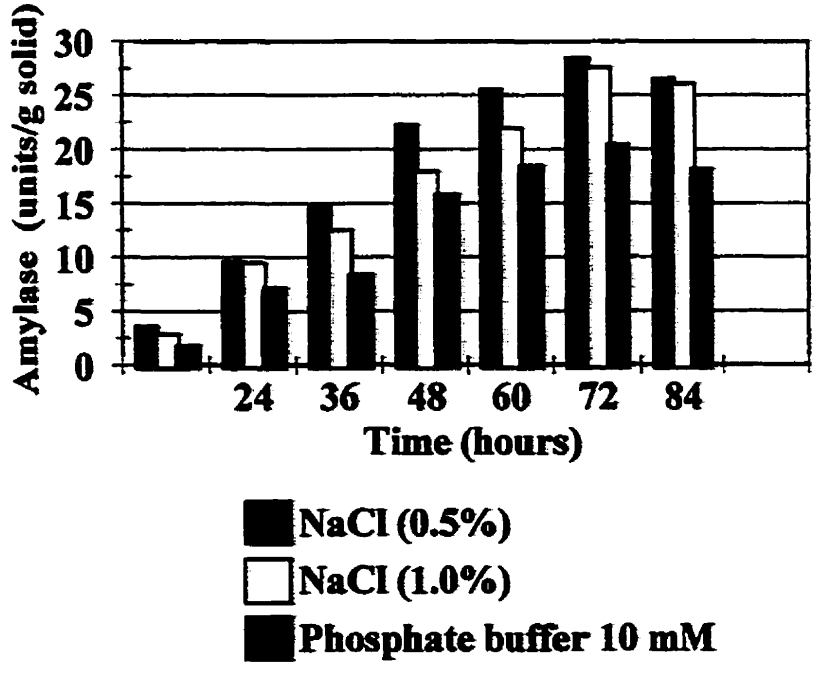
Once the process was well controlled, the effect of solvents on bacterial α-amylase recovery was evaluated. The leaching process was established after studying three different solvents for recovery of enzyme. Among these solvents were 0.5% (w/v) NaCl, 1.0% (w/v) NaCl and 10mM phosphate buffer. In Figure 4.6, the results from the various leaching processes are shown. Among the three solvents tested for enzyme recovery, NaCl provided the highest enzyme recovery, 28.5 units/g solid. There was no significant difference in the results obtained from 0.5% NaCl and 1.0% NaCl but there was a 28% increase in the enzyme yields when compared to phosphate buffer. For all subsequent α-amylase analyses with *B. subtilis*, 1.0% NaCl was used for enzyme recovery.

4.2.2.2 Bacterial growth

The bacterial growth based on plate counts of viable cells could not be determined due to difficulties in separating the biomass from the fermented substrate. Several attempts were made using different approaches but still, the most frequent result obtained, even with dilutions up to 10^{-10}, was that the number of colonies was too numerous to count (TNTC).

Figure 4.6 Moisture content and pH measurements (top) and enzyme yield from leaching with NaCl and phosphate buffer (bottom) during a SSF with *Bacillus subtilis* ATCC 21556 on potato peel.


8
6
4
2
0
pH
80
60
40
20
0
Moisture (%)
0 12 24 36 48 60 72 84
Time (hours)
pH
Moisture (%)

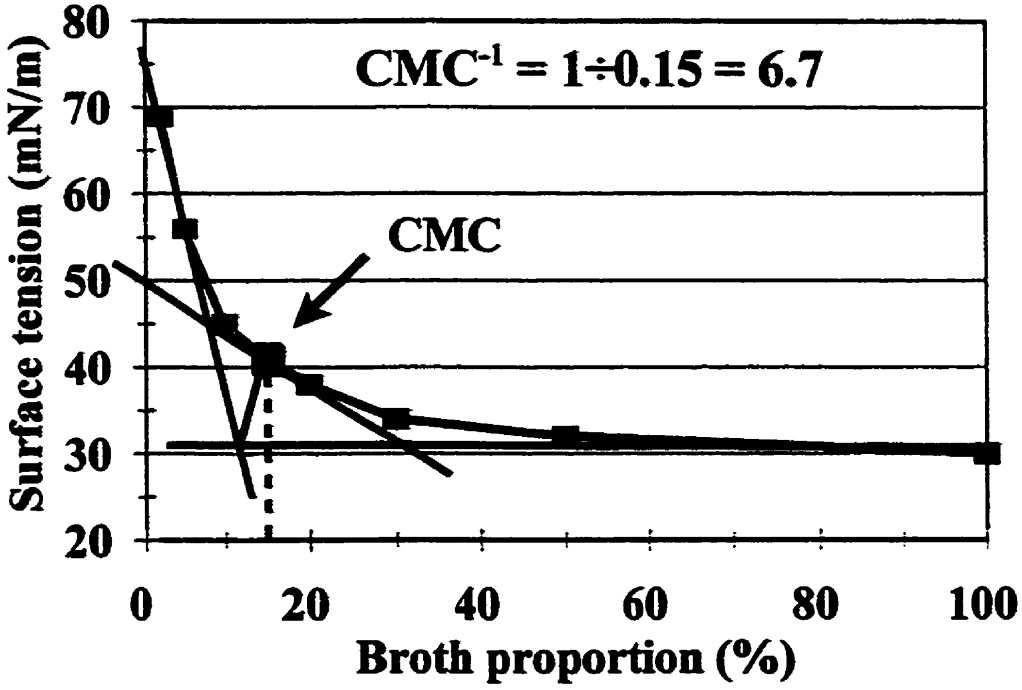

Amylase (units/g solid)
30
25
20
15
10
5
0
24 36 48 60 72 84
Time (hours)
NaCl (0.5%)
NaCl (1.0%)
Phosphate buffer 10 mM

4.2.3 *Bacillus subtilis* ATCC 21332

This particular strain of *Bacillus subtilis* is a known producer of the biosurfactant known as surfactin. Two of the objectives of this work are completely related to this microorganism. Surfactin as a component of cell-free broth was added to the SSF processes using either *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21556 to verify its effect on enzyme production, and this was compared to using this strain as a co-culture with *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21556.

Surfactin was produced by *B. subtilis* ATCC 21332 grown in a submerged fermentation of a glucose and mineral salts medium for 72 hours in 500 mL flasks containing 200 mL of medium shaken in a temperature controlled incubator. After separation of the biomass the amount of surfactin present in the cell-free broth, was determined by the procedure described in Section 3.2.6. The surface tension (mN/m) was plotted as a function of the percentage of cell-free broth after dilution with distilled water. The dilution factor that corresponded to the CMC value was a measurement of the amount of surfactin in the original cell-free broth and was designated as CMC^{-1}. The CMC^{-1} value in this Figure 4.7 corresponded to 6.7 and was used for the conversion of the volume of cell-free broth into mg of surfactin/g of solid based on a concentration of 10 mg/L of surfactin at the CMC (Sheppard, 1989). The CMC^{-1} was determined for every submerged fermentation of *B. subtilis* ATCC 21332 and was found to vary between 6.25 and 7.0 (with a CMC^{-1} of 6.7 the estimated concentration of surfactin in the broth would be 67 mg/L and if 10 mL were added to 250 g of substrate this corresponds to 0.00268 mg/g or 2.68×10^{-4}% (w/w)).

Figure 4.7 Determination of the critical micelle concentration (CMC) in a sample of cell-free broth from *B. subtilis* ATCC 21332.


$CMC^{-1} = 1 \div 0.15 = 6.7$
CMC
Surface tension (mN/m)
80
70
60
50
40
30
20
0
20
40
60
80
100
Broth proportion (%)

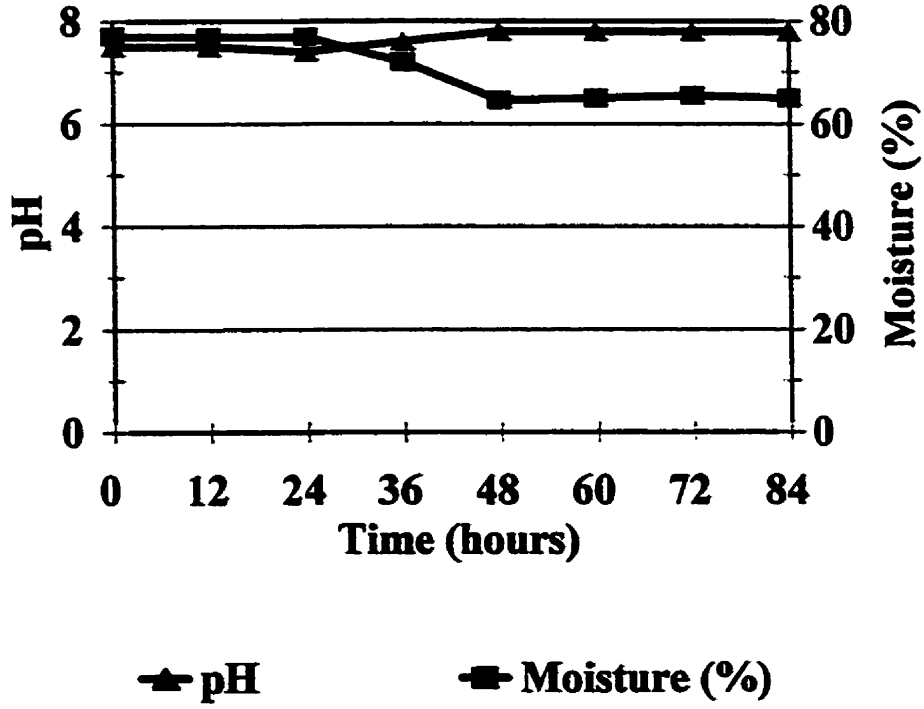
This strain of *Bacillus* is not well known as an amylase producer but, it was decided to test this strain during a SSF process by monitoring pH and moisture content, α-amylase production as well as the production of surfactin .

Variations in pH and moisture content and surfactin recovery are shown in Figure 4.8 These results are the mean values of two experiments. In the fermentation process using *B. subtilis* ATCC 21332 the variation in pH was between 7.5 and 7.8. The variation in moisture content was between 77% and 65%. For *B. subtilis* ATCC 21332 the drop in moisture content of about 11% which can be explained by the hypothesis that the surfactant produced by this strain released adsorbed moisture from the substrate.

Figure 4.9 shows the recovery of surfactin from 0.5, 1, 2, 5 and 10 g of fermented solid. This methodology was developed for the determination of surfactin production in SSF according to the amount of water used to recover the surfactin per g of substrate. It shows the results obtained by measuring surface tension as a function of proportion of extract, calculating the CMC^{-1} and converting these values to mg surfactin/g substrate. This particular graph shows the results obtained after 24 hours of fermentation using a pure culture of *B. subtilis* ATCC 21332.

The results for α-amylase production show that there was little production of this enzyme by this strain. The highest enzyme activity was obtained after 72 hours of fermentation, with a mean value from three replicate experiments of 12.7 units/g solid.

Figure 4.8 Change in pH and moisture content during the SSF of *B. subtilis* ATCC 21332 on potato peel.


8
6
4
2
0
pH
80
60
40
20
0
Moisture (%)
0
12
24
36
48
60
72
84
Time (hours)
pH
Moisture (%)

4.2.3.1 Surfactin production by *B. subtilis* ATCC 21332 in an SSF

The production of surfactin by a pure culture of *B. subtilis* ATCC 21332 is also shown in Figure 4.9. At zero hour of SSF,10% (v/w) *B. subtilis* broth containing 62 mg/L of surfactin was added. This value was the amount of surfactin produced after 72 hours of growth of *B. subtilis* ATCC 21332 in a submerged culture of glucose and mineral salts medium. During the SSF, surfactin production reached 1.1 mg/g of substrate after 72 hours.

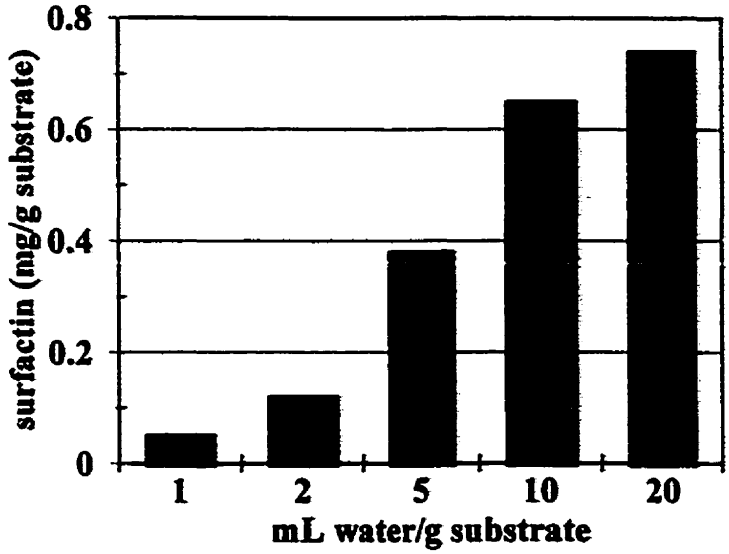
4.2.4 Effect of synthetic surfactants

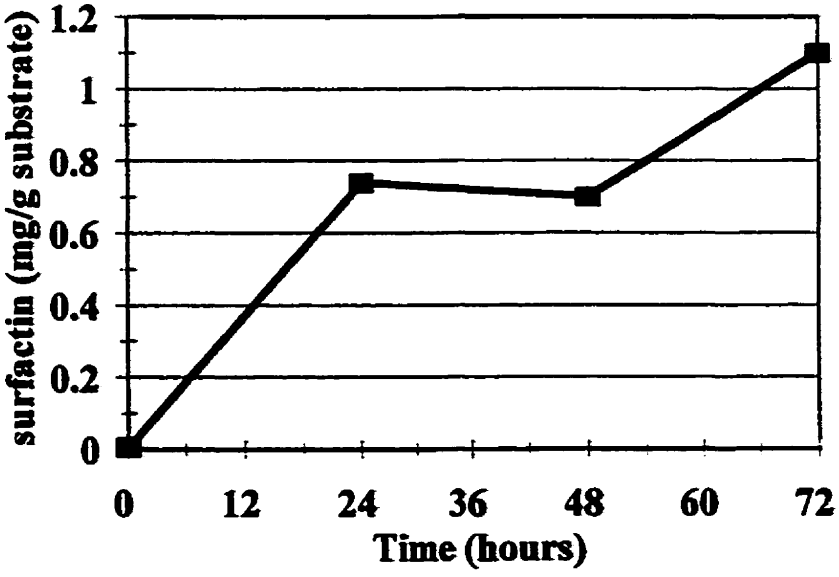
The synthetic surfactants used were Tween 80, Tween 20 and SDS. The purpose of using these commercial surfactants was to verify their effect on enzyme production in an SSF process using ground potato peel with *B. subtilis* ATCC 21556 or with *A. oryzae* ATCC 1011. Two different concentration of surfactants were tested, 0.05% and 0.10% based on v/w of the substrate. They were added to the inoculum under sterile conditions, mixed for 30 minutes for complete solubilization. Table 4.1 shows the surface tension of the inoculum after the addition of the various surfactants.

Table 4.1 Surface tension (mN/m) after addition of surfactants.

Inoculum:	*A. oryzae* ATCC 1011		*B. subtilis* ATCC 21556	
Surfactant conc. (v/v):	0.5%	1.0%	0.5%	1.0%
Tween 20	42	40	40	39
Tween 80	47	44	44	44
SDS	42	42	42	38

Figure 4.9 Surfactin recovery from a sample obtained after 24 hours (top) and production (bottom) during a SSF with *Bacillus subtilis* ATCC 21332.


0.8
0.6
0.4
0.2
0
surfactin (mg/g substrate)
1
2
5
10
20
mL water/g substrate


1.2
1
0.8
0.6
0.4
0.2
0
surfactin (mg/g substrate)
0
12
24
36
48
60
72
Time (hours)

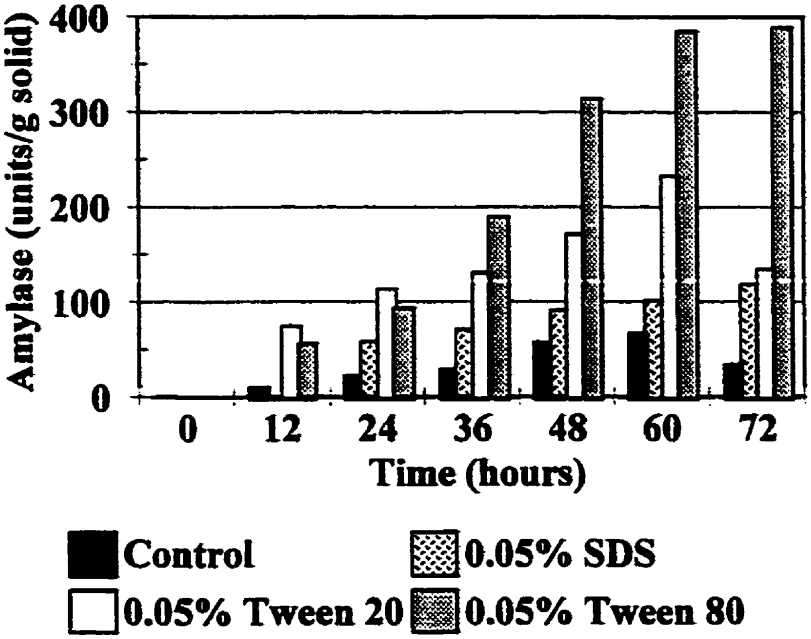
The results from the addition of 0.05% and 0.10% Tween 20, Tween 80 and SDS with *A. oryzae* are shown in Figure 4.10. The data shown are the mean values obtained from three experiments. Refer to Appendix 1 for the standard deviations.

When added to *A. orzyae* ATCC 1011 at 0.05% concentration, the results show that Tween 80 is the surfactant which provided the highest enzyme activity. The maximum α-amylase activity found was 390 units/g solid, about 6 times higher when compared to no surfactant addition. Using Tween 20, the maximum activity found was 233 units/g solid, a 3.5 fold increase and with SDS the amount of enzyme produced was doubled. It was found that at 0.10% concentration of surfactant there was no significant difference between the results obtained at 0.05% concentration.

Figure 4.11 shows the results of the addition of Tween 20, Tween 80 and SDS added to a SSF with *B. subtilis* ATCC 21556. These results are also the mean values obtained from three experiments. When added to *B. subtilis* ATCC 21556 Tween 80 at 0.05% and 0.10% (v/w) resulted in the highest enzyme activity which corresponded to a 6 fold increase over that of the control, which corresponds to the growth of *B. subtilis* ATCC 21556 with no surfactant addition. Tween 20 and SDS at 0.5% and 1.0% were also able to increase the enzyme activity by 5 and 4 fold respectively.

Tween 80 and Tween 20 have been in use for sometime in submerged fermentation to assist bacterial growth by increasing the permeability of the cells. It has been suggested that surfactants may act in the cell membrane affecting the production and release of microbial enzymes. The anionic surfactant, SDS, resulted in the lowest enzyme activity, perhaps due to its protein denaturing ability.

Figure 4.10 Amylase production from an SSF with *Aspergillus oryzae* in the presence of Tween 20, Tween 80 and SDS at 0.05% (v/w) (top) and 0.10% (v/w) bottom.


Amylase (units/g solid)
400
300
200
100
0
0
12
24
36
48
60
72
Time (hours)
Control
0.05% SDS
0.05% Tween 20
0.05% Tween 80

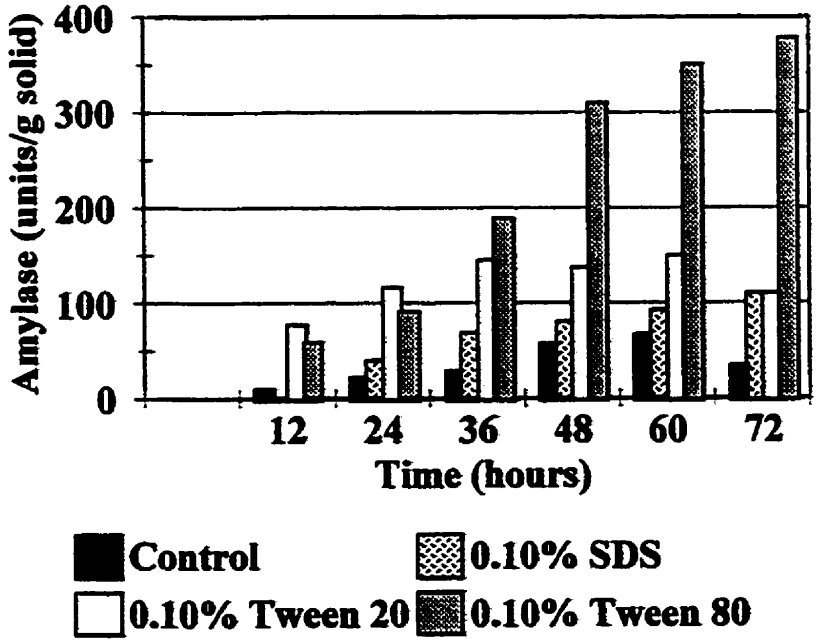
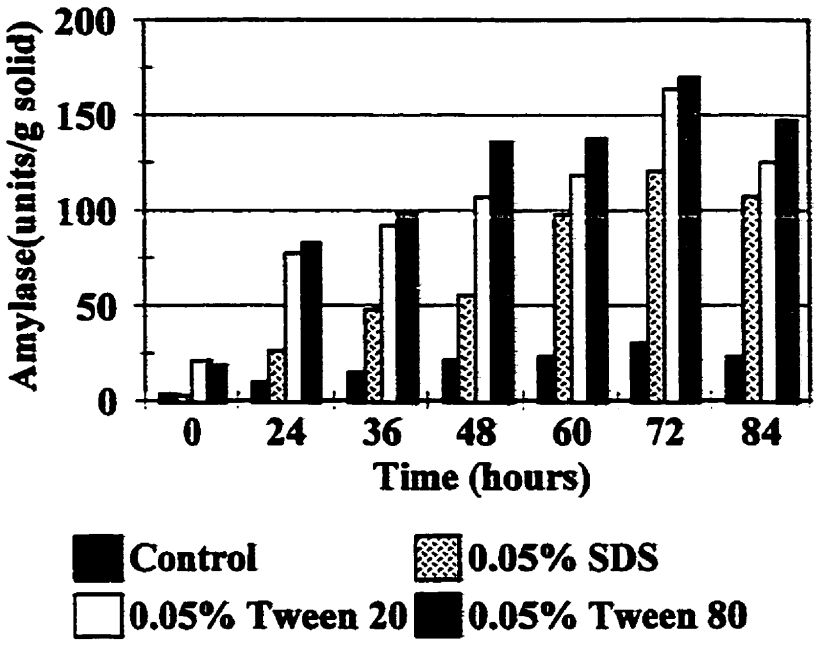
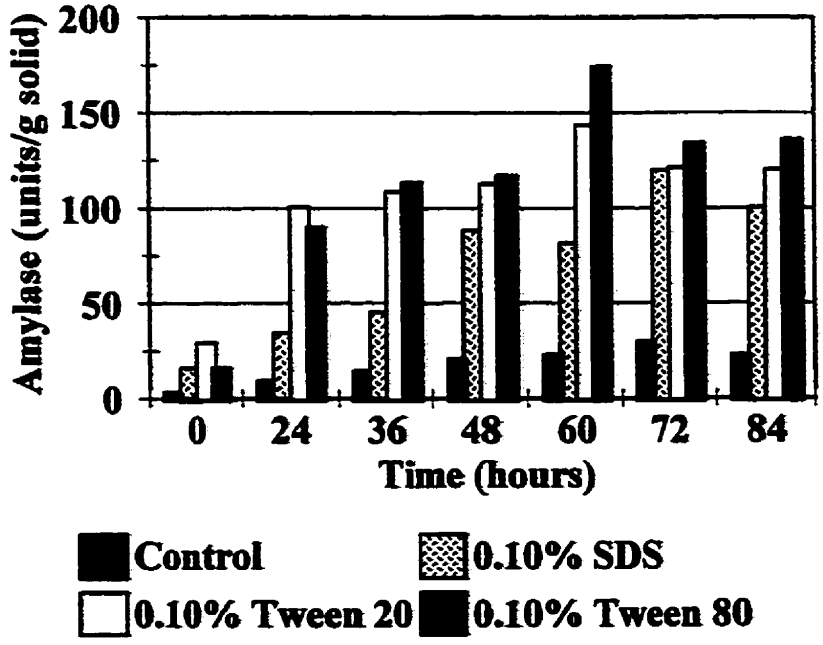

Amylase (units/g solid)
400
300
200
100
0
12
24
36
48
60
72
Time (hours)
Control
0.10% SDS
0.10% Tween 20
0.10% Tween 80

Figure 4.11 Amylase production from an SSF with *B. subtilis* ATCC 21556 in the presence of Tween 20, Tween 80 and SDS at 0.05% (v/w) (top) and 0.10% (v/w) (bottom).


Amylase(units/g solid)
200
150
100
50
0
0
24
36
48
60
72
84
Time (hours)
Control
0.05% SDS
0.05% Tween 20
0.05% Tween 80


Amylase (units/g solid)
200
150
100
50
0
0
24
36
48
60
72
84
Time (hours)
Control
0.10% SDS
0.10% Tween 20
0.10% Tween 80

4.2.5 Effect of surfactin concentrations

Surfactin was previously produced in a submerged fermentation of glucose and mineral salts medium and recovered from the broth after removing the cells. To be added in the SSF process, the cell-free broth was autoclaved and added in the inoculum at 0.5%, 1.0%, 2.0% and 5.0% (v/v). These values have been converted to the real amount of surfactin present in the broth based on surface tension measurements. The calculation of the amount of surfactin added to the process has already been described in section 3.2.6. After the conversion, these values were 5 ng/g, 11 ng/g, 20 ng/g and 54 ng/g (w/w) of substrate and correspond to the amount of surfactin added in the SSF process. Table 4.2 shows the surface tension measurements of the inoculum after addition of the different concentrations of cell-free broth containing surfactin were added to the inocula of *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21556.

Table 4.2 Surface tension (mN/m) of inocula after addition of surfactin broth.

Broth added (v/v%)	*A. oryzae*	*B. subtilis*
0.5	66	40
1.0	64	39
2.0	54	36
5.0	52	37

Figure 4.12 shows the effect of surfactin concentration on the enzyme yield by *A. oryzae* ATCC 1011, the data points averaged from three experiments. There was an increase in fungal amylase activity with surfactin addition but less if compared to the addition of synthetic surfactant Tween 80. The highest enzyme activity was found when 11 ng/g of surfactin was added to the process, 196 units/g solid corresponding to an increase of 2.8 times over the control. This modest, although significant increase compared to the control, could be explained by the fact that surfactin is known to possess fungicidal activity and this could have inhibited the production of the enzyme (Lin, 1996).

On the other hand, when surfactin was added to the process with *B. subtilis* ATCC 21556, it was found to be very effective (Figure 4.13). The highest enzyme activity was obtained with 20 ng/g of surfactin up to 72 hours of fermentation which corresponded to an increase of 10 fold when compared to the control. The other concentrations also increased α-amylase activity 8.5 to 9.5 fold which indicated that the use of the biosurfactant, surfactin had a greater effect with *B. subtilis* than the synthetic surfactants.

Figure 4.12 Effect of the addition of 5 ng/g, 11 ng/g, 20ng/g and 54 ng/g of surfactin on amylase activity in an SSF with *A. oryzae*.

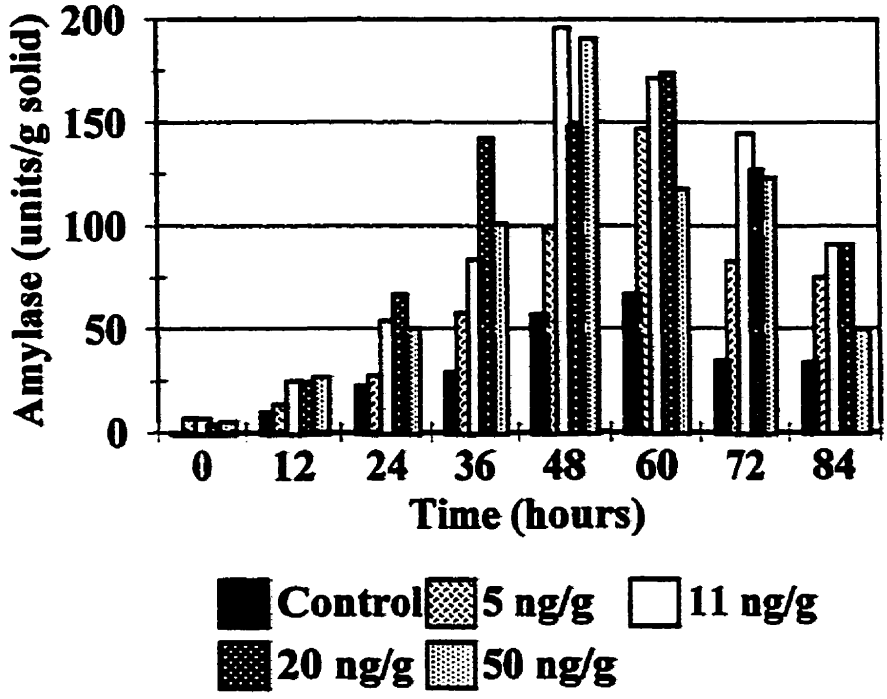
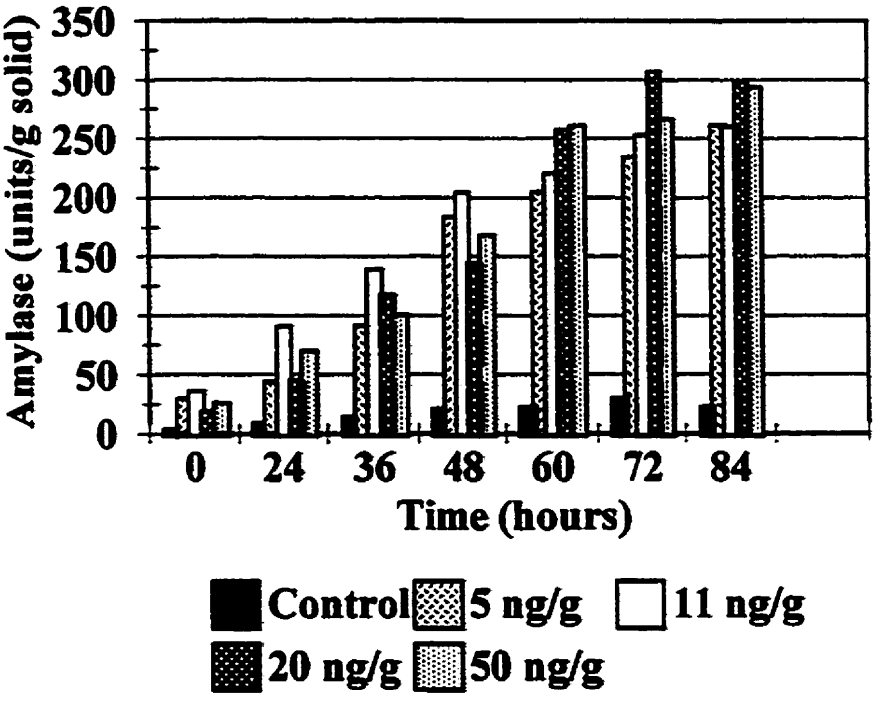

Amylase (units/g solid)
200
150
100
50
0
0
12
24
36
48
60
72
84
Time (hours)
Control
5 ng/g
11 ng/g
20 ng/g
50 ng/g

Figure 4.13 Effect of the addition of 5 ng/g, 11 ng/g, 20 ng/g and 54 ng/g of surfactin on amylase activity in an SSF with *B. subtilis* ATCC 21556.


Amylase (units/g solid)
350
300
250
200
150
100
50
0
0
24
36
48
60
72
84
Time (hours)
Control
5 ng/g
11 ng/g
20 ng/g
50 ng/g

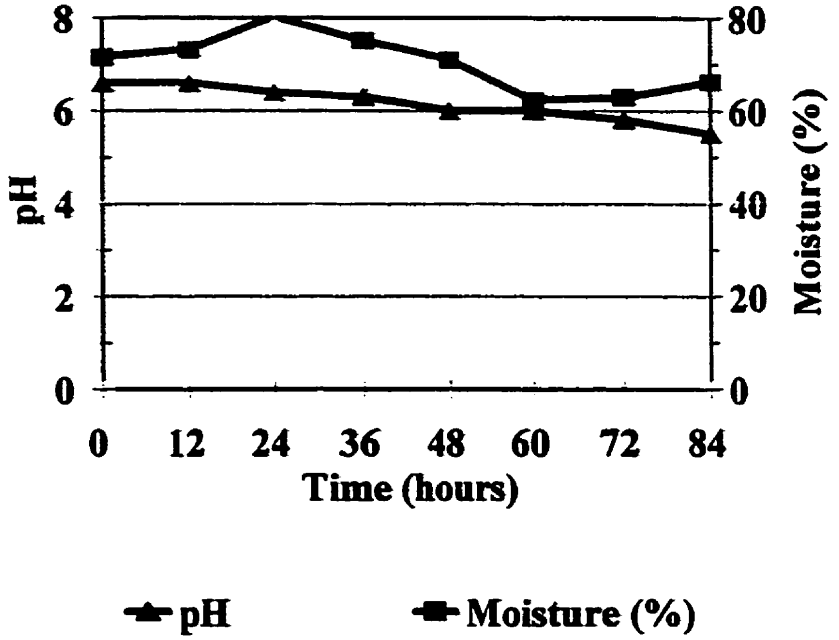
4.3 Mixed culture

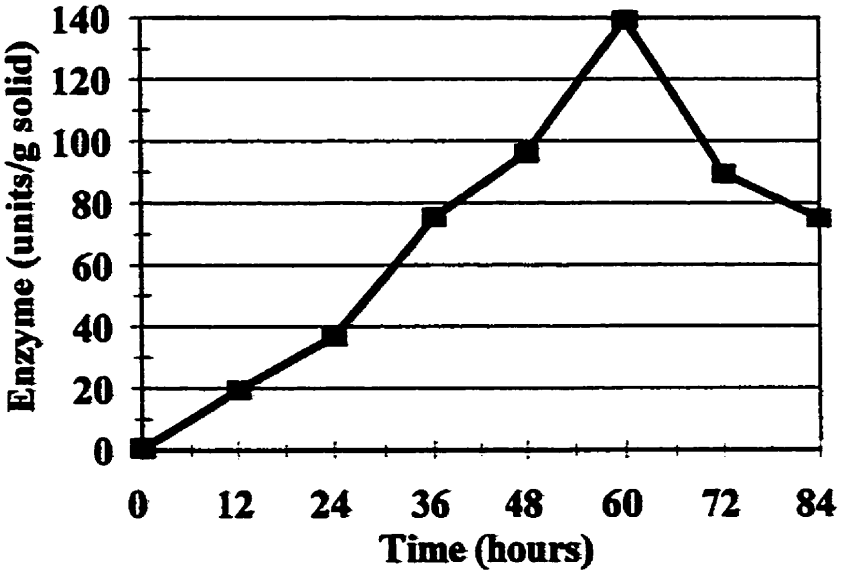
The addition of surfactin did result in an increase in enzyme activity with both *A. oryzae* and *B. subtilis* and there are only few reports in the literature describing the use of a mixed culture in a SSF to stimulate enzyme production. Taking into consideration these factors, it was decided to study the effect of adding the surfactin-producing strain of *B. subtilis* to both fermentation processes, with *Aspergillus* and *Bacillus*. A 250 g quantity of ground potato peel (80% initial moisture content and pH 6.0) was added to the tray fermentor and the trays were autoclaved at 121°C for 60 min, cooled, inoculated with a combination of either *A. orzyae* and *B. subtilis* ATCC 21332 or *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 for the mixed culture condition. The trays were incubated in closed chambers with automatic control of temperature (30°C), relative humidity (90%) and with uniform upward air flow. Samples were taken every 12 hours.

4.3.1 *A. oryzae* and *B. subtilis* ATCC 21332

Figure 4.14 demonstrates the variations in pH and moisture content. The initial pH was 6.6, dropping to 5.5 after 84 hours of fermentation. The moisture content was 71.6% in the beginning of the process, increasing to 80.5% in 24 hours of fermentation time and then dropping to a final value of 66.3%. Figure 4.14 also shows the profile for α-amylase production. The maximum enzyme yields doubled when a mixed culture was used, going from 67 to 139 units/g solid.

Figure 4.14 pH and moisture content of the substrate (top) and amylase activity (bottom) obtained from a mixed culture SSF of *A. oryzae* and *B. subtilis* ATCC 21332.


8
6
4
2
0
pH
80
60
40
20
0
Moisture (%)
0 12 24 36 48 60 72 84
Time (hours)
pH
Moisture (%)


140
120
100
80
60
40
20
0
Enzyme (units/g solid)
0 12 24 36 48 60 72 84
Time (hours)

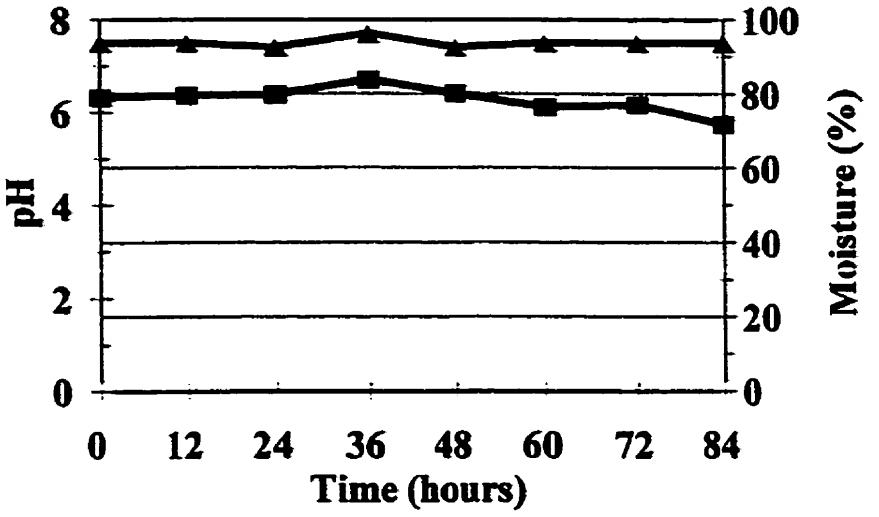
4.3.2 *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332

In this SSF process with a co-culture of two *Bacillus* strains the potential for enhancing both α-amylase production and surfactin was investigated.

Figure 4.15 shows the changes in pH and moisture content for this co-culture as well as the values obtained for α-amylase activity. The pH of the substrate inoculated with this mixed culture was about 7.5 during the entire process. The moisture content was initially 79% increasing up to 84% at 36 hours of fermentation and then decreasing to reach a final moisture content of 72%. The highest enzyme activity was found after 72 hours of fermentation, 288 units/g solid, which corresponds to a 9.5 fold increase in comparison to the highest value obtained in a SSF process with a pure culture of *B. subtilis* ATCC 21556.

The yields obtained from enzyme activity indicated that α-amylase production by a defined mixed bacterial culture of two strains of *B. subtilis* was much greater than by the pure cultures of the same species of *B. subtilis* (Figure 4.16). A defined mixed culture involves the inoculation of the substrate with more than one pure culture, so that both microorganisms grow simultaneously (Mitchell, 1992). By using a defined mixed bacterial culture there may be a synergistic effect providing better growth and product formation. In this particular case, one of the *B. subtilis* strains, ATCC 21556 is a well known α-amylase producer and the other strain, ATCC 21332 is better known as surfactin producer.

Figure 4.15 pH and moisture content of the substrate (top) and amylase activity (bottom) obtained from a mixed culture of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332.


8
6
4
2
0
pH
100
80
60
40
20
0
Moisture (%)
0
12
24
36
48
60
72
84
Time (hours)
pH
Moisture (%)

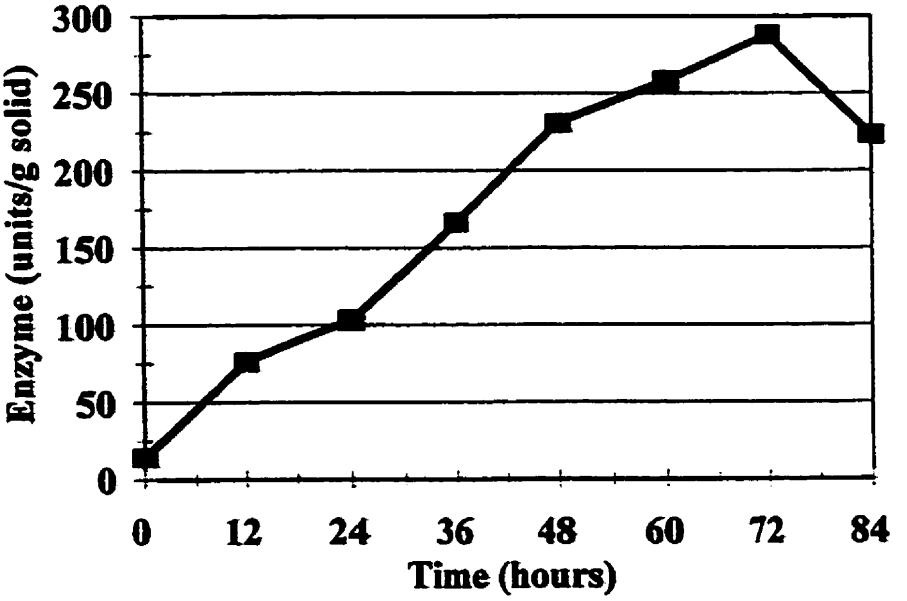
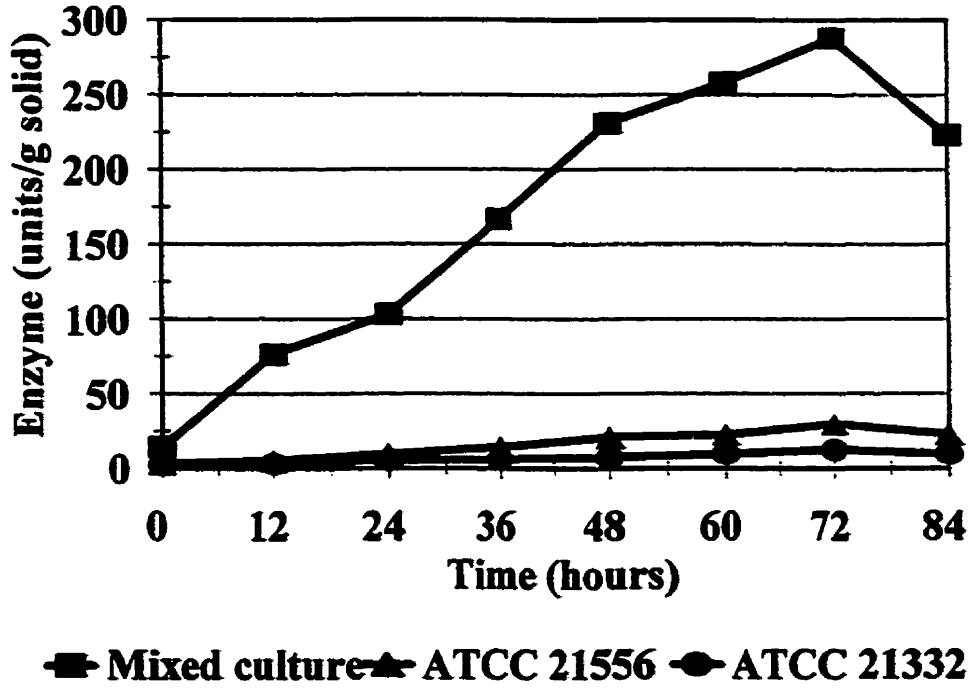

300
250
200
150
100
50
0
Enzyme (units/g solid)
0
12
24
36
48
60
72
84
Time (hours)

Figure 4.16 Comparison in enzyme production between a co-culture of two strains of *Bacillus* and the individual strains in SSF.


300
250
200
150
100
50
0
Enzyme (units/g solid)
0
12
24
36
48
60
72
84
Time (hours)
Mixed culture
ATCC 21556
ATCC 21332

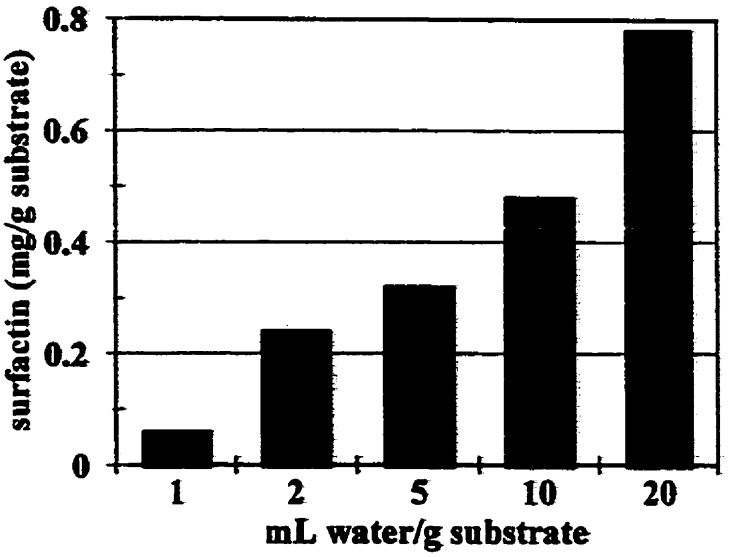
4.3.2.1 Surfactin production by a co-culture of *Bacillus* strains

The recovery of surfactin from this co-culture was done by the same procedure with the pure culture of *B. subtilis* ATCC 21332. Figure 4.17 shows the results for the recovery of surfactin after 24 hours of fermentation from 0.5, 1.0, 2.0, 5.0 and 10 g of fermented substrate (using 10 mL of water in each case) as well as the increase in surfactin production by the mixed culture during the fermentation time. The results in Figure 4.17 show an increase in surfactin production in a mixed culture from 62 mg/L at zero hour of fermentation (inoculum) to 1.41 mg/g substrate at 72 hours. Both processes, mixed and pure cultures produced biosurfactant in quantities comparable to and even higher than that of surfactin production in submerged culture which was on average 0.8 mg/mL of broth (Cooper *et al.*, 1981). The yields of surfactin obtained by the mixed culture in SSF were higher than that of the pure culture of *B. subtilis* ATCC 21332 in SSF, however, these yields were lower than that of SSF using Okara as substrate, reported to be between 1.8-2.0 mg/g wet weight of substrate (Ohno *et al.* 1995a, 1995b).

4.3.3 Co-culture of *Bacillus* with Tween 80

This experiment was done in order to evaluate if the addition of a synthetic surfactant in the co-culture process would also help to improve enzyme activity. The values shown in Figure 4.18 are the mean values of two experiments. It was observed that enzyme yields were increased even more in comparison to a co-culture with no addition of surfactant. The enzyme activity reached the value of 342 units/g solid, a further increase of 18%. This

Figure 4.17 Surfactin recovery from a SSF process after 24 hours (top) and surfactin production (bottom) using a mixed culture of *Bacillus subtilis* strains.


surfactin (mg/g substrate)
0.8
0.6
0.4
0.2
0
1
2
5
10
20
mL water/g substrate

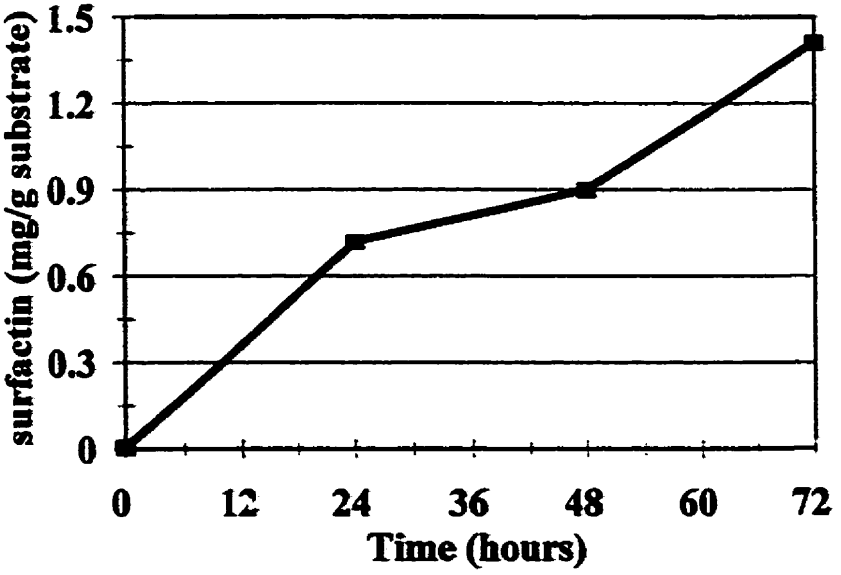
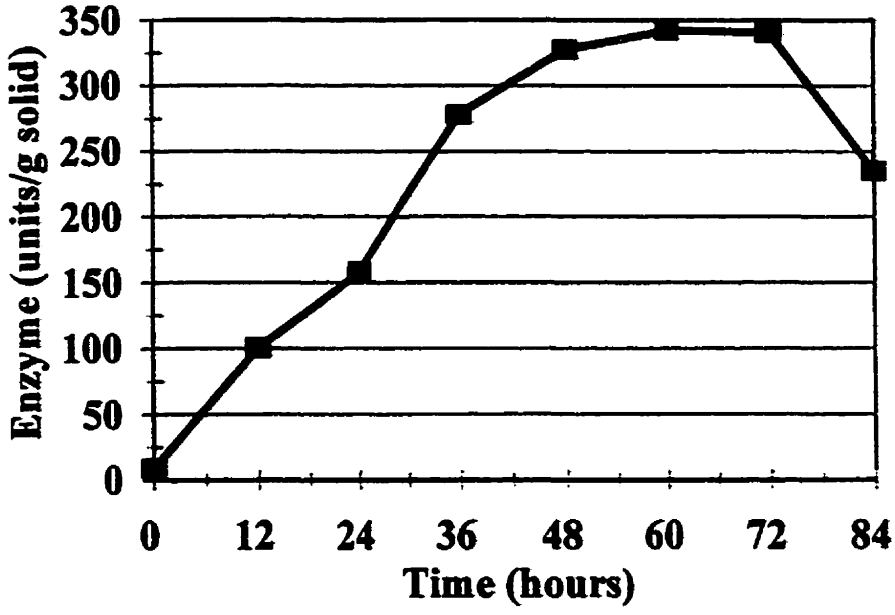

surfactin (mg/g substrate)
1.5
1.2
0.9
0.6
0.3
0
0
12
24
36
48
60
72
Time (hours)

Figure 4.18 α-amylase activity obtained from a SSF with a mixed culture of two *Bacillus* strains after the addition of 1.0% (v/v) Tween 80 to the inocula.


Enzyme (units/g solid)
350
300
250
200
150
100
50
0
0
12
24
36
48
60
72
84
Time (hours)

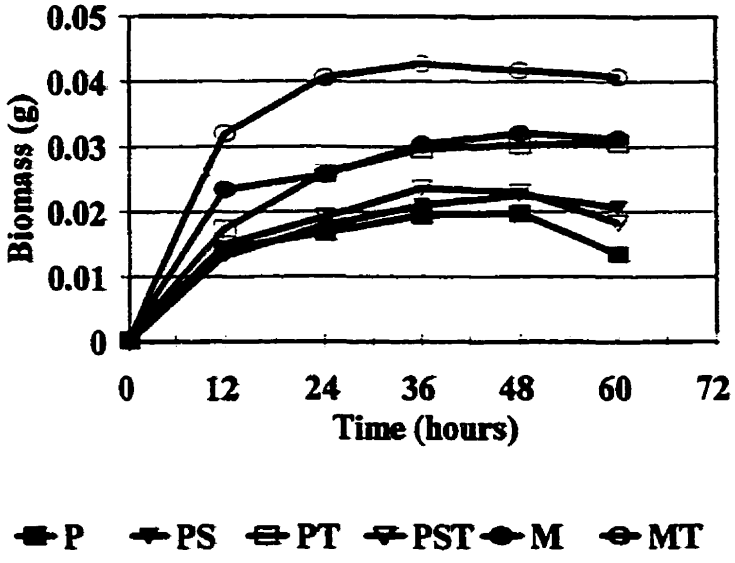
could be due to a cumulative effect of the biosurfactant which was released in the process, with the addition of a synthetic surfactant.

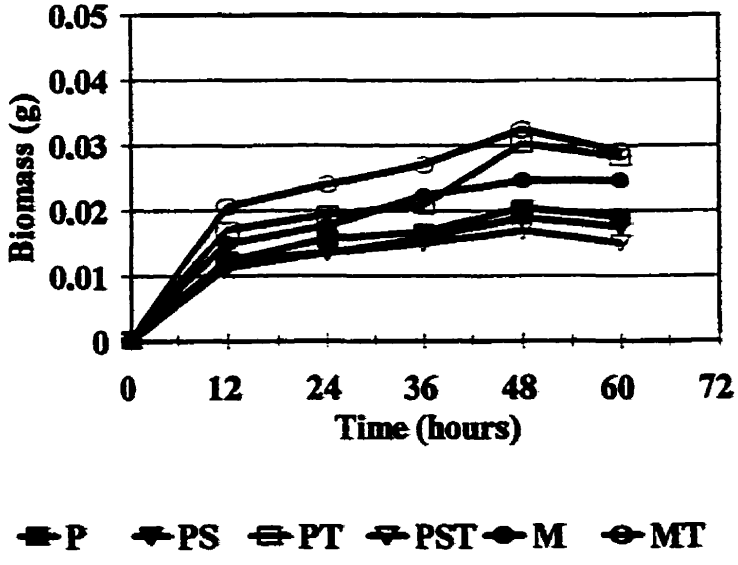
4.4 Bacterial growth on agar plates

Estimation of the biomass for the SSF with bacteria could not be established due to the difficulty of separating the cells from the substrate. This information would be of great value for establishing a relationship between surfactant addition and growth, enzyme production and also biofilm formation. The alternative used, was to quantify the effects of growth conditions on biomass production using more uniform solid substrates. The substrates used were plate count agar (PCA) and potato dextrose agar (PDA). The procedure for these experiments was described in Section 3.3.5. The experiments were done in triplicate and samples for evaluation of biofilm formation were kept for SEM analyses.

Figure 4.19 shows the kinetics of growth of *B. subtilis* ATCC 21556, *B. subtilis* ATCC 21332 and a mixed culture of this two *Bacillus* with or without addition of Tween 80. The results on both PCA and PDA plates have shown that the highest amount biomass was obtained with mixed culture with Tween 80 addition, followed by *B.subtilis* ATCC 21556 with Tween 80 and then the mixed culture with no surfactant added. Unfortunately, the evaluation of biofilm formation by SEM analyses was not possible due to problems in the sample preparation. A thick biofilm could be seen very clearly on the agar plates by eye but, during the sample preservation in the buffer, the biofilm became detached from the agar.

Figure 4.19 Growth kinetics on PDA plates (top) and PCA plates (bottom) with a pure culture of *B. subtilis* ATCC 21556 (P), *B. subtilis* ATCC 21556 plus Tween 80 (PT), *B. subtilis* ATCC 21332 (PS), *B. subtilis* ATCC 21332 plus Tween 80 (PST), mixed culture of the two *Bacillus* strains (M) and the mixed culture plus Tween 80 (MT).


0.05
0.04
0.03
0.02
0.01
0
Biomass (g)
0
12
24
36
48
60
72
Time (hours)
P
PS
PT
PST
M
MT


0.05
0.04
0.03
0.02
0.01
0
Biomass (g)
0
12
24
36
48
60
72
Time (hours)
P
PS
PT
PST
M
MT

4.5 Qualitative results

These qualitative experiments were done in order to investigate the mechanism of surfactant action. A number of approaches were taken. The diffusivity tests were done to evaluate the effect of the presence of surfactant in a solution, on plate count agar and on potato slices. The experiment on plate count agar with *B. subtilis* ATCC 21556 was done to verify the effect of surfactant on colony formation. Other qualitative experiments done were scanning electron microscopy to observe the growth of the cells and their interaction with the substrate and starch film formation to study various interactions in the process.

4.5.1 Diffusivity tests

The results obtained from these tests were generally limited to observations, with few or no quantitative results. These observations will be described with the maximum of details. The first experiment was done on petri dishes containing water and/or surfactin solution and by adding dyes. This solution was the cell-free broth obtained from the growth of *B. subtilis* ATCC 21332. Before the beginning of the experiment, the surface tension of the broth was measured and it was found to be 30 mN/m. When the dyes, either safranin or crystal violet, were added to the surfactin solution, the diffusion was much slower in comparison to the diffusion in water. When the dyes were added to the aqueous solutions, the dyes would diffuse immediately, whereas, for the dyes to diffuse completely over the surface of the surfactin solution would take around 20 minutes.

The other experiment was done by adding dye on plate count agar with and without surfactant. After 24 hours it was observed that the dyes did not move when injected on the agar with no surfactant or lower concentration of surfactants, such as 0.5% and 1.0% for surfactin and 0.3% and 0.5% for Tween 80. However, as the concentration of surfactant in the agar plates were increased, a clear zone was formed around the point at which the dye was injected. The plates were kept for at least 24 hours more and it was observed that the diameter of the clear zone increased over time. These results were confirmed by performing all experiments in triplicate.

The last diffusivity test was done on slices of potato. The potatoes were sliced and weighed for the addition of the surfactant on a v/w basis. Surfactin broth at 1.0%. 2.0% and 5.0% and Tween 80 at 0.3%, 0.5% and 1.0% were added on the surface of the sliced potatoes. The results were observed and compared to slices of potatoes without surfactant addition. Two mL of crystal violet were added to the surface of the sliced potatoes. On the slices with surfactin, the dye not only spread all over the surface but also infiltrated into the starch material. The same results were obtained with Tween 80. On the contrary, on the slices with no surfactant the dye seemed to infiltrate only on the injection point and did not spread.

4.5.2 Colony formation of *B. subtilis* ATCC 21556 on plate count agar (PCA)

The experiments on plate count agar (PCA) were done to verify the effect of different surfactant concentrations on the growth of *B. subtilis* ATCC 21556 when the culture was inoculated at the center of each PCA plate. The same experiment was done under two different conditions. One experiment was on a shaker (New Brunswick Scientific, New Jersey) at 30°C and 200 rpm and the other at 30°C with no rotation of the plates.

When there was no shaking movement, from the point where the inoculum was added, the colonies would grow forming a circle. The diameter of this circle was measured over 24, 48, 72 and 96 hours of incubation. Table 4.3 shows the values obtained. The presence of surfactants had a positive effect on the growth of this microorganism, especially surfactin after 72 hours, with the diameter of the colony over 7 times that of the control.

Table 4.3 The diameter of colonies *B. subtilis* ATCC 221556 grown on agar plates

		Diameter of colonies (mm)			
	Conc (w/w)	24 hours	48 hours	72 hours	96 hours
No surfactant	-	5	6	9	10
Tween	0.1%	7	11	12	12
	0.2%	9	18	30	40
	0.3%	10	10	11	11
Surfactin	0.5%	4	7	20	23
	1.0%	8	10	40	45
	2.0%	9	8	60	65
	5.0%	10	10	68	72

When the plates were shaken, it was not possible to measure the diameter of the colonies, since they grew in different shapes all over the plates. One example of this growth pattern can be seen in Figure 4.20.

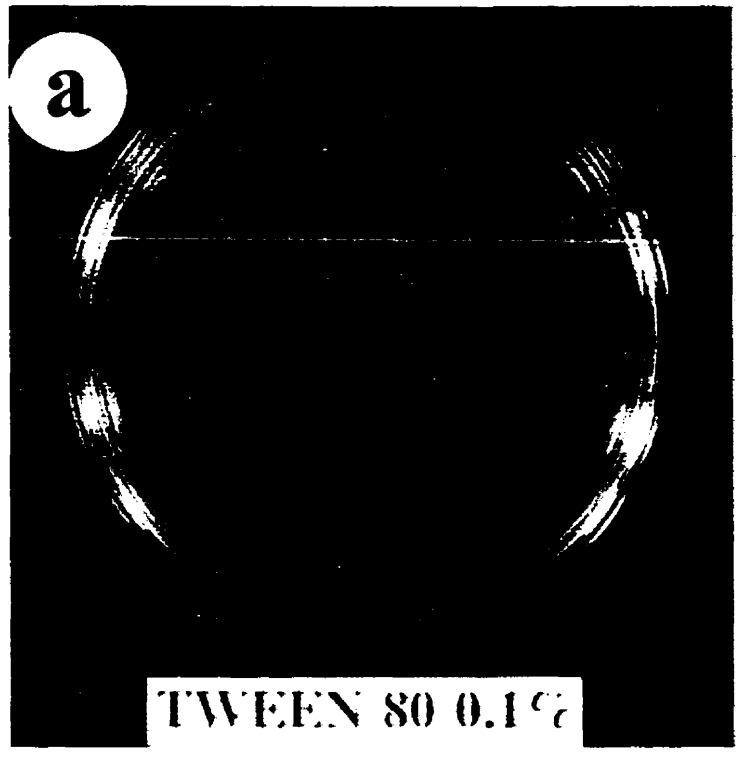
4.5.3 Scanning electron microscopy

The purpose of the work done with the scanning electron microscope was an attempt to observe the physical interaction between the microbes and the fermented substrate over the fermentation time. One important aspect of this study was the sample preparation which has already been described in Section 3.3.1. Scanning electron micrographs were made on control substrates without bacteria, such as potato starch and potato skin to verify the main components of these two substrates and to better understand the components of the fermented samples. All the samples were scanned at 10 kV accelerating voltage and with magnification of x200, x500, 1000x and x3500. The samples were composed of the controls, the controls with addition of either Tween 80 or surfactin, ground or sliced fermented substrates inoculated with a pure culture of *B. subtilis* ATCC 21556 with and without surfactant addition, a pure culture of *B. subtilis* ATCC 21332, or a mixed culture of these two *Bacillus* strains with and without surfactant addition.

4.5.3.1 SEM of ground potato skins

Micrographs of the ground potato skins are shown in Figures 4.21 and 4.26. Figure 4.21 shows the growth of *B. subtilis* ATCC 21556. Picture (a) is the micrograph taken at x500 magnification and picture (b) is the one taken at x3500 magnification. It is evident that

Figure 4.20 After 48 hours of growth of *B. subtilis* ATCC 21556 on Plate Count Agar (PCA) with 0.1% Tween 80 (a) and 0.2% Tween 80 (b) added and the plates shaken at 200 rpm.


a
TWEEN 80 0.1%

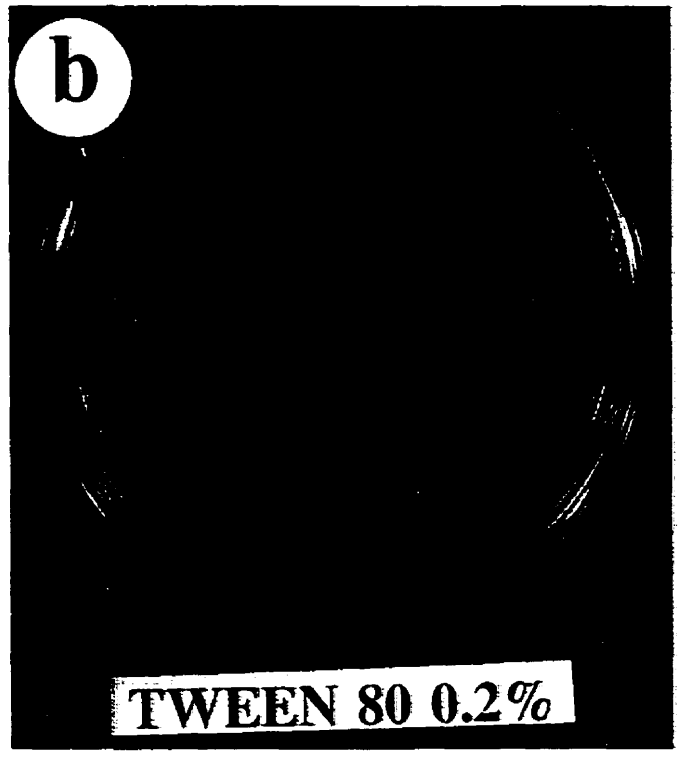
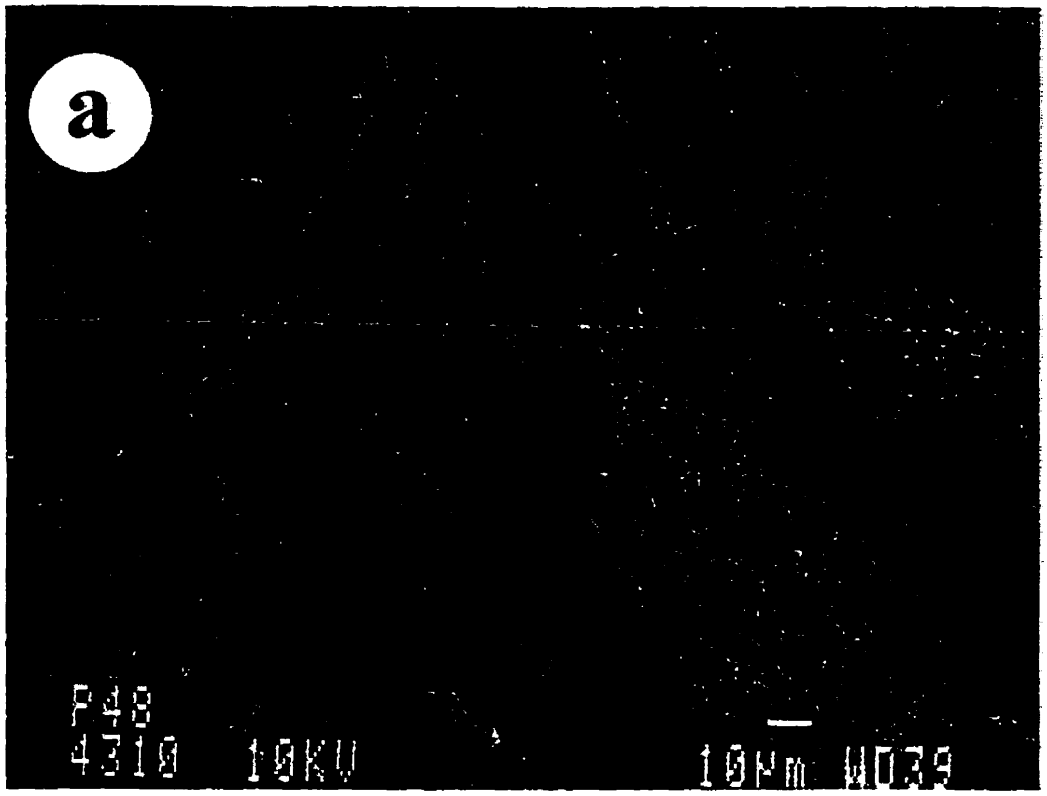
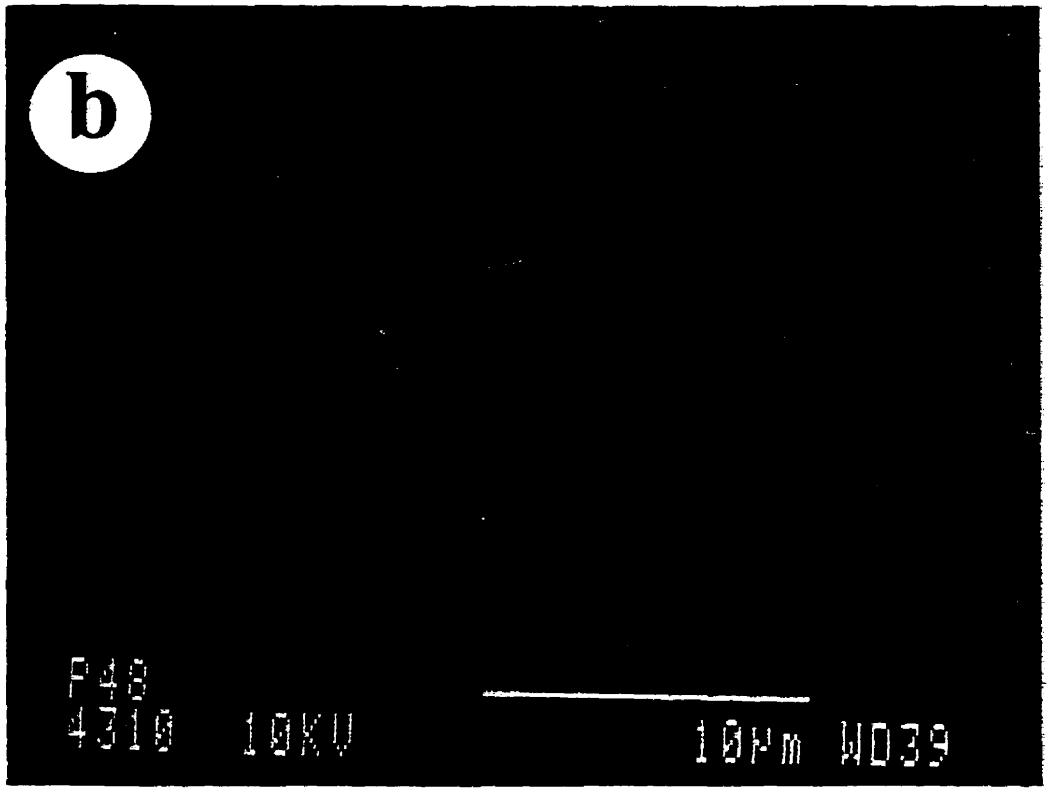

b
TWEEN 80 0.2%

Figure 4.21 Scanning electron micrographs of sample obtained after 48 hours of SSF of ground potato peel inoculated with *B. subtilis* ATCC 21556 at magnification of x500 (a) and x3500 (b).


a
P48
4310 10KV
10μm W039


b
P48
4310 10KV
10μm W039

the bacteria are covering the entire surface of the substrate. This was observed even after 24 hours of fermentation. Figures 4.22 to 4.24 show the accumulation of *B. subtilis* ATCC 21556 cells with addition of Tween 80 and surfactin. At this point, an interesting pattern starts to be evident. In Figure 4.22, which is the one with Tween 80 added to the process, there is clearly a formation of a thin biofilm covering the bacterial mass. The same characteristic could be seen in Figures 4.23 and 4.24, the samples in which cells were grown in the presence of surfactin, where a biofilm is also evident, after both 48 hours and 72 hours of fermentation.

The mixed culture micrographs are shown in Figures 4.25 and 4.26. The first figure shows two different fermentation times, indicating that significant growth is evident 24 hours after the beginning of the process. The bacteria are growing very close to each other and no biofilm was visible until 72 hours. Figure 4.26 shows the mixed culture after 72 hours of fermentation. One important characteristic of the culture seen in the micrograph taken at x3500 magnification (Figure 4.26 (b)) is the presence of long chains of bacteria, which is one of the characteristics of the growth of *B. subtilis* ATCC 21332.

The micrographs of the ground peel provided evidence of an unexpected phenomenon occurring in the SSF process, the biofilm formation. Unfortunately, it was not clear whether the bacteria were growing on the skin or if the growth was confined to the starchy material. Also, it could not be seen if there was penetration of the bacteria under the surface of the substrate. To clarify some of these questions, it was decided to conduct an SSF process on the surface of sliced potatoes. By making longitudinal cuts of the samples, it was possible to check for penetration, to examine the skin separately from the starch

Figure 4.22 Scanning electron micrographs of the ground potato peel, showing the growth of *B. subtilis* ATCC 21556 with Tween 80 after 48 hours of fermentation at magnification of x1000 (a) and x3500 (b).


a
T48
4310 10KV
10μm WD39

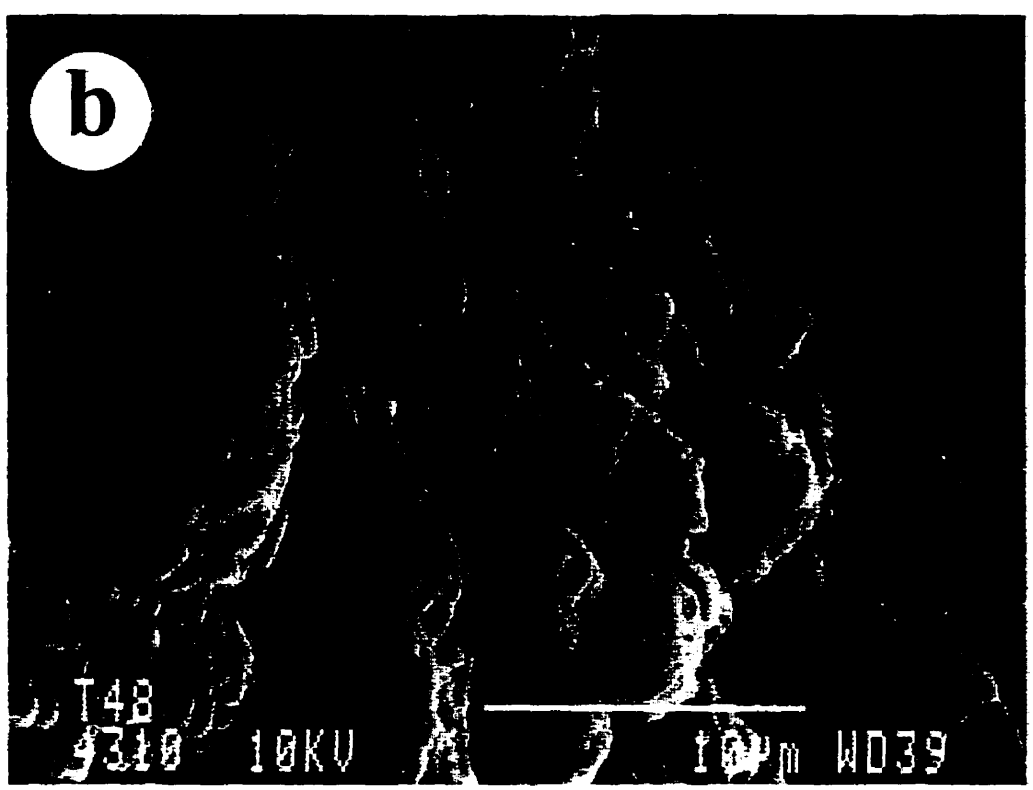
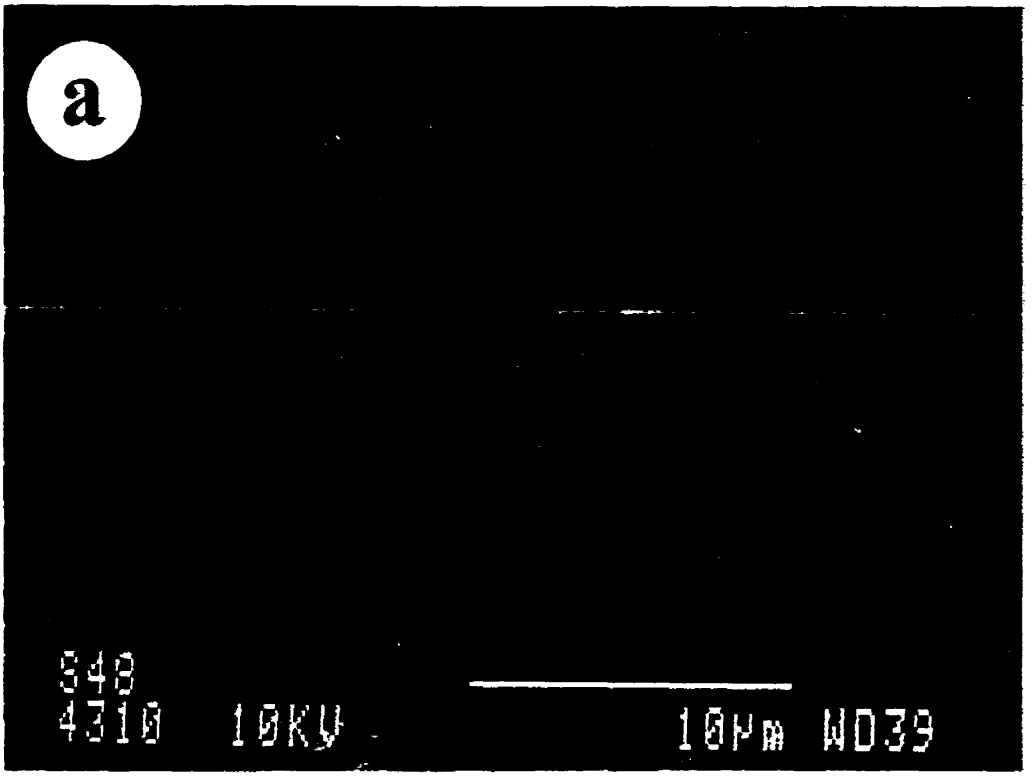
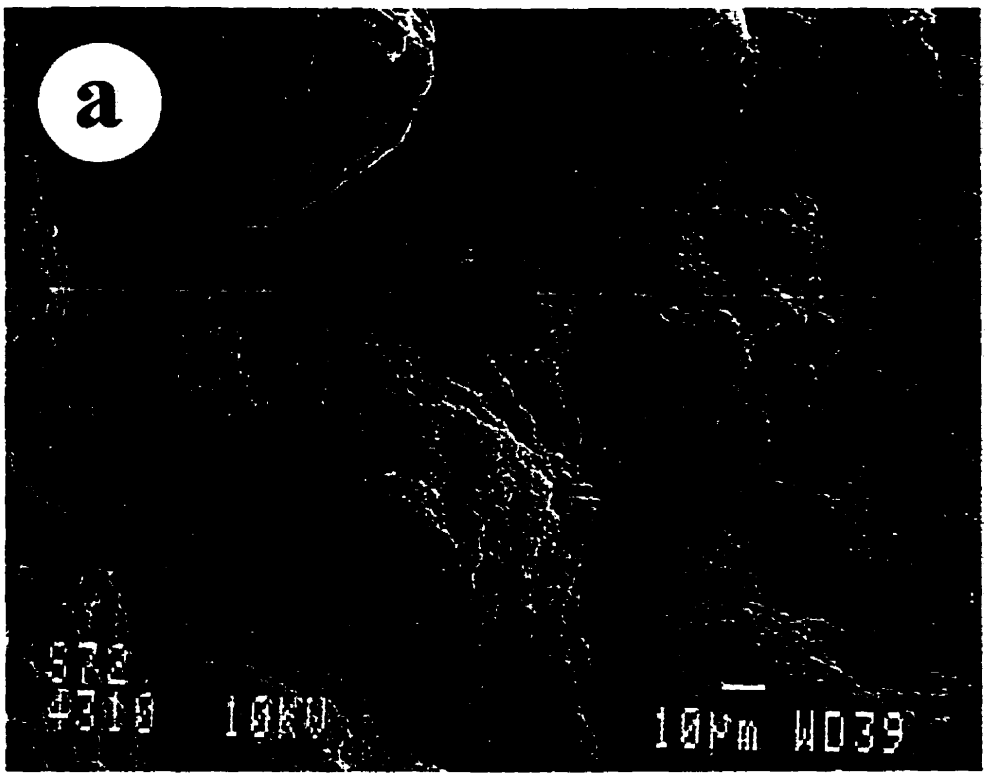

b
T48
4310 10KV
10μm WD39

Figure 4.23 Scanning electron micrographs of the ground potato peel, showing the growth of *B. subtilis* ATCC 21556 with surfactin after 48 hours of fermentation at x3500 magnification.


a
S48
4310 10KV
10μm WD39


b
S48
4310 10KV
10μm WD39

Figure 4.24 Scanning electron micrographs of the ground potato peel, showing the growth of *B. subtilis* ATCC 21556 with surfactin after 72 hours of fermentation at x500 magnification (a) and x3500 magnification (b).


a
10KV
10μm WD39

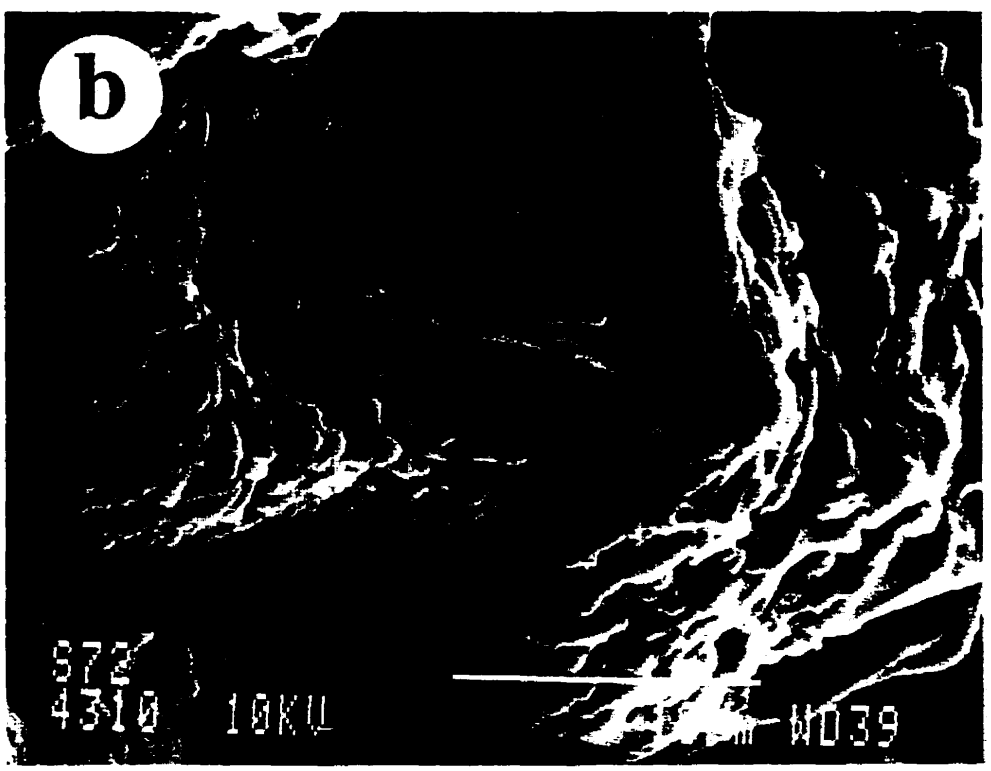
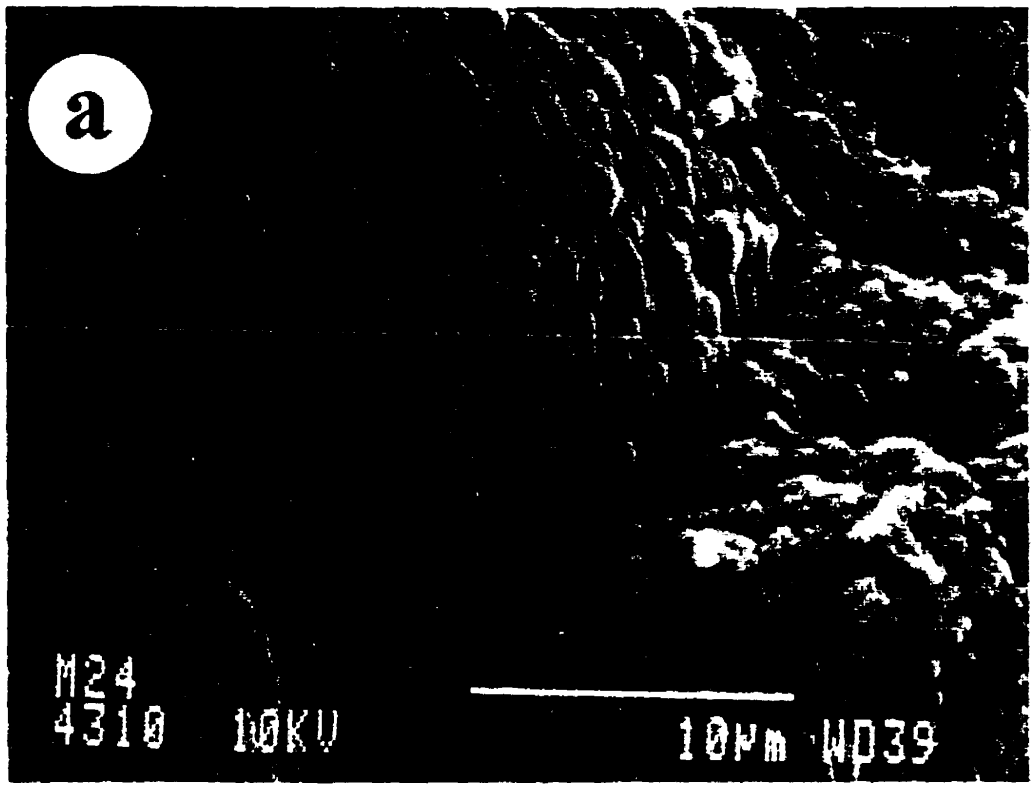

b
10KV
WD39

Figure 4.25 Scanning electron micrographs of the ground potato peel, showing the growth of a mixed culture of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 after 24 hours (a) and 48 hours (b) of fermentation, both at x3500 magnification.


a
M24
4310 10KV
10µm WD39

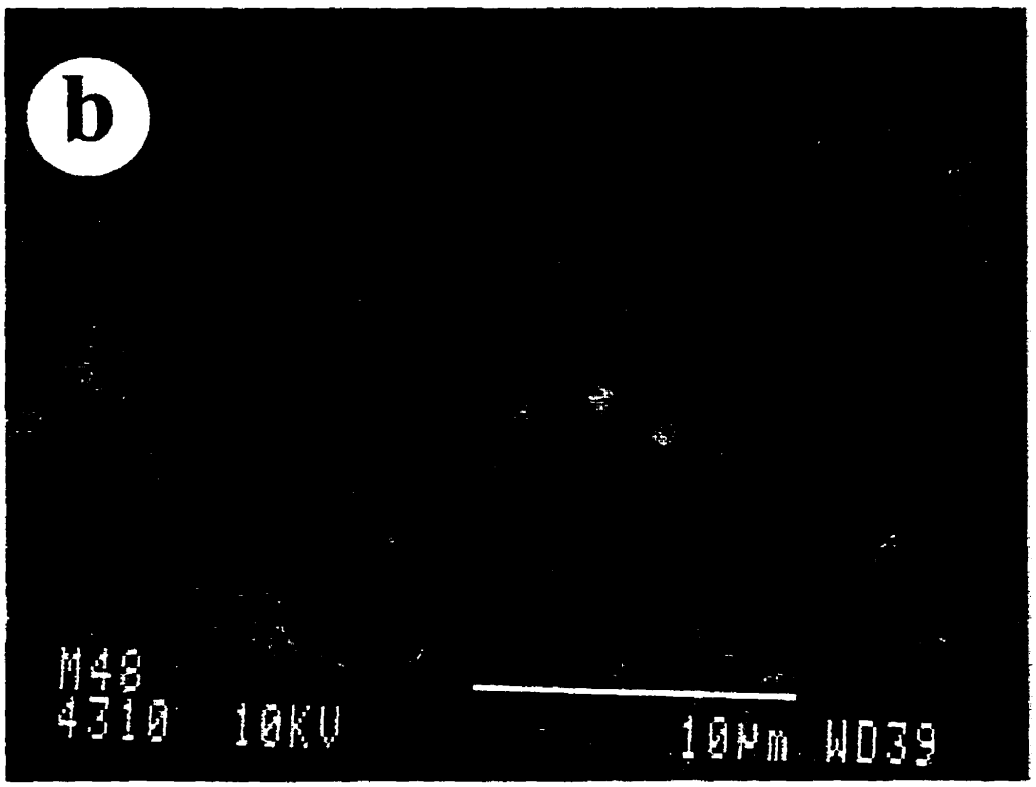
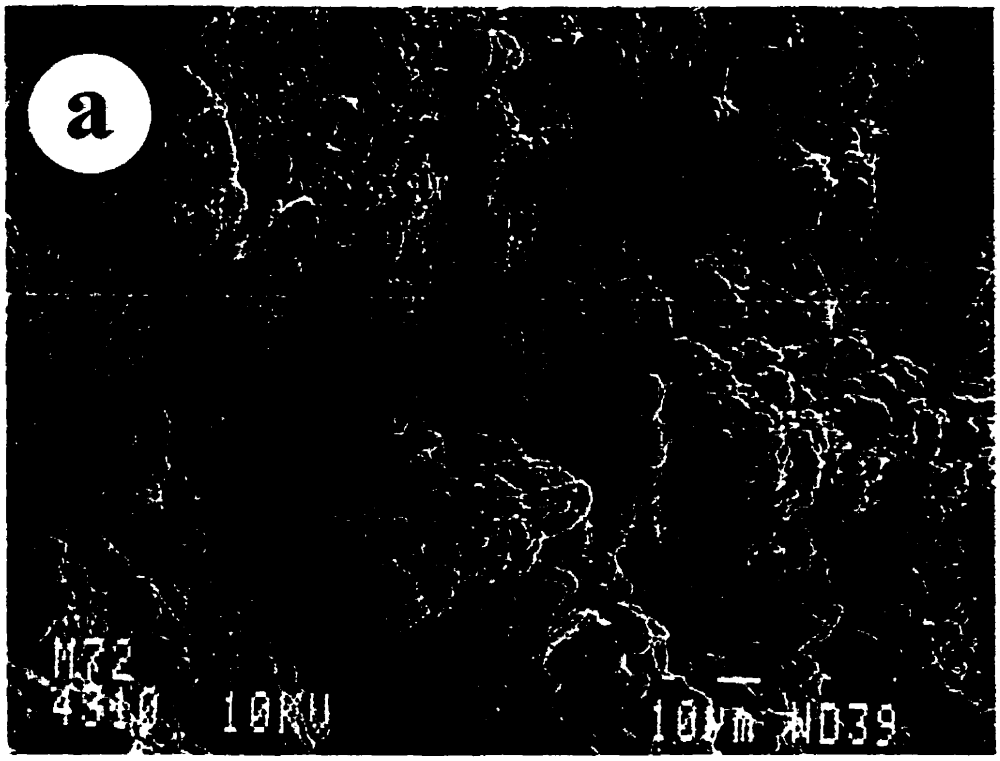
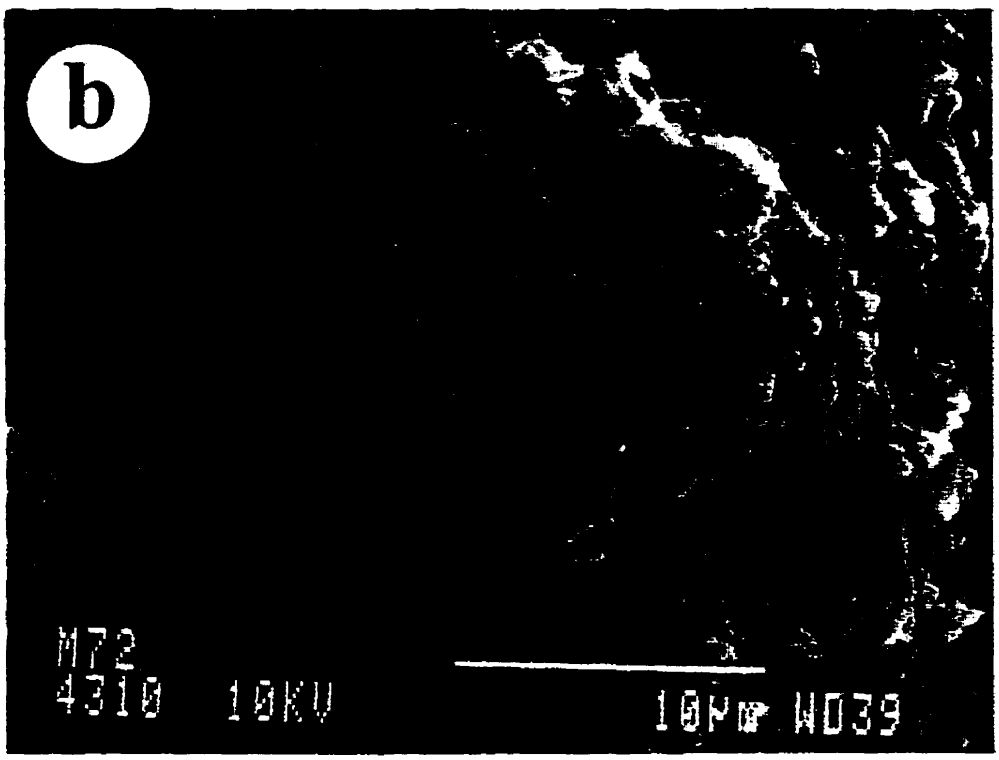

b
M48
4310 10KV
10µm WD39

Figure 4.26 Scanning electron micrographs of ground potato peel, showing the growth of a mixed culture of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 after 72 hours of fermentation at x500 magnification (a) and x3500 magnification (b).


a
M72
4310 10KV
10μm WD39


b
M72
4310 10KV
10μm WD39

granules, to compare with controls that had not been inoculated and, finally, to have micrographs taken from two other conditions: the growth of a pure culture of *B. subtilis* ATCC 21332 and the addition of Tween 80 to an SSF with a mixed bacterial culture.

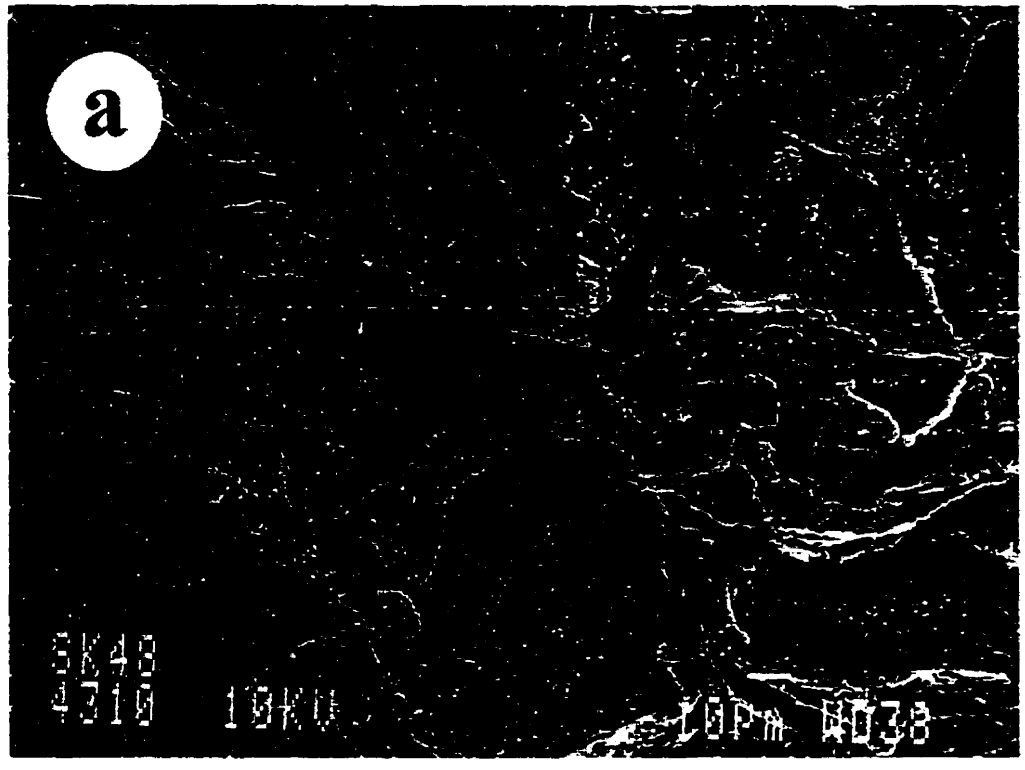
4.5.3.2 SEM of the controls

Micrographs of the controls (not inoculated) are shown in Figures 4.27 and 4.28. Figure 4.27 shows the surface of a potato skin and the surface of the cut potato slice. The skin seems to be very irregular, with cracks. The small white dots are likely to be artefacts from the SEM preparation, for example during the placement of the samples in the thermanox slide, or ice crystals from the freeze drying. The surface of the potato slice, by contrast, is smooth and regular and the cells are more compact with small spaces between them. This pattern could be noticed all over the surface of the sample. Figure 4.28 shows the same control with addition of Tween 80 and surfactin on the surface of the potato slices. The control with Tween 80 reveals some cell degradation and larger spaces between the cells. This degradation of the structure of the cells is even more evident on the micrograph of the control with surfactin (4.28 (b)).

4.5.3.3 SEM of sliced potatoes

A slice of potato fermented by *B. subtilis* ATCC 21556 is presented in Figure 4.29. The micrograph on the top (a) shows a longitudinal cut. On the left hand side of the picture, is the view of the surface and the other half is the cut down through the slice, revealing that there was no bacterial penetration. The growth is evident only on the surface of the samples.

Figure 4.27 Scanning electron micrographs of the surface of a potato skin (a) and of the potato slice (b) at x500 magnification without inoculation of bacteria.


a
5K48
4310 10KV
10Pm WD38

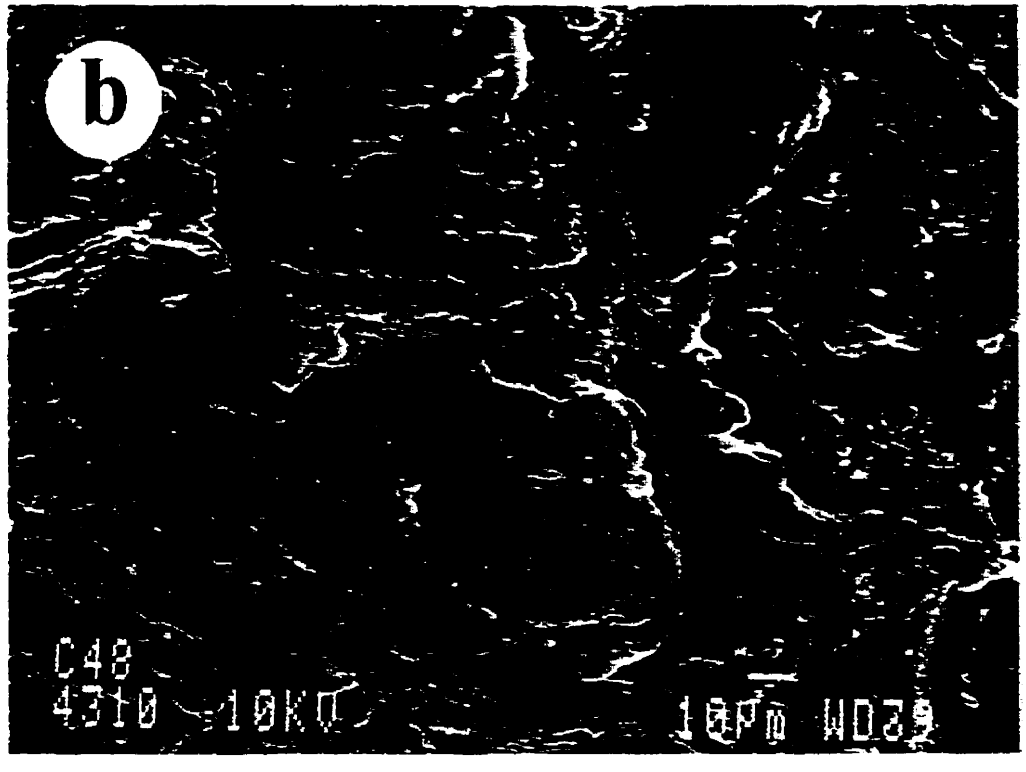
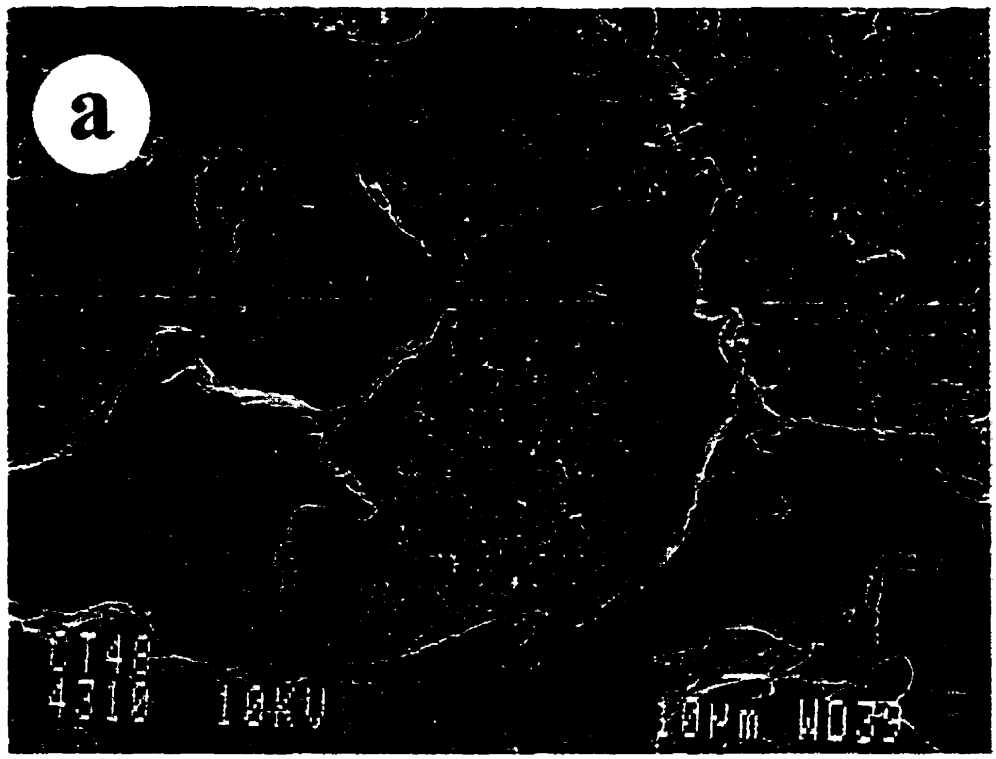
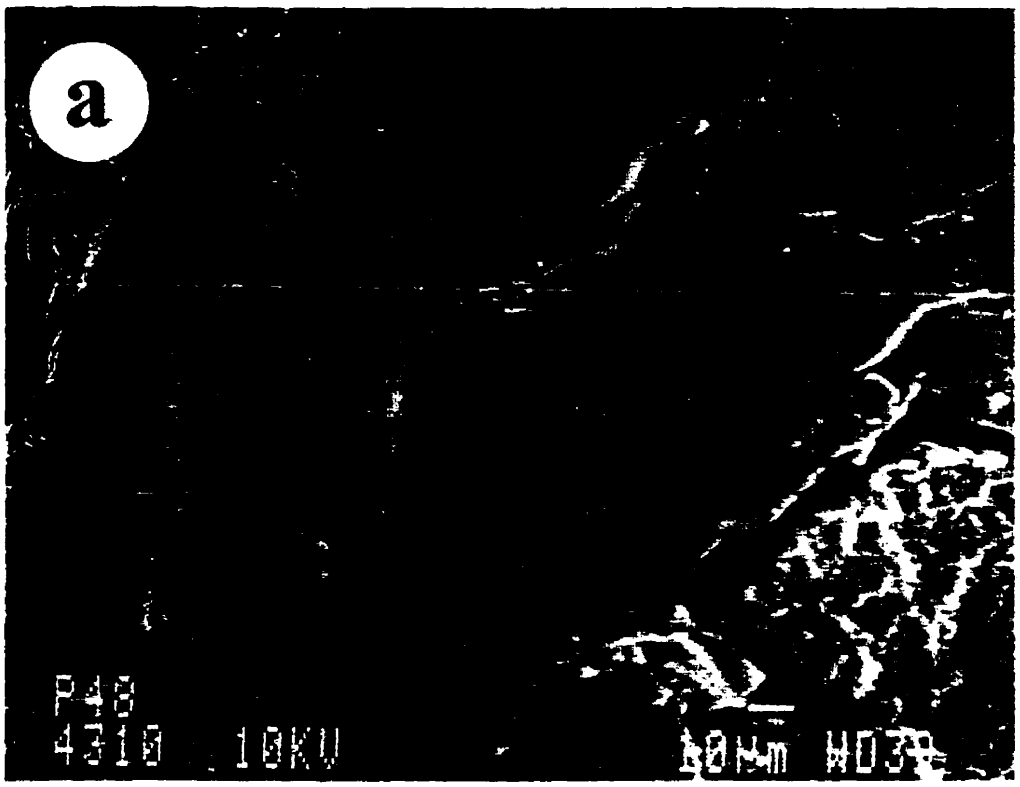

b
C48
4310 10KV
10Pm WD39

Figure 4.28 Scanning electron micrographs of the surface of potato slices with addition of Tween 80 (a) and surfactin (b) at x500 magnification without inoculation of bacteria.


a
4310 10KV
10Pm W039


b
4310 10KV
10Pm W039

Figure 4.29 Scanning electron micrographs of the surface of potato slices inoculated with *B. subtilis* ATCC 21556 after 48 hours of fermentation showing: (a) vertical cut with top of substrate on the left hand side of the picture at x500 magnification and (b) top view at x1000 magnification.


a
P48
4310 10KV
10μm WD39

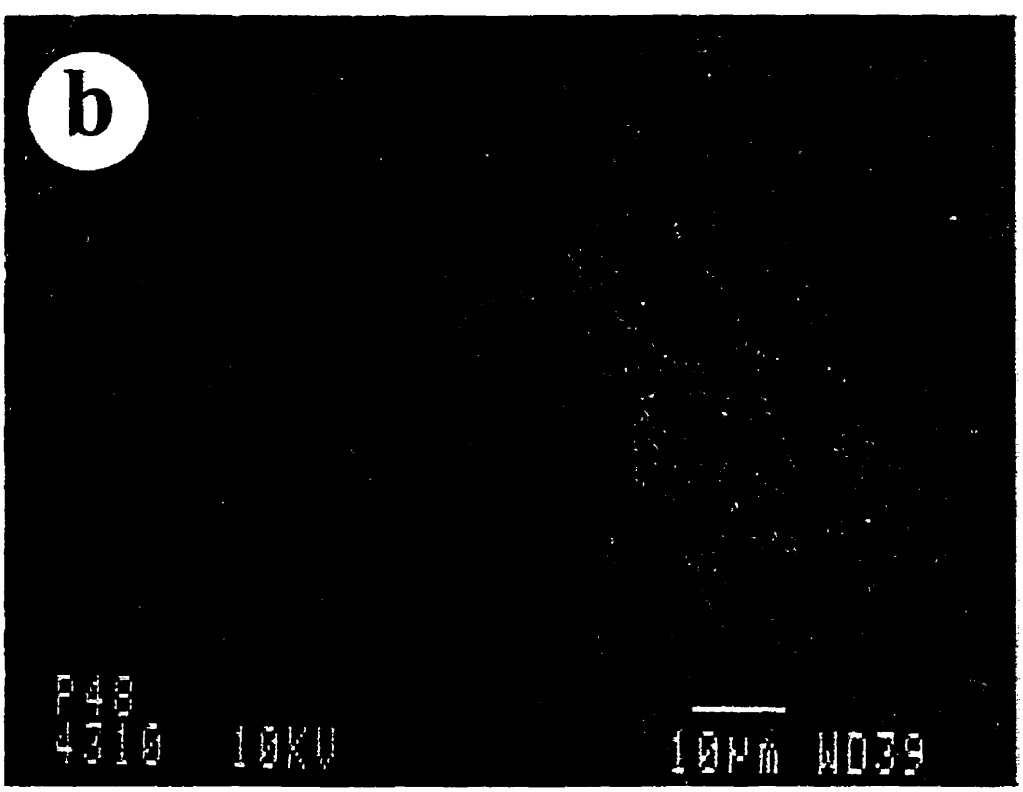

b
P48
4310 10KV
10μm WD39

Figure 4.30 shows micrographs taken of the potato slices inoculated with *B. subtilis* ATCC 21332. The pictures reveal extensive biofilm formation after both 48 and 72 hours. The bacteria are apparently attached to each other and embedded in the substrate underneath the biofilm. The same phenomenon is revealed in Figure 4.31, which shows a longitudinal cut of the potato inoculated with *B. subtilis* ATCC 21556 plus Tween 80, as well as the surface view. Figure 4.32 shows two more micrographs of the same two conditions, illustrating the very dense cell packing on the surface after 48 hours of fermentation.

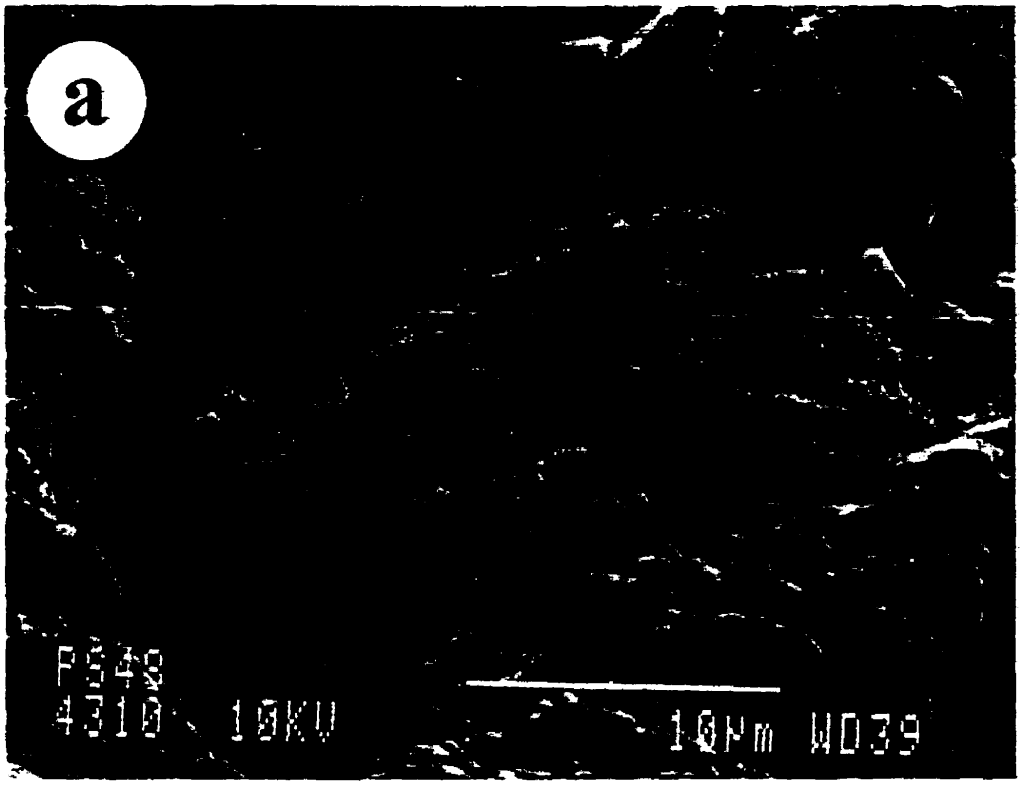
The addition of surfactin to the potato inoculated with *B. subtilis* ATCC 21556, also resulted in significant biofilm formation and the micrographs reveal how the bacteria grow under this biofilm (Figure 4.33 (b)). Seen here, are the surface views that also show degradation of the structure similar to the control with surfactant addition.

Figures 4.34 and 4.35 shown the growth of the mixed culture in the absence and presence of Tween 80 respectively. In Figure 4.34 the micrographs are of samples taken at 24 and 48 hours of fermentation. At 24 hours, the bacterial growth covers the starch granules and again here, the bacteria grew very close together, although at x500 magnification the biofilm is not clearly visible.

On the other hand, with the presence of Tween 80 on the sample in Figure 4.35 the biofilm is spread and shown to be over the entire surface, embedding the bacteria. The side view of this sample (a) demonstrates once more that there was no bacterial penetration and growth was limited to the surface.

The phenomenon of biofilm formation was observed by SEM on the samples that were associated with the presence of surfactants, such as Tween 80 and the addition or

Figure 4.30 Scanning electron micrographs of the surfaces of potato slices inoculated with *B. subtilis* ATCC 21332 after 48 hours (a) and 72 hours (b) of fermentation, both at x3500 magnification.


a
P548
4310 10KV
10Nm WD39

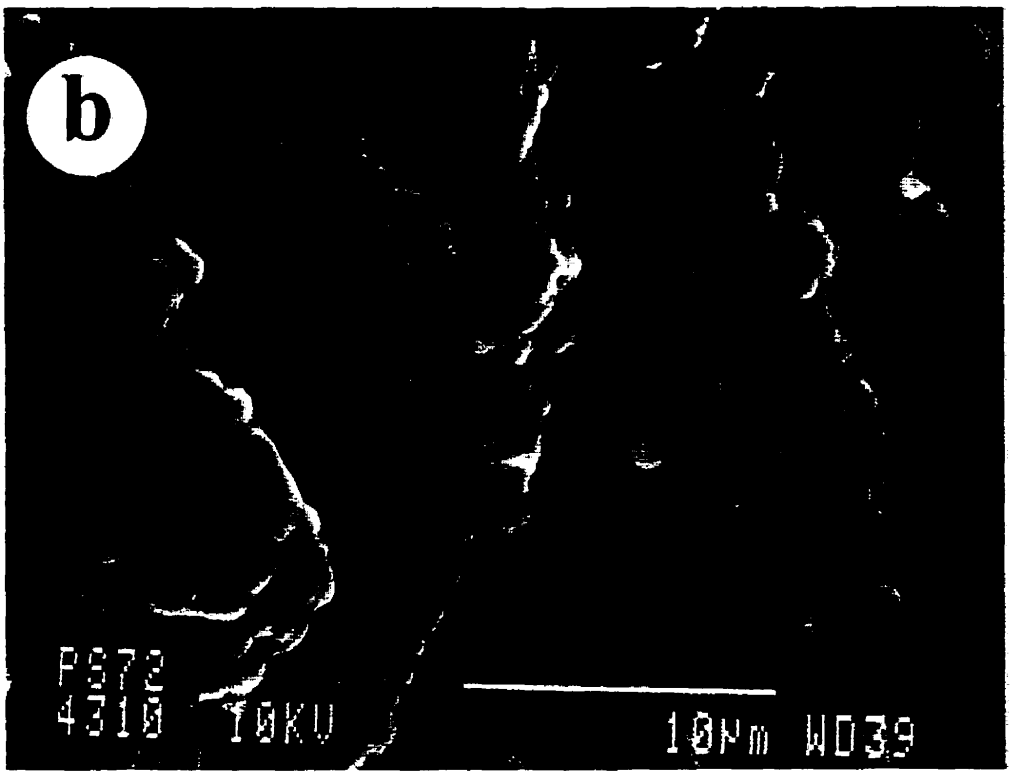
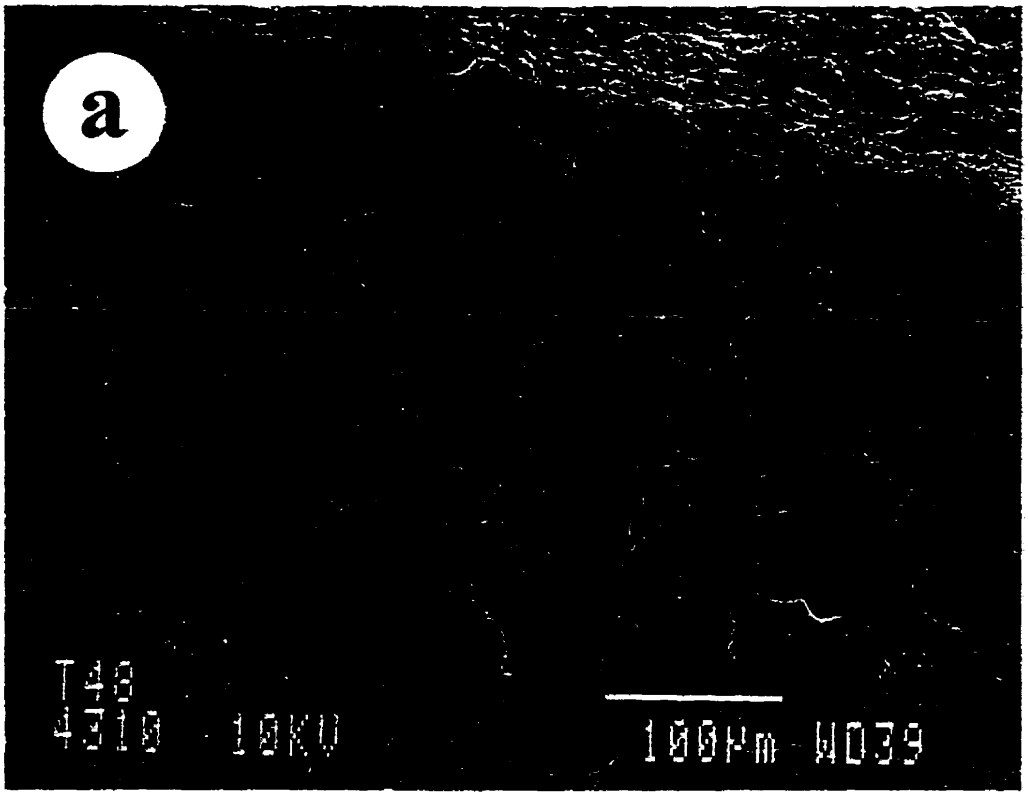

b
P572
4310 10KV
10Nm WD39

Figure 4.31 Scanning electron micrographs of the surfaces of potato slices inoculated with *B. subtilis* ATCC 21556 plus Tween 80 after 48 hours of fermentation showing: (a) side view at x200 magnification and (b) top view at x3500 magnification.


a
T48
4310 10KV
100Pm W039

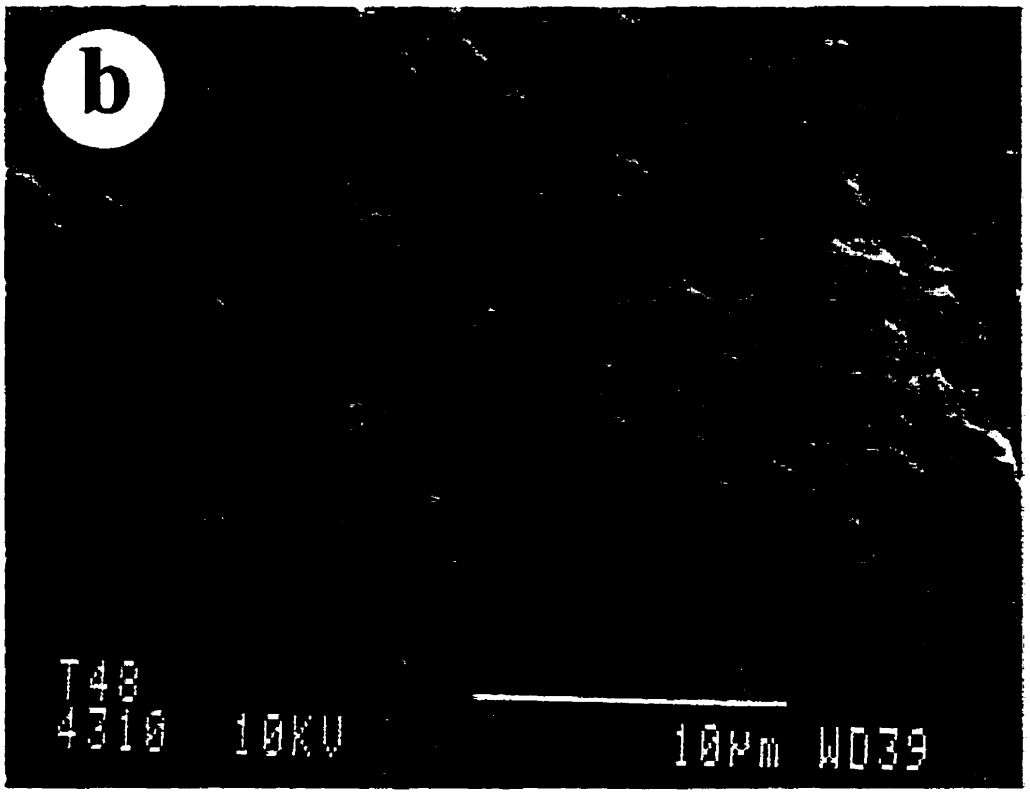
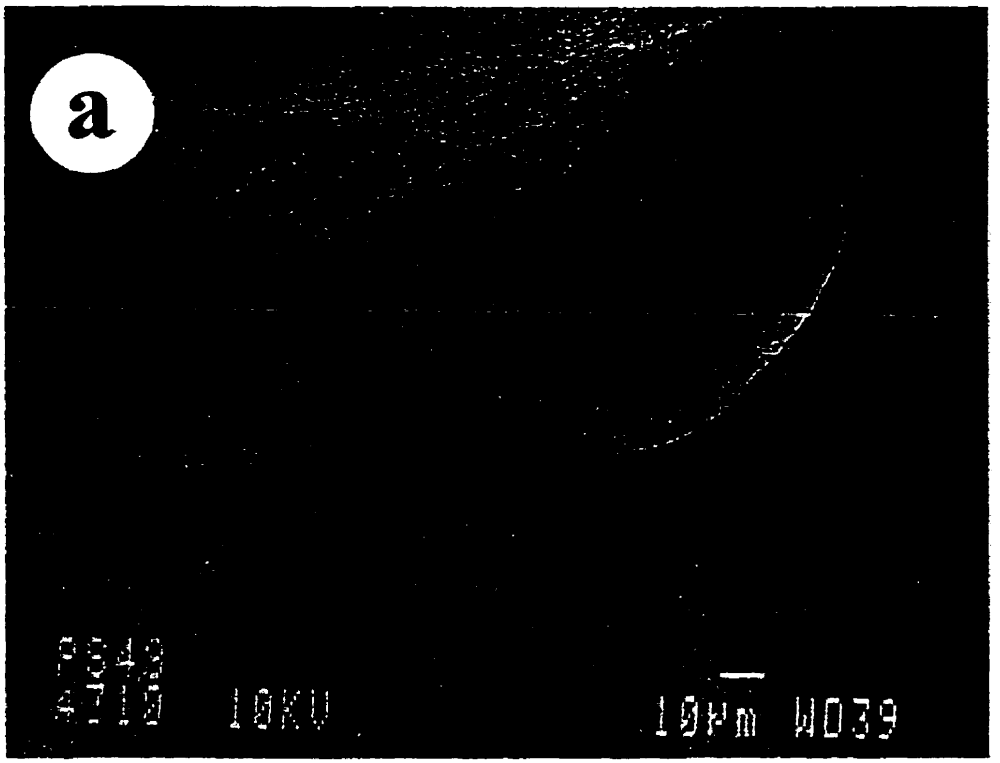

b
T48
4310 10KV
10Pm W039

Figure 4.32 Scanning electron micrographs of the surfaces potato slices inoculated with *B. subtilis* ATCC 21332 (a) and *B. subtilis* ATCC 21556 with Tween 80 (b) after 48 hours of fermentation, both at x500 magnification.


a
10KV
10μm WD39

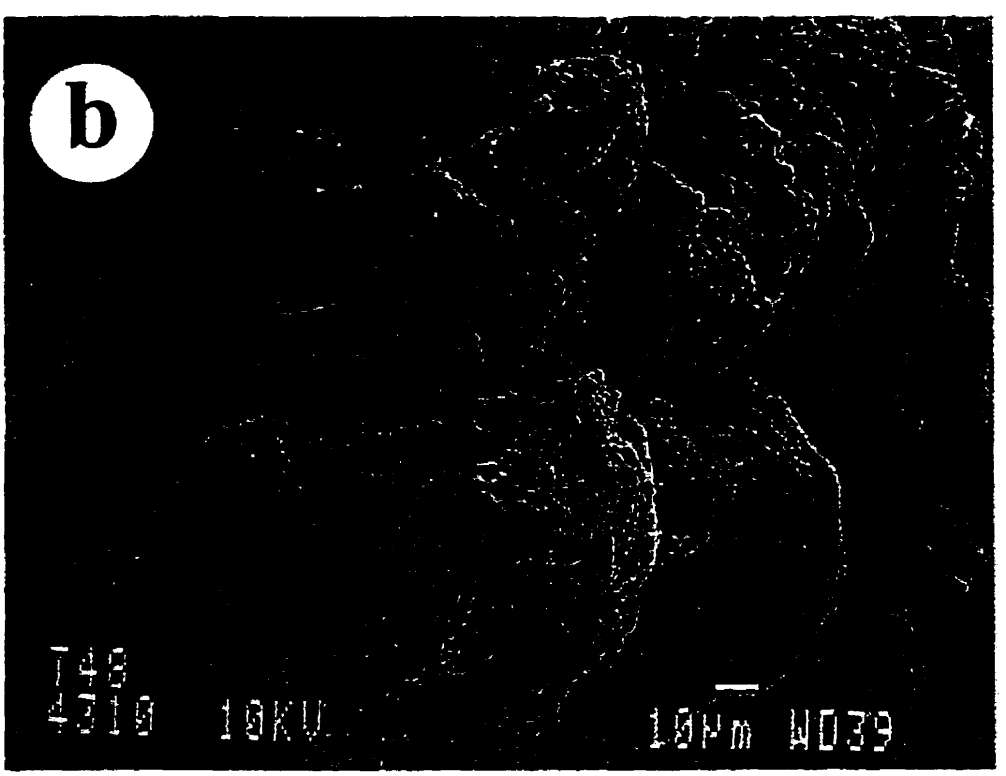
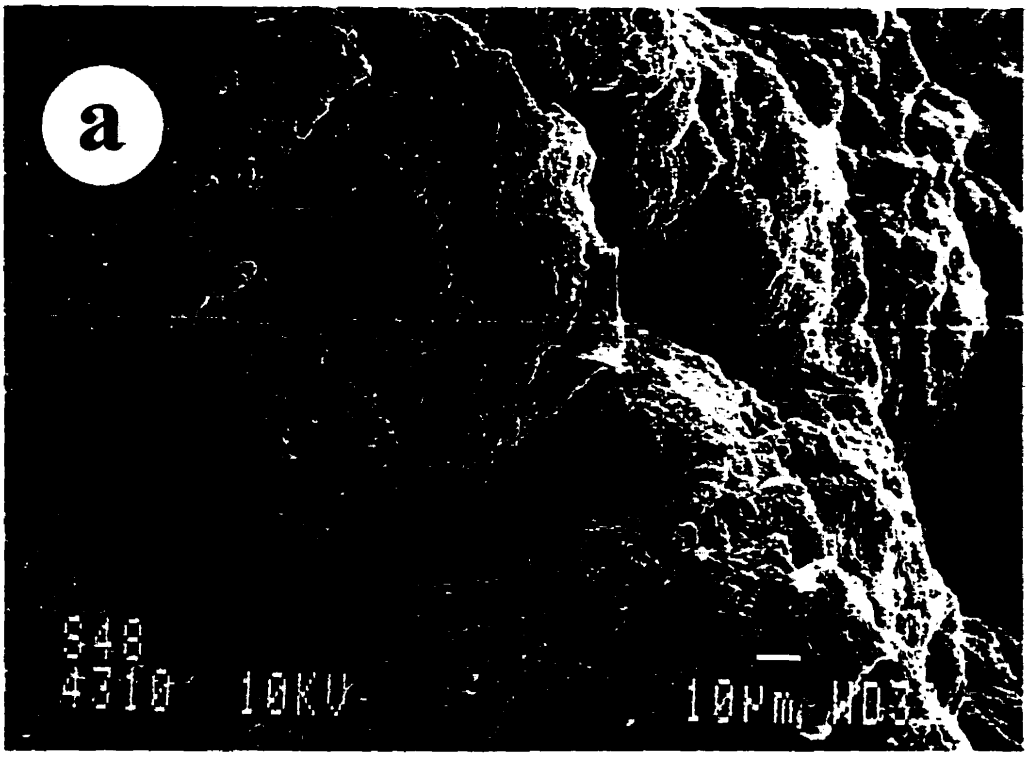

b
10KV
10μm WD39

Figure 4.33 Scanning electron micrographs of the surface of potato slices inoculated with *B. subtilis* ATCC 21556 plus surfactin after 48 hours of fermentation at x500 magnification (a) and x3500 magnification (b).


a
S48
4310 10KV
10μm WD3

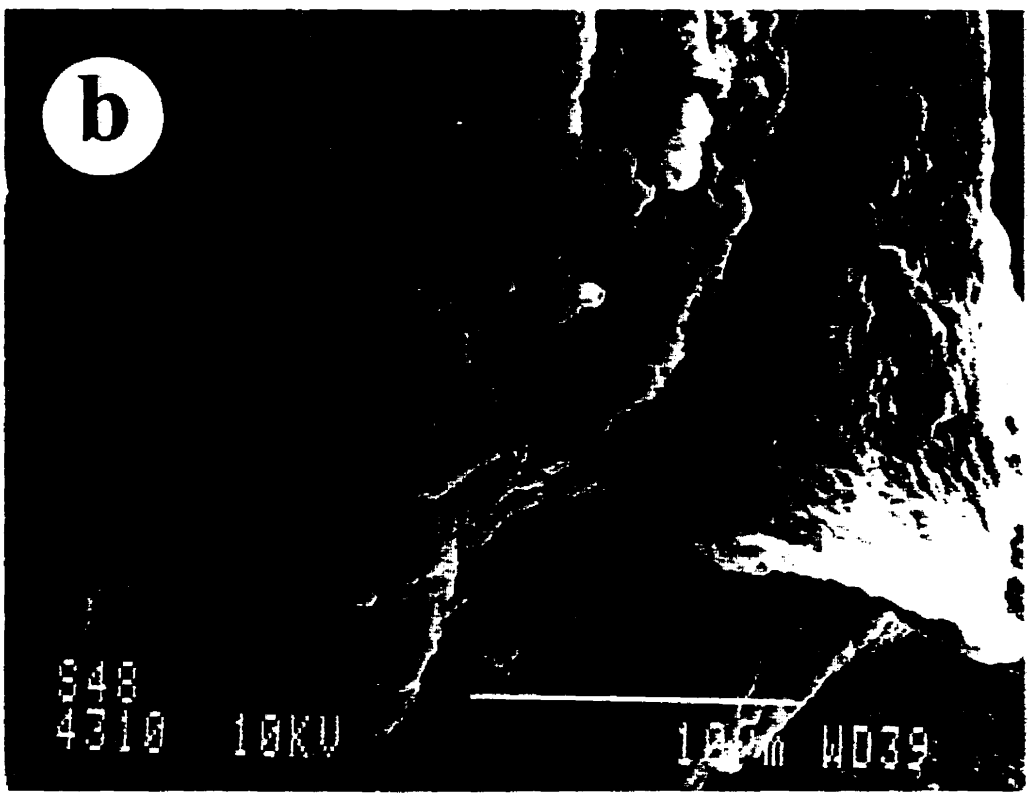
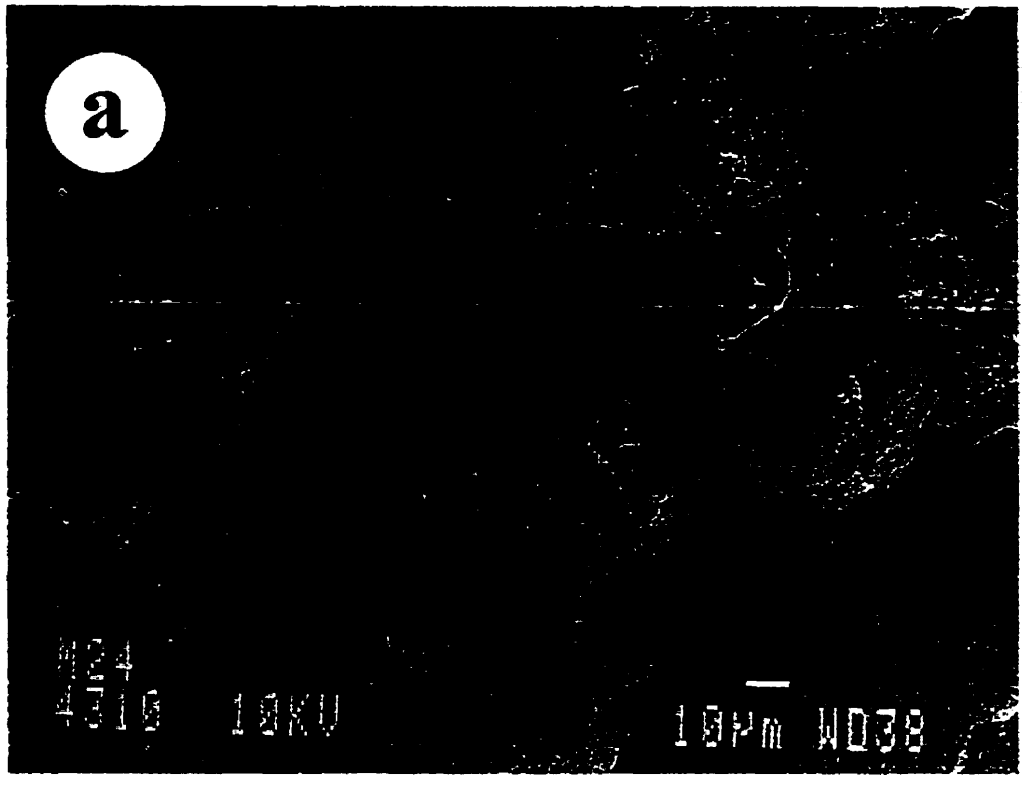

b
S48
4310 10KV
10μm WD39

Figure 4.34 Scanning electron micrographs of the surface of potato slices inoculated with a mixed culture of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 after 24 hours (a) and 48 hours (b) of fermentation, both at x500 magnification.


a
4310 10KV
10μm WD38

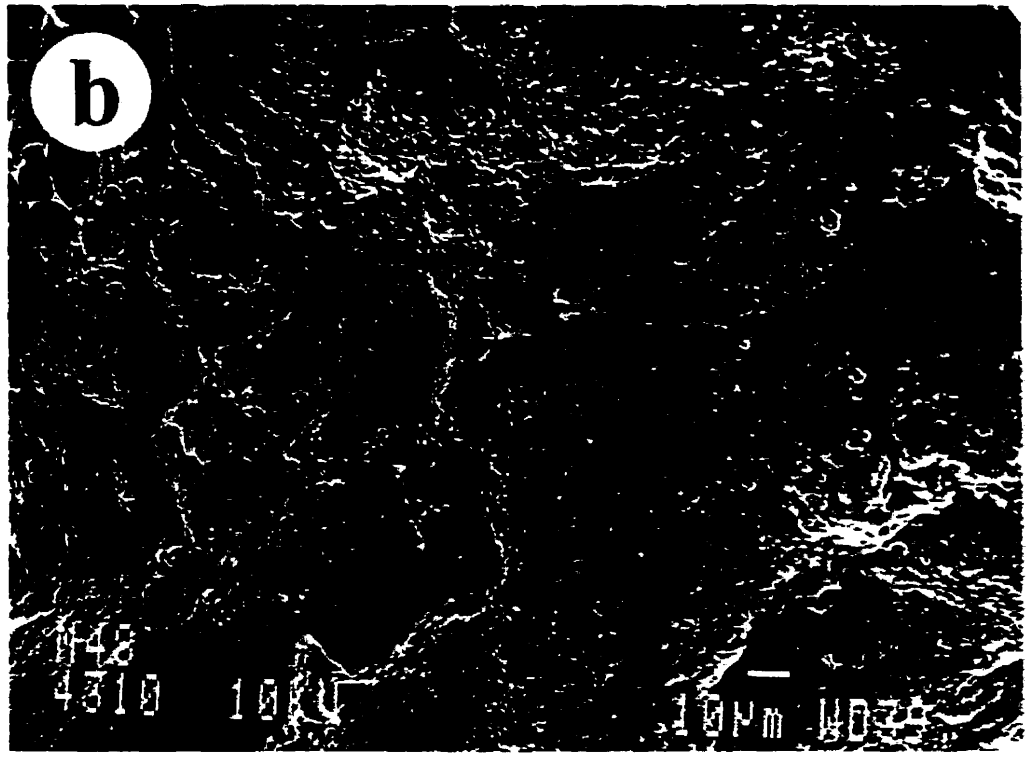
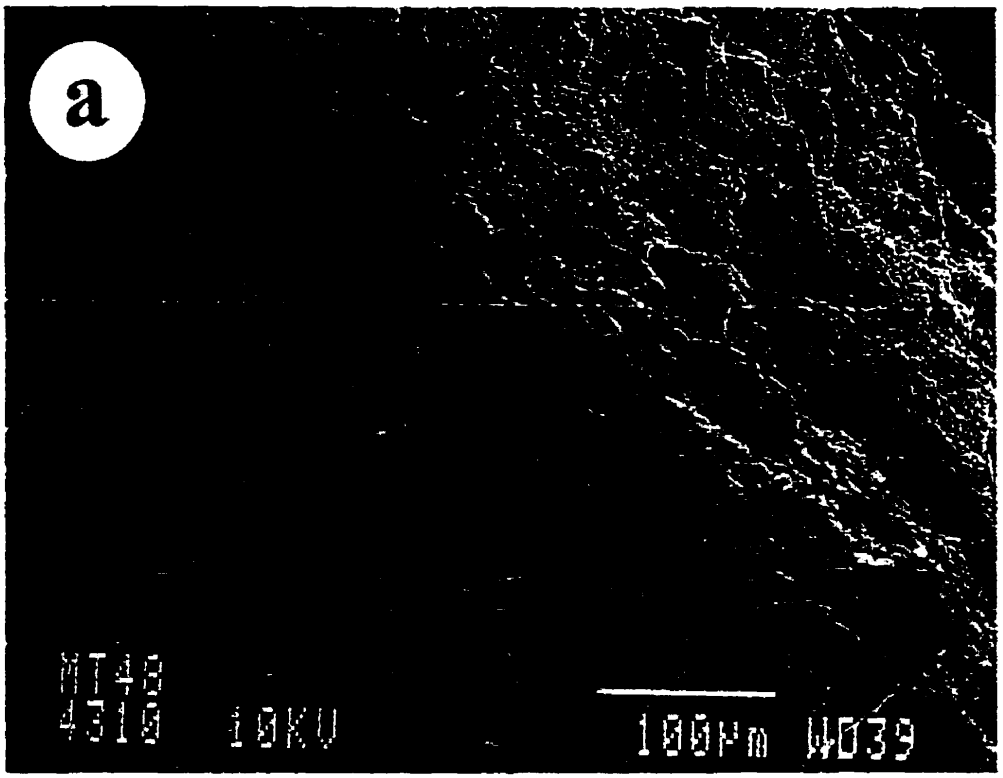
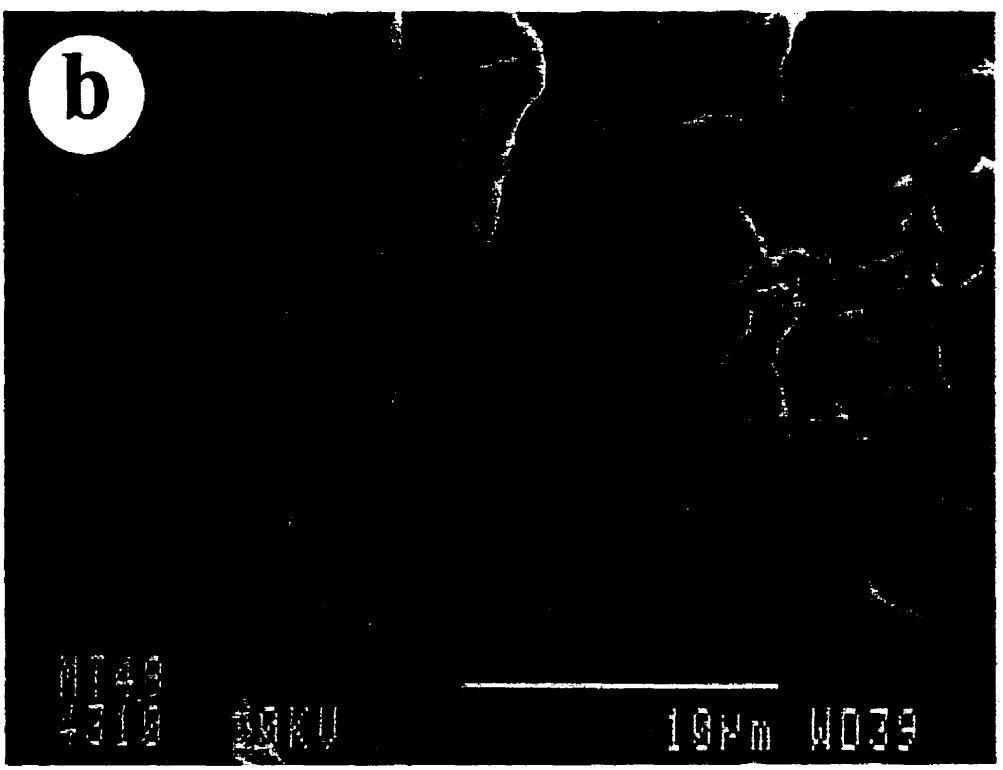

b
4310 10KV
10μm WD38

Figure 4.35 Scanning electron micrographs of potato slices with a mixed culture of *B. subtilis* ATCC 21556 and *B. subtilis* ATCC 21332 and Tween 80 after 48 hours of fermentation showing:

(a) side view at x200 magnification and

(b) top view, at x3500 magnification.


a
MT48
4318
10KU
100Pm W039


b
MT48
4318
10KU
10Pm W039

production of surfactin. Thus, there is an apparent correlation between biofilm formation and the SSF conditions in which the enzyme activity reached high levels.

The mechanism of how the surfactants increased enzyme production could be related to the formation of this biofilm. In the next results to be shown, the interactions between starch, enzyme, water and surfactant in the absence of microbial cells will be discussed.

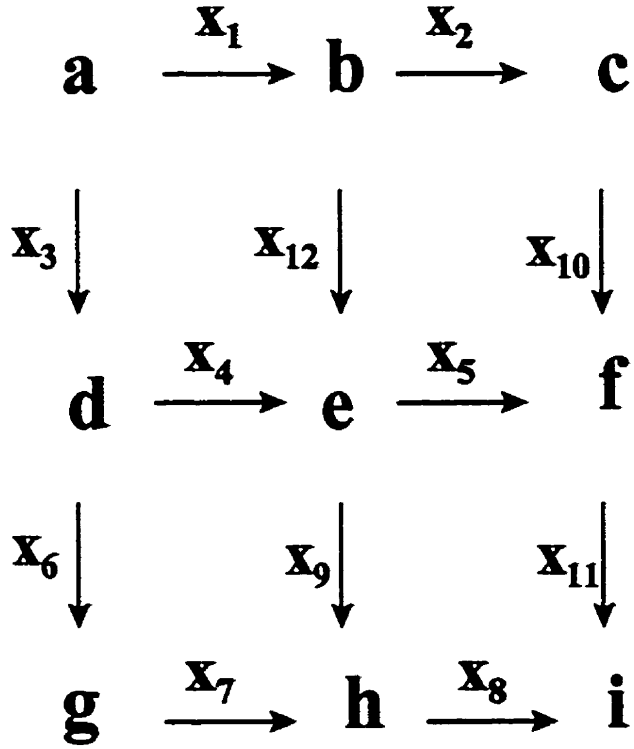
4.5.4 Starch film formation

The procedure for this experiment was described in Section 3.3.4. The analyses of these experiments were done by using matrices to evaluate pictures taken of the starch films which were observed at x300 magnification with a phase contrast light microscope. The films were produced using different conditions to study the interactions between starch/enzyme, starch/surfactant, enzyme/surfactant, enzyme/water, surfactant/water, starch/water at 100°C and at 30°C, with 1.0% and 10% starch solutions.

The matrixes were constructed in a 3x3 format and the study was done by comparing the variations in two parameters at a time and assigning points to relative differences that were observed. There were twelve possible comparisons per matrix. Table 4.4 describes an example of the composition of a matrix by showing how the amount of surfactant and enzyme increase on horizontal and vertical lines respectively with, a constant concentration of starch. Figure 4.36 shows the diagram of a matrix as well as the how the grade points were distributed (from x_1 to x_{12}). The matrices were analyzed by comparing two pictures side by side on the matrix in either a horizontal or vertical direction. For example, x_1

Figure 4.36 Diagram of a 3x3 matrix with the nine pictures (a to i)

showing the grade point distribution (x_1 to x_{12}).


a
b
c
d
e
f
g
h
i
x1
x2
x3
x12
x10
x4
x5
x6
x9
x11
x7
x8

represents the points given as a result of the comparison between picture (a) and picture (b). The grades and values given are shown in Table 4.5. A complete list with the description of the conditions used to produce individual films (a-i) for each matrix is presented in Tables 4.6 and 4.7.

Table 4.4 - Example of the elements contained in a 3x3 matrix

(a)	1.0% Starch (control)	(b)	1.0 % Starch 1.0% Tween 80	(c)	1.0% Starch 2.0% Tween 80
(d)	1.0% Starch 10 µL Amylase	(e)	1.0% Starch 1.0% Tween 80 10 µL Amylase	(f)	1.0% Starch 2.0% Tween 80 10 µL Amylase
(g)	1.0% Starch 20 µL Amylase	(h)	1.0% Starch 1.0% Tween 80 20 µL Amylase	(i)	1.0% Starch 2.0% Tween 80 20 µL Amylase

Table 4.5 Grade points according to the degree of difference between the observed films.

	Grade Point
No difference	0
Slight difference	1
Significant difference	2
Large difference	3

Table 4.6 Individual films (a-i) from Figures 4.37 to 4.40

	Figure 4.37 (with heating)	Figure 4.38 (at 30°C)	Figure 4.39 (with heating)	Figure 4.40 (at 30°C)
a	1.0% starch	1.0% starch	10% starch	10% starch
b	1.0% starch 1.0% Tween 80	1.0% starch 1.0% Tween 80	10% starch 1.0% Tween 80	10% starch 1.0% Tween 80
c	1.0% starch 2.0% Tween 80	1.0% starch 2.0% Tween 80	10% starch 2.0% Tween 80	10% starch 2.0% Tween 80
d	1.0% starch 10 μL amylase	1.0% starch 10 μL amylase	10% starch 10 μL amylase	10% starch 10 μL amylase
e	1.0% starch 1.0% Tween 80 10 μL amylase	1.0% starch 1.0% Tween 80 10 μL amylase	10% starch 1.0% Tween 80 10 μL amylase	10% starch 1.0% Tween 80 10 μL amylase
f	1.0% starch 2.0% Tween 80 10 μL amylase	1.0% starch 2.0% Tween 80 10 μL amylase	10% starch 2.0% Tween 80 10 μL amylase	10% starch 2.0% Tween 80 10 μL amylase
g	1.0% starch 20 μL amylase	1.0% starch 20 μL amylase	10% starch 20 μL amylase	10% starch 20 μL amylase
h	1.0% starch 1.0% Tween 80 20 μL amylase	1.0% starch 1.0% Tween 80 20 μL amylase	10% starch 1.0% Tween 80 20 μL amylase	10% starch 1.0% Tween 80 20 μL amylase
i	1.0% starch 2.0% Tween 80 20 μL amylase	1.0% starch 2.0% Tween 80 20 μL amylase	10% starch 2.0% Tween 80 20 μL amylase	10% starch 2.0% Tween 80 20 μL amylase

Table 4.7 Individual films from Figures 4.41 to 4.44

	Figure 4.41 (with heating)	Figure 4.42 (at 30°C)	Figure 4.43 (with heating)	Figure 4.44 (at 30°C)
a	1.0% starch	1.0% starch	10% starch	10% starch
b	1.0% starch 10% surfactin	1.0% starch 10% surfactin	10% starch 10% surfactin	10% starch 10% surfactin
c	1.0% starch 20% surfactin	1.0% starch 20% surfactin	10% starch 20% surfactin	10% starch 20% surfactin
d	1.0% starch 10 μL amylase	1.0% starch 10 μL amylase	10% starch 10 μL amylase	10% starch 10 μL amylase
e	1.0% starch 10% surfactin 10 μL amylase	1.0% starch 10% surfactin 10 μL amylase	10% starch 10% surfactin 10 μL amylase	10% starch 10% surfactin 10 μL amylase
f	1.0% starch 20% surfactin 10 μL amylase	1.0% starch 20% surfactin 10 μL amylase	10% starch 20% surfactin 10 μL amylase	10% starch 20% surfactin 10 μL amylase
g	1.0% starch 20 μL amylase	1.0% starch 20 μL amylase	10% starch 20 μL amylase	10% starch 20 μL amylase
h	1.0% starch 10% surfactin 20 μL amylase	1.0% starch 10% surfactin 20 μL amylase	10% starch 10% surfactin 20 μL amylase	10% starch 10% surfactin 20 μL amylase
i	1.0% starch 20% surfactin 20 μL amylase	1.0% starch 20% surfactin 20 μL amylase	10% starch 20% surfactin 20 μL amylase	10% starch 20% surfactin 20 μL amylase

Figures 4.37 to 4.40 show the films produced with 1.0 and 2.0% (v/v) Tween 80 added to the starch solutions. The effect of heating and no heating with 1.0% starch is illustrated in Figures 4.37 and 4.38. Figures 4.39 and 4.40 show the effect of 10% starch with heating and no heating. Table 4.8 shows an example of how the grade points were attributed on the matrix with 1.0% starch, Tween 80 with heating and no heating (Figures 4.37 and 4.38) to study the different interactions. A summary of grade points for each of the other matrices are given in Appendix 2. The interaction between starch/surfactant/water is represented by the sum of the grade points x_1, x_2, x_4, x_5, x_7 and x_8 and the interaction between starch/enzyme/water is represented by the sum of x_3, x_6, x_9, x_{10}, x_{11} and x_{12}.

Table 4.8 Scores attributed to the interactions (x_1 to x_{12}) on the matrix with 1.0% starch, Tween 80 and heating.

Starch/surfactant/water			Starch/enzyme/water		
	Heating	30°C		Heating	30°C
x_1	3	2	x_3	2	2
x_2	1	2	x_6	1	1
x_4	3	3	x_9	1	2
x_5	2	1	x_{10}	2	1
x_7	3	3	x_{11}	3	1
x_8	2	1	x_{12}	2	2
Total	**14**	**12**	**Total**	**11**	**9**

Figure 4.37 Matrix showing films produced from 1.0% starch solution and Tween 80 with heating. Concentration of Tween 80 increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no Tween 80 and no amylase (bar = 100 μm).

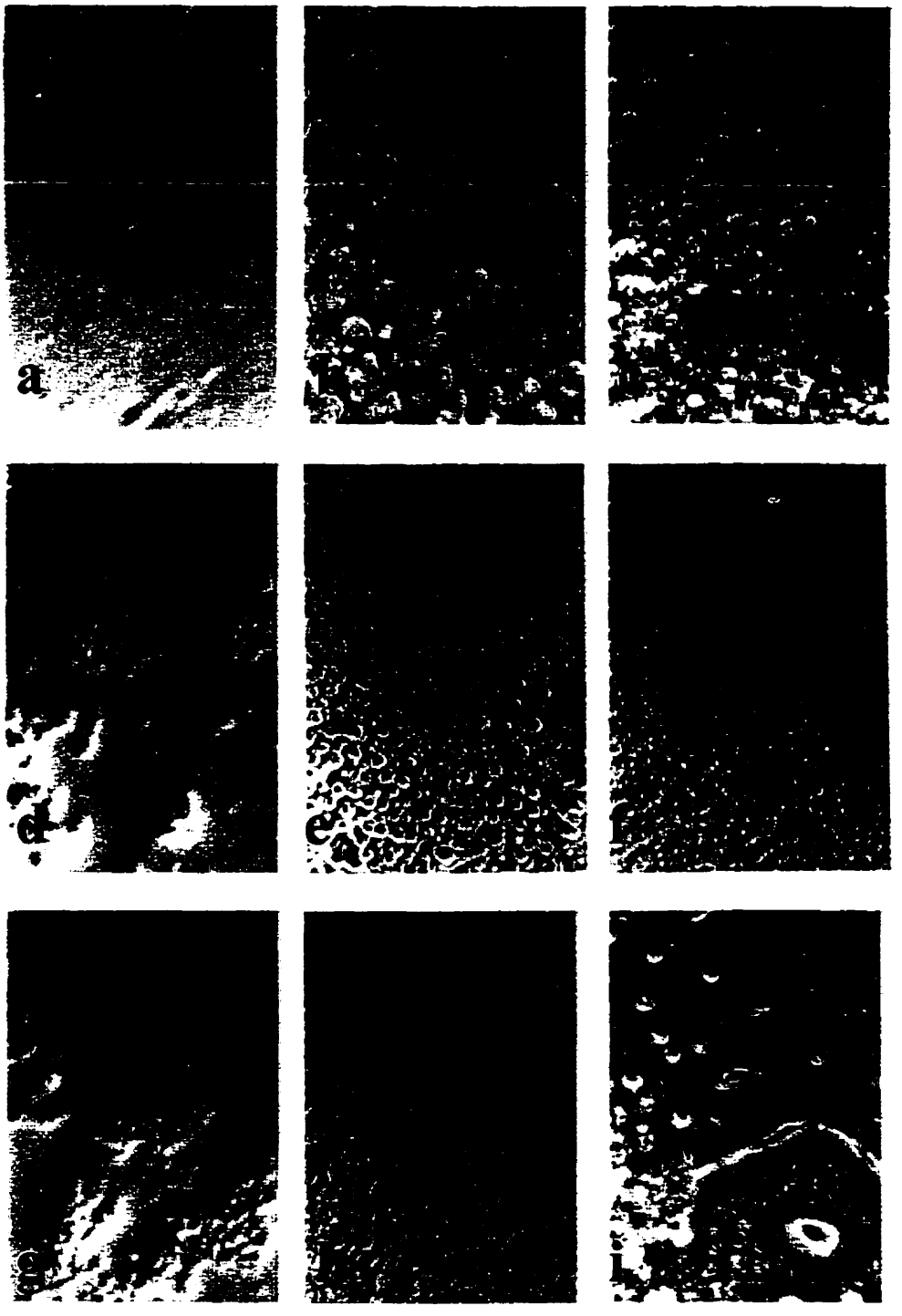

a
d
e

Figure 4.38 Matrix showing films produced from 1.0% starch solution and Tween 80 at 30°C. Concentration of Tween 80 increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no Tween 80 and no amylase (bar = 100 μm).

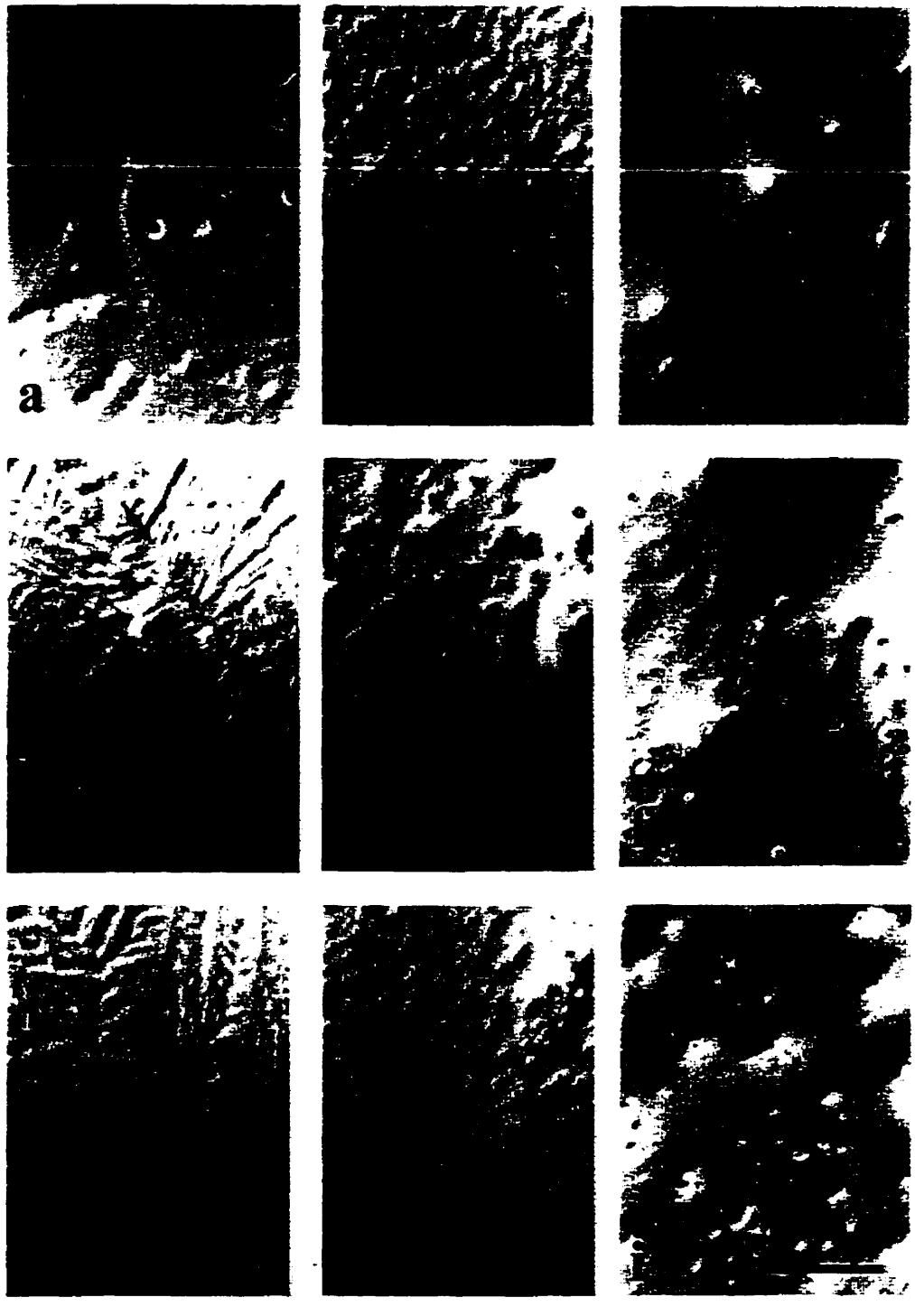

a

Figure 4.39 Matrix showing films produced from 10% starch solution and Tween 80 with heating. Concentration of Tween 80 increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no Tween 80 and no amylase (bar = 100 μm).

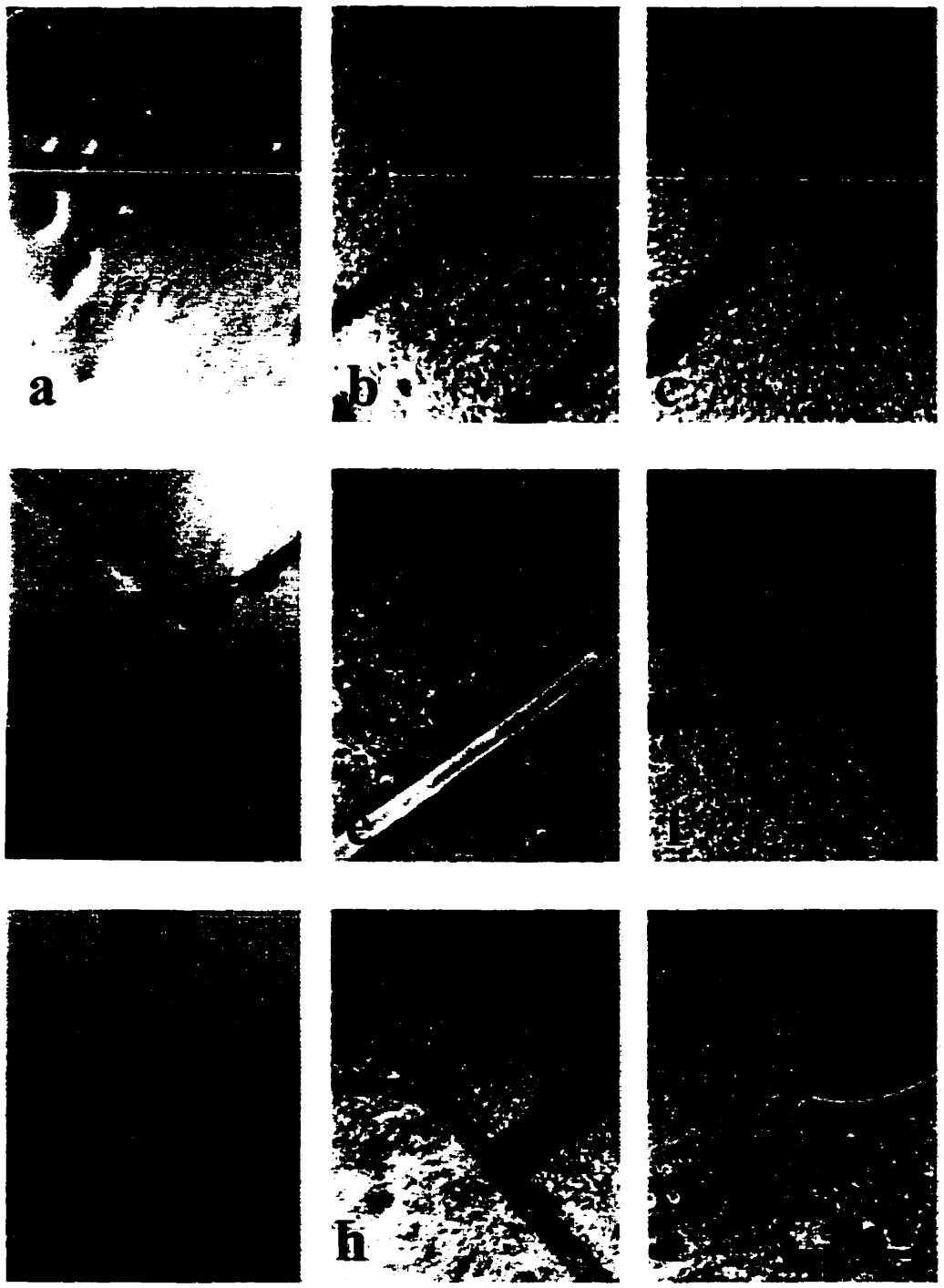

a
b
c
e
h

Figure 4.40 Matrix showing films produced from 10% starch solution and Tween 80 at 30°C. Concentration of Tween 80 increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no Tween 80 and no amylase (bar = 100 μm).

a
d
g

For this particular example, the final score obtained in the matrix from Figure 4.37 for the interaction between starch/surfactant/water was 14 and for the interaction between starch/enzyme/water the final score was 11. It should be noted that for each interaction the minimum score that can be obtained is zero and the maximum is 18. The total scores obtained from the sum of x_1 to x_{12} for the interactions on all the matrices with Tween 80 are shown in Table 4.9.

Table 4.9 Scores obtained from the matrices with Tween 80
by comparing their components

Interaction:	Starch/surfactant/water	Starch/enzyme/water
1.0% starch with heating	14	11
1.0% starch with no heating	12	9
10% starch with heating	12	6
10% starch with no heating	10	8
Totals	**48**	**34**

Table 4.10 shows an example of the individual scores given when the matrix from Figure 4.37 with 1.0% starch, Tween 80 and heating was compared to the matrix from Figure 4.39 with 10% starch, Tween 80 and heating and the matrix from Figure 4.38 with 1.0% starch, Tween 80 and no heating was compared to the matrix from Figure 4.40 with 10%

starch and no heating, to verify the effect of starch concentration on film formation with heating and no heating.

Table 4.10 Comparison between 1.0% starch and 10% starch with Tween 80

	Grade points	
Pictures compared	100°C	30°C
a	0	0
b	3	2
c	3	0
d	2	2
e	3	1
f	2	2
g	2	2
h	3	2
i	2	2
Total	**20/27**	**13/27**

Table 4.11 shows the total scores for the comparison between pictures of films prepared using heating/no heating and between pictures of films prepared using different starch concentrations. For example, picture (a) in Figure 4.37 which corresponds to the matrix with 1.0% starch, Tween 80 and heating was compared to picture (a) in Figure 4.38 which shows the matrix with 1.0% starch, Tween 80 and no heating to verify the effect of heating on the starch film formation. This table shows the overall scores of the effect of starch

concentration on film preparation by comparing the matrices with 1.0% starch and 10% starch, Tween 80 with heating and the matrices with 1.0% starch and 10% starch with no heating. The effect of heating on film preparation was done by comparing the matrices with 1.0% starch and Tween 80, with heating and with no heating and the matrices with 10% starch and Tween 80 with heating and with no heating. The maximum score that can be obtained in this comparison is 27.

Table 4.11 Scores of the 3x3 matrices comparing the matrices with Tween 80

Effect of starch concentration on film preparation		Effect of heating on film preparation	
100°C	30°C	1.0% Starch	10% Starch
20/27	13/27	22/27 -	20/27

The matrices representing the films produced in the presence of surfactin are shown in Figures 4.41 to 4.44. Figures 4.41 and 4.42 correspond to heating and no heating treatments on a 1.0% starch solution and Figures 4.43 and 4.44 show the effect of heating and no heating on a 10% starch solution. Tables 4.12 and 4.13 summarize the scores obtained from an analysis of these matrices and they were constructed following the same procedure described in the examples above with Tween 80.

Figure 4.41 Matrix showing films produced from 1.0% starch solution and surfactin with heating. Concentration of surfactin increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no surfactin and no amylase (bar = 100 μm).

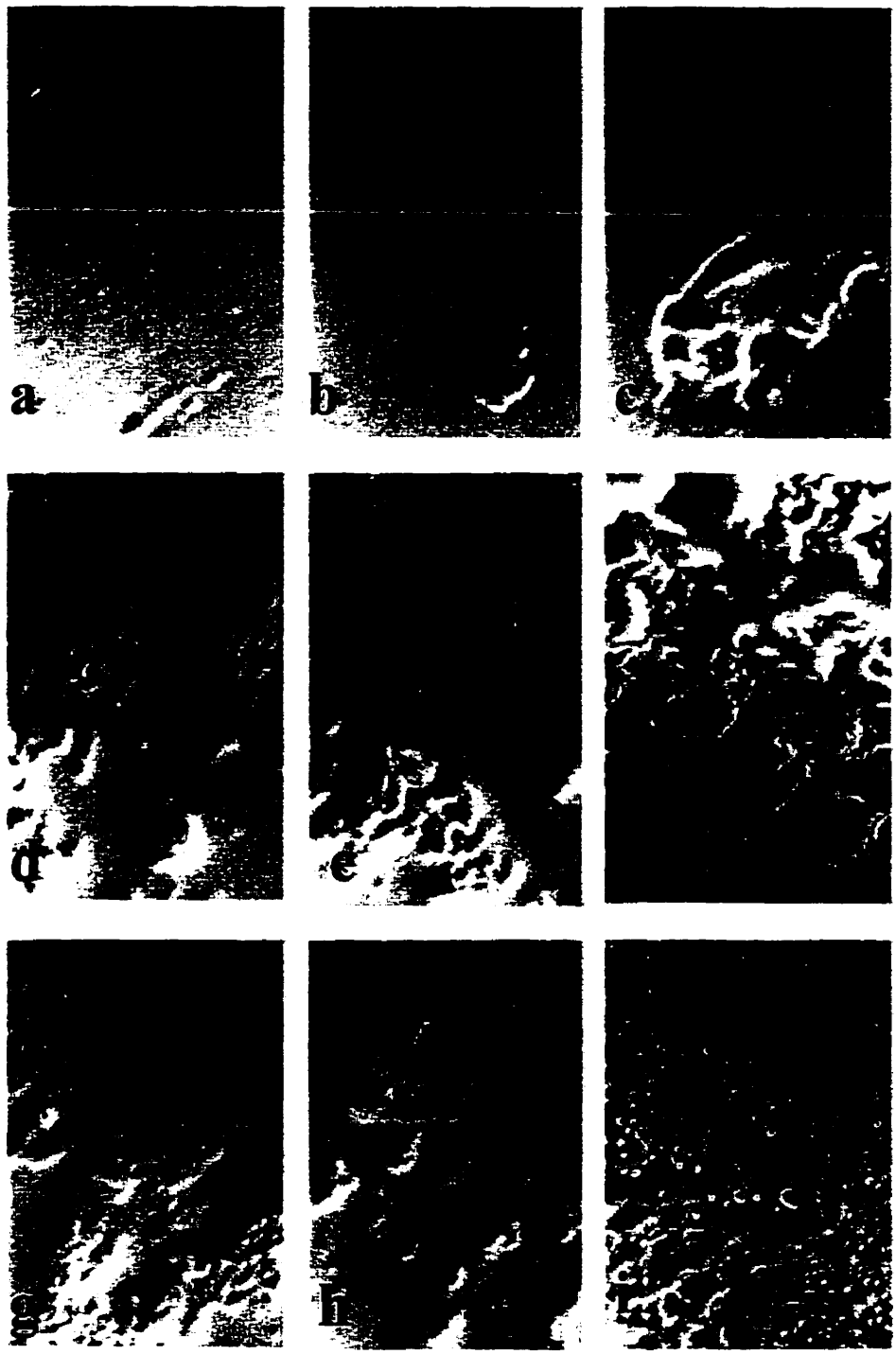

a
b
c
d
e
f
g
h
i

Figure 4.42 Matrix showing films produced from 1.0% starch solution and surfactin at 30°C. Concentration of surfactin increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no surfactin and no amylase (bar = 100 μm).

a
b
c

Figure 4.43 Matrix showing films produced from 10% starch solution and surfactin with heating. Concentration of surfactin increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no surfactin and no amylase (bar = 100 μm).

Figure 4.44 Matrix showing films produced from 10% starch solution and surfactin at 30°C. Concentration of surfactin increases from right to left and concentration of amylase increases from top to bottom. Picture (a) represents the control, no surfactin and no amylase (bar = 100 μm).

a
d

Table 4.12 Scores obtained from the matrices with surfactin by comparing their components

Interaction:	Starch/surfactant/water	Starch/enzyme/water
1.0% starch with heating	10	10
1.0% starch with no heating	13	11
10% starch with heating	9	9
10% starch with no heating	10	9
Totals	**42**	**39**

Table 4.13 Scores of the 3x3 matrices comparing the matrices with surfactin

Effect of starch concentration on film preparation		Effect of heating on film preparation	
100°C	30°C	1.0% Starch	10% Starch
21/27	17/27	19/27	21/27

The results from the matrices of films formed from 1.0% starch solution and Tween 80 with and without heating, have shown that the interactions between starch/ surfactant/water present higher scores than that of the interactions between starch/enzyme/water (a total of 48 compared to 34). During the film formation with heating, overall the scores demonstrated a higher value in comparison to no heating. On the other

hand, the difference in the scores for starch/surfactant/water and starch/enzyme/water were about the same, differing by only 3 points. On the matrices with films formed from a 10% starch solution the difference between the interaction of starch/surfactant/water and starch/enzyme/water was more clearly shown in the heating treatment with a difference of 6 points. The evaluation of heating on the effect of the concentration of starch have shown a difference of 3 points in the scores between 1.0% and 10% starch solutions. During the evaluation of starch concentration on the effect of heating, a larger difference between heating and no heating was found, a total of 7 points.

On the matrices showing films produced in the presence of surfactin, the results have shown little difference between the interactions of starch/enzyme/water and starch/surfactant/water and also between heating and no heating. For the studies on the influence of heating on the effect of starch concentrations, the same pattern found in the evaluation of the matrices with Tween 80 was observed.

From these results it can be said that film formation when no heating was used was more sensitive to relative interactions in the presence of Tween and surfactin. The interactions which resulted in the highest scores were starch/surfactant/water.

4.6 SUMMARY AND SYNTHESIS

The major experimental findings of this study can be summarized as follows:

- The addition of surfactants to the solid substrate fermentation of potato peel significantly increased α-amylase production from both *B. subtilis* ATCC 21556 and *A. oryzae* ATCC 1011. Synthetic sufactants were effective in increasing the enzyme yields of both fungal and bacterial α-amylase. On the other hand, surfactin was much more effective in increasing the yields of bacterial α-amylase in comparison to fungal α-amylase and this can be justified by the fungicidal activity of surfactin.

- Co-culturing with a biosurfactant producer, *B. subtilis* ATCC 21332 with or without Tween 80 added, also significantly increased α-amylase production, especially from the other strain of *B. subtilis*. During the SSF with the co-culture it was possible to verify and to compare the production of surfactin with a SSF of a pure culture of *Bacillus subtilis* ATCC 21332. The potential of SSF for the production of this biosurfactant in yields comparable to those obtained in submerged fermentation was demonstrated.

- Surfactants did not improve the diffusivity of dye at air-water interfaces but did increase diffusivity of dye at solid potato-water interfaces. In the

experiments done with the plate count agar and potato slices, the results have shown that the various concentrations of surfactants did increase the diffusivity of the dye.

- Surfactants increased the rate of colony growth and biomass accumulation on agar plates.

- SEM indicates that surfactants were associated with the production of biofilm during the SSF of potato starch and a partial degradation of the surface of the starch granules.

- Surfactants appeared to interact strongly with starch in solution and affected the structure of starch films produced in the absence of microbial cells.

Based on these findings, the mechanism by which surfactants affect the solid substrate fermentation process is proposed to involve two phenomena: an increase in the rate of spreading or mobility of the colonies over the surface of the substrate and a degradation of the surface of the starch, liberating substrate for microbial attack. The results of these two phenomena are increased biomass, increased enzyme production and the formation of a thin film over the surface of the bacterial colonies.

Different surfactants can produce a greater or lesser effect in the system studied here. However, the biosurfactant, surfactin, is the most effective of those tested and there is the distinct advantage of producing surfactin and enzyme simultaneously from a co-culture of the two strains of *B. subtilis*.

The association between surfactants and the presence of a biofilm is of particular interest since it may be that interfacial phenomena play an important role in the establishment of biofilms. Further insight into this phenomenon may help not only in improvements in the rate of solid substrate degradation, but also in the design or modification of surface conditions to prevent biofilm formation.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

5.1 Costs Analysis

One of the advantages of using SSF for the production of enzymes is said to be the low cost of the process. This could be true, as some costs are likely to be lower (Table 5.1). The reports available on this subject usually discuss the economics of this process in developing countries except for Japan which has experience with large scale production of koji. Relative advantages for the European or North American enzyme industry to adopt SSF technology must be evaluated on a case by case basis.

Table 5.1 Approximate costs of an SSF process in comparison to submerged fermentation

Costs	Lower	Moderate	Higher
Substrate	x		
Nutrients	x		
Plant		x	
Capital equipment	x		
Operating costs			x
Waste disposal	x		
Product recovery			x

The substrate for solid substrate fermentation may be a waste, although it will generally have some value, for example, as animal feed. For its use in the SSF process, it is necessary to pretreat the potato peel, such as with size reduction, grinding and/or the removal of chemicals that will add costs to the process. Addition of nutrients may not be necessary in this process, although yields may be further improved. On the other hand the cost for the construction of the industrial plant may be a problem for this process. Depending on the type of SSF equipment selected, it may be necessary to build a larger plant in comparison to the space used in a submerged fermentation, although the cost of equipment used in an SSF should be lower. Another drawback of an SSF process are the operating costs including labor and maintenance. The SSF process is generally labor intensive and requires more human resources. One of the main positive features is the reduction in waste disposal even though, the product recovery could be more expensive. There is still the need for a purification of the product but in the SSF leaching is a necessary extra unit operation. Enzyme recovery could be eliminated if the enzyme could be used in conjunction with the fermented substrate and biomass.

5.2 Summary and Conclusions

This study was aimed at the evaluation of the use of an SSF process for the production of fungal and bacterial α-amylases using potato wastes as the starchy substrate. The principal emphasis was placed on investigating the effects of surfactants on this process, including the production of the biosurfactant surfactin, the formation of a biofilm and the

development of a theory that could explain the mechanism of surfactant action in an SSF. The following conclusions may be drawn:

1. There is a potential for the production of both bacterial and fungal amylases using an SSF process with ground potato peels as the sole substrate. The values obtained for the production of α-amylase from *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21556 were found to be in the same range of values obtained in previous works using different strains and substrates.

2. The addition of 0.05% and 0.10% (v/w) of the synthetic surfactants Tween 80, Tween 20 and SDS increased the enzyme activity up to 6 times in comparison to no addition. When the same synthetic surfactants were added to the process with *B. subtilis* ATCC 21556 the enzyme activity increased by up to 5.7 times. In the presence of different concentrations of surfactin solution, from 5 ng/g to 54 ng/g (w/w), α-amylase activity from *B. subtilis* ATCC 21556 was even higher, with up to a 10 fold increase in the enzyme yields when 20 ng/g (w/w) surfactin was added to the process. However, when surfactin was added to the fermentation of *A. oryzae* ATCC 1011, the enzyme yields only increased a maximum of 2.5 fold, perhaps due to the fact that surfactin possesses fungicidal properties. This property was evident from the reduction in problems with contamination in the processes containing surfactin.

3. The use of a mixed culture is an alternative process to adding surfactants. From the two mixed cultures studied, the two strains of *B. subtilis* with and without the addition of Tween 80 have shown to be very effective in increasing the enzyme activity. The yields of α-amylase increased between 10 to 11.5 fold in comparison to a pure culture of *B. subtilis* ATCC 21556. On the other hand, the enzyme production obtained with mixed culture of *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21332 only doubled in comparison to a pure culture of *A. oryzae* ATCC 1011.

4. In the SSF processes that either used both strains of *B. subtilis* or a pure strain of *B. subtilis* ATCC 21332, there was also the production of the biosurfactant surfactin. The yields of surfactin obtained by the mixed culture were higher than that of the pure culture of *B. subtilis* ATCC 21332 and were comparable to the ones obtained from the production of surfactin in a submerged fermentation with the same strain.

5. The studies done with the SEM have shown that biofilm formation seems to be associated with an the increase in enzyme activity and is related to the presence of surfactants in the process. The biofilm appears to entrap the bacteria and, consequently, a more intimate relationship between the cells and the substrate may develop, resulting in higher nutrient availability for bacterial growth and higher production of α-amylase.

6. The mechanism of action of the surfactant is most likely related to the interaction between the surfactant and starch as mediated by the absorbed water on the substrate, and an increase in the rate of formation of the colonies over the surface of the substrate.

5.3 Contributions to knowledge

1. Investigation of the addition of surfactants on an SSF process using *B. subtilis* ATCC 21556. The synthetic surfactants Tween 20, Tween 80 and SDS as well as the biosurfactant surfactin, were tested. The interaction between the surfactants and the substrate, a starchy material, have shown to improve the enzyme yields considerably.

2. The use of a mixed bacterial culture has suggested that there is a synergistic effect between the two *Bacillus* strains increasing both the α-amylase and the production of surfactin in comparison to pure cultures.

3. Based on the analyses using Scanning Electron Microscopy (SEM) it is proposed that biofilm formation occurring in the SSF is associated with the presence of surfactants, which consequently, results in higher biomass and enzyme activity.

5.4 Recommendations for further study

This work does not claim to have optimized α-amylase production. Even though in this work, enzyme yields increased as much as 10 fold when surfactants were added and/or when using a mixed culture, there is a lot of potential for further work in the area of SSF with starchy material. For example, there are opportunities for studies with different starchy materials, different types of surfactants as well as, the use of mixed bacterial cultures from different sources to enhance enzyme productivities.

The determination of a reliable method for growth kinetics with bacteria in an SSF should be studied in more detail. This parameter would give further insight into how to increase enzyme yields and for purposes of process control.

Biofilm formation is an extremely important phenomenon in many diverse systems. The mechanism of formation as well as the composition of the biofilm in an SSF process with starchy material should be verified and studied in relation to the complex physical-chemical interactions occurring between the cells, substrate and surface active agents.

REFERENCES

Abate, C., Callieri, D., Rodriguez, E., Garro, O. (1996). Ethanol production by a mixed culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces* sp. Appl. Microbiol. Biotechnol., **45**:580-583

Abdullah, A.L., Tengerdy, R.P., Murphy, V.G. (1985). Optimization of solid substrate fermentation of wheat straw. Biotechnol. Bioeng, **27**:20-27

Agriculture Canada Report, 1997/1998 Canadian Potato Crop, Situation and Trends.

Ahmed, S.Y., Lonsane, N.P., Ghildyal, N.P., Ramakrishna, S.V. (1987). Design of solid state fermentor for production of fungal metabolites on large scale. Biotechnol. Techn., **1**(2):97-102

Aidoo, K. E., Hendry, R., Wood, B.J.B. (1982). Solid substrate fermentations. Adv. Appl. Microbiol., **28**:201-237

Aidoo, K.E., Hendry, R., Wood, B.J.B. (1981). Estimation of fungal growth in a solid state fermentation system. Eur. J. Appl. Microbiol. Biotechnol., **12**:6-9

Alazard, D., Baldensperger, J.F. (1983). Amylolytic enzymes from *Aspergillus hennebergi* (*A. niger* group): purification and characterization of amylases from solid and liquid cultures. Carbohydrate Res., **107**: 231-241

Arora, A., Zhao, J., Camire, M.E. (1993). Extruded potato peel functional properties affected by extrusion conditions. J. Food Sci., **58**(2):335-337

Auria, R., Morales, M., Vilegas, E., Revah, S. (1993). Influence of mold growth on the pressure drop in aerated solid state fermentors. Biotechnol. Bioeng., 41:1007-1013

Babu, K.R., Satyanarayana, T. (1995). α-Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation. Process Biochem., 30(4):305-309

Bandelier, S., Renaud, R., Durand, A. (1997). Production of gibberellic acid by fed-batch solid state fermentation in an aseptic pilot-scale reactor. Process Biochem., 32(2):141-145

Barreto de Menezes, T.J., Salva, J.G., Baldini, V.L., Papini, R.S., Sales, A.M. (1989). Protein enrichment of citrus wastes by solid substrate fermentation. Process Biochem., October

Benjamin, S., Pandey, A., (1997). Coconut cake - A potent substrate for the production of lipase by *Candida rugosa* in solid-state fermentation. Acta Biotechnol., 17(3):241-251

Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. Adv. Enzymol., 12:380

Besson, L., Creuly, C., Gros, J.B., Larroche, C. (1997). Pyrazine production by *Bacillus subtilis* in solid-state fermentation of soybeans. Appl. Microbiol. Biotechnol., 47:489-495

Beukema, H.P., Van der Zaag, D.E. 1990. Introduction to Potato Production, Pucoc Wageningen

Brading, M.G., Fass, F., Lappin-Scott, H.M. (1995). In: Microbial Biofilms (Eds. Lappin-Scott, H.M., Costerton, J.W.). Cambridge University Press, p. 46

Bryers, J.D. (1994). Biofilms and the technological implications of microbial cell adhesion. Colloids Surf., 2:9-23

Budiatam, S., Lonsane, B.K. (1987). Cassava fibrous waste residue: a substitute to wheat bran in solid state fermentation. Biotechnol. Lett., 9(8):597-600

Bull, A.T. (1985). In: Comprehensive Biotechnology: the principles, applications and regulations of biotechnology in industry, agriculture and medicine (Eds. Moo-Young, M, Bull, A.T., Dalton, H.), Pergamon Press, p. 281

Camire, M.E., Violette, D., Dougherty, M., McLaughlin, M.A. (1997). Potato peel dietary fiber composition: Effects of peeling and extrusion cooking processes. J. Agric. Food. Chem., 45:1404-1408

Cannel, E., Moo-Young, M. (1980). Solid-state fermentaion systems. Process Biochem., June/July.

Carlsen, M., Marcher, J., Nielsen, J. (1994). An improved FIA-System for measuring α-amylase in cultivation media. Biotechnol. Tech., 8(7):479-482

Carrizales, V., Jaffe, W. (1986). Solid state fermentation an appropriate biotechnology for developing countries. Intersciencia, 11(1):9-15

Carsen, M., Nielsen, J., Villadsen, J. (1996). Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivation. J. Biotechnol., 45:81-93

Castillo, M.R., Gutierrez-Correa, M., Linden, J.C., Tengerdy, R.P. (1994). Mixed culture solid substrate fermentation for cellulolytic enzyme production. Biotechnol. Lett., 16(9):967-972

Characklis, W.G., Marshall, K.C. (1989). In: Biofilms (Eds. Characklis, W.G., Marshall, K.C.). John Wiley & Sons, Inc., p 3.

Chou, C, Rwan, J. (1995). Mycelial propagation and enzyme production in *koji* prepared with *Aspergillus oryzae* on various rice extrudates and steamed rice. J. Ferment. Bioeng., 79(5):509-512

Christensen, D.H., Madsen, M.H. (1996) Changes in potato starch quality during growth. Potato Res., 39:43-50

Cochet, N., Nonus, M., Lebeault, J.M. (1988). Solid-state fermentation of sugar-beet. Bitoechnol. Lett., 10(7):491-496

Cooper, D.G. (1986). Biosurfactants. Microbiol. Sci., 3(5):145-149

Cooper, D.G., Macdonald, C.R., Duff, S.J.B., Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. App. Environ. Microbiol., 42:408-412

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M.(1995). Microbial biofilms. Annu. Rev. Microbiol., 49:711-745

Daubresse, P., Ntibashirwa, S., Gheysen, A., Meyer, J.A. (1987). A process for protein enrichment of cassava by solid substrate fermenation in rural conditions. Biotechnol. Bioeng., 29:962-968

Desai, J.D., Banat, I.M. (1996). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biot. Rev., 61(1):47-64

Desgranges, C., Vergoignan, C., Georges, M., Durand, A. (1991a). Biomass estimation in solid state fermentation. Manual biochemical methods. Appl. Microbiol. Biotechnol., 35:200-205

Desgranges, C., Vergoignan, C., Georges, M., Durand, A. (1991b). Biomass estimation in solid state fermentation. On-line measurements. Appl. Microbiol. Biotechnol., 35:206-209

Duran, A., Renaud, R., Maratray, J., Almanza, S., Diez, M. (1996). INRA-Dijon reactors for solid state fermentation: designs and applications. J. Sci. Ind. Res., 55:317-332

Ebune, A., Al-Asheh, S., Duvnjak,Z. (1995). Effects of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid-state fermentation. Bioresour. Technol., 54:241-247

Echevarria, J., Rodriguez Leon, J.A., Espinosa, M.E., Delgado, G. (1991). Optimization of solid state fermentation of sugar cane by *Aspergillus niger* considering particles size effect. Acta Bitoechnol., 11:15-22

Eriksen, S., Jensen, B., Olsen, J. (1998). Effect of n-linked glycosylation on secretion, activity and stability of α-amylase from *Aspergillus oryzae*. Curr. Microbiol., 37:117-122

Fields, M.L., Al-Shoshan, A., Poosiri, Y. (1988). Mixed culture fermentation to improve nutritional value of corn meal. J. Food Prot., 51(11):866-888

Fogarty, W.M., Griffin, P.J., Joyce, A.M. (1974). Enzymes of *Bacillus* species - Part 1. Proc. Biochem., July/August, 11-18

Garzón, C.G., Hours, R.A. (1992). Citrus waste: an alternative substrate for pectinase production in solid-state culture. Bioresour. Technol., 39:93-95

Gashe, B.A. (1992). Cellulase production and activity by *Trichoderma* sp. A-001. J. Appl. Bacteriol., 73: 79-82

Gervais, P., Bensoussan, M. (1994). In: *Aspergillus* (Ed. Smith, J.E.), Plenum Press, New York, p. 101

Gervais, P., Sarrette, M. (1990). Influence of age of mycelium and water activity of the meidum on aroma production by *Trichoderma viride* grown on solid substrate. J. Ferment. Bioeng., 69(1):46-50

Gervais, P., Molin, P., Grajek, W., Bensoussan, M. (1988b). Influence of the water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. Biotechnol. Bioeng., 31:457-463

Gervais, P., Bensoussan, M., Grajek, W. (1988a). Water activity and water content: comparative effects on the growth of *Penicillium roqueforti* on solid substrate. Appl. Microbiol. Biotechnol., 27:389-392

Ghildyal, N.P., Ramakrishna, M., Lonsane, B.K. and Karanth, N.G. (1992). Gaseous concentration gradients in tray type solid state fermentors - Effect on yields and productivity. Bioprocess Eng., 8:67-72

Ghurye, G.L., Viipulanandan, C., Wilson, R.C. (1994). A pratical approach to biosurfactant production using nonaseptic fermentation of mixed cultures. Biotechnol. Bioeng., 44:661-666

Gowthaman, M.K., Raghava Rao, K.S.M.S., Ghildyal, N.P., Karanth, N.G. (1995). Estimation of *Kla* in solid-state fermentation using a packed-bed bioreactor. Process Biochem., **30**(1): 9-15

Gowthaman, M.K., Raghava Rao, M.S., Ghildyal, N.P., Karanth, N.G. (1993). Gas concentration and temperature gradients in a packed bed solid-state fermentor. Biotechnol. Adv., **11**:611-620

Grajek, W., Gervais, P. (1987). Effect of the sugar-beet pulp water activity on the solid-state culture of *Trichoderma viride* TS. Appl. Microbiol. Bioetchnol., **26**:537-541

Gutierrez-Correa, M., Tengerdy, R.P. (1998). Xylanase production by fungal mixed culture solid substrate fermentation on sugar cane bagasse. Biotechnol. Lett., **20**(1):45-47

Gutierrez-Correa, M., Tengerdy, R.P. (1997). Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnol. Lett., **19**(7):665-667

Guzmánn-Maldonado, H., Paredes-López, O. (1995). Amylolytic enzymes and products derived from starch: a review. Crit. Rev. Food Sci. Nutr., **35**(5):373-403

Haas, C.N., Bungay, H.R., Bungay, M.L. (1980). In: Annual Reports on Fermentation Processes (Eds. Tsao, G.T., Flickinger, M.C., Finn, R.K.), Academic Press, p. 1

Hawkes, J.G. (1992). In: The Potato Crop (Ed. Harris, P.M.), Chapman & Hall, p 13

Helle, S.S., Duff, S.J.B., Cooper, D.G. (1993). Effect of surfactants on cellulose hydrolysis. Biotechnol. Bioeng., **42**:611-617

Jäguer, A., Croan, S., Kent Kirk, T. (1985). Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., **50**(2):1274-1278

Kargi, F., Curme, J.A. (1985). Solid-state fermentation of sweet sorghum to ethanol in a rotary-drum fermentor. Biotechnol. Bioeng., **27**:1122-1125

Krishna, C., Chandrasekaran, M. (1996). Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation. Appl. Microbiol. Biotechnol., **46**:106-111

Kurakake, M., Ooshima, H., Kato, J., Harano, Y. (1994). Pretreatment of bagasse by nonionic surfactant for the enzymatic hydrolysis. Bioresour. Technol., **49**:247-251

Lachmund, A., Urmann, U., Minol, K., Wirsel, S., Ruttkowski, E. (1993). Regulation of α-amylase formation in *Aspergillus oryzae* and *Aspergillus nidulans* transformants. Curr. Microbiol., **26**:47-51

Ladd, T.I., Costerton, J.W. (1995). Methods for studying biofilm bacteria. Methods in Microbiology, **22**:285-307

Laukevics, J.J., Apsite, A.F., Vierturs, U.E., Tengerdy, R.P. (1984) Solid substrate fermentation of wheat straw to fungal protein. Biotechnol. Bioeng., **26**:1465-1474

Lee, B.H. (1991). In: Bioconversion of wastes materials to industrial products. (Ed: Martin, A.M.), Elsevier Applied Science, New York, p. 265

Lekha, P.K., Lonsane, B.K. (1994). Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. Process Biochem., 29(6):497-503

Lin, S. (1996). Biosurfactants: recent advances. J. Chem. Tech. Biotechnol., 66:109-120

Lindsay, D., Holy, A. (1997). Evaluation of dislodging methods for laboratory-grown bacterial biofilms. Food Microbiol., 14:383-390

Lisinska, G. and Leszczynski, W. 1989. Potato Science and Technology. Elsevier Applied Science.

Little, B., Wagner, P., Ray, R., Pope, R., Scheetz, R. (1991). Biofilms: an ESEM evaluation of artifacts introduced during SEM preparation. J. Ind. Microbiol., 8:213-222

Lonsane, B.K., Ghildyal, N.P., Budiatman, S., Ramakrishna, S.V. (1985). Engineering aspects of solid state fermentation. Enzyme Microb. Technol., 7:258-265

Lonsane, B.K., Ramesh, M.V. (1990). Production of bacterial thermostable α-amylase by solid-state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis. Adv. Appl. Microbiol., 35:1-56

Lonsane, B.K., Kriahnarah, M.M. (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 147

Lonsane, B.K., Ghildyal (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 191

Lu, M.Y., Maddox, I.S., Brooks, J.D. (1998). Application of a multi-layer packed-bed reactor to citric acid production in solid-state fermentation using *Aspergillus niger*. Process Biochem., 33(2):117-123

Makkar, R.S.,Cameotra, S.S. (1997). Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. J. Am. Oil Chem. Soc., 74(7):887-889

Mamma, D., Koullas, D., Fountoukidis, G., Kekos, D., Macris, J., Koukios, E. (1996). Bioethanol from sweet sorghum: simultaneous saccharification and fermentation of carbohydrates by a mixed microbial culture. Process Biochem., 31(4):377-381

Matcham, S.E., Jordan, B.R., Wood, D.A. (1985). Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol., 21:108-112

Meyrath, J. (1966). Fungal amylase production. Process Biochem., July

Michelena, V.V., Castillo, F.J. (1984). Production of amylase by *Aspergillus foetidus* on rice flour medium and characterization of the enzyme. J. Appl. Bacteriol., 56:395-407

Mitch, E.L. (1984). In: Starch: Chemistry and Technology (Eds. Whistler, R.L., Bemille, J.N., Paschall, E.F.). Academic Press, London, p. 479

Mitchell, D.A., Lonsane, B.K. (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 1

Mitchell, D.A., Lonsane, B.K., Durant, A., Renaud, R., Almanza, S., Maratray, J., Desgranges, C., Crooke, P.S., Hong., K., Tanner, R.D., Malaney, G.W. (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 115

Mitchell, D.A. (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 17

Moo-Young, M., Moreira, A.R., Tengerdy, R.P. 1983. In: The Filamentous Fungi, v. 4: Fungal Technology (Eds. Smith, J.E., Berry, D.R., Kristansen, B), Edward Arnold Publishers, London, p. 117

Mudget, R.E. (1986). In: Manual of Industrial Microbiology and Biotechnology (Eds. Demain, A.L., Solomon, A.), American Society for Microbiology, Washington, p. 66

Mulligan, C.N., Gibbs, B.F. (1993). In: Biosurfactants (Ed. Kosaric, N.). Marcel Dekker, New York, **48**, p. 419

Murado, M.A., González, M.P., Torrado, A., Pastrana, L.M. (1997). Amylase production by solid state culture of *Aspergillus oryzae* on polyurethane foams. Some mechanistic approaches from an empirical model. Process Biochem., **32**(1):35-42

Nakadai, T, Nasuno, S. (1988). Culture conditions of *Aspergillus oryzae* for production of enzyme preparation. J. Ferment. Technol., **66**(5):525-533

Narahara, H., Koyama, Y., Yoshida, T., Pichangkura, S., Ueda, R, Taguchi, H. (1982). Growth and enzyme production in a solid-state culture of *Aspergillus oryzae*. J. Ferment. Technol., **60**(4):311-319

Nigam, P, Singh, D. (1995). Enzyme and microbial systems involved in starch processing. Enzyme Microb. Technol., 17:770-778.

Ohno, A., Ano, T., Shoda, M. (1995a). Production of a lipopetide antibiotic, surfactin, by recombinant *Bacillus subtilis* in solid state fermentation. Biotechnol. Bioeng., 47:209-214

Ohno, A., Ano, T., Shoda, M. (1995b). Effect of temperature on production of lipopetide antibiotics, iturin A and surfactin by a dual producer, *Bacillus subtilis* RB14 in solid-state fermentation. J. Ferment. Bioeng., 80(5):517-519

Ooshima, H., Sakata, M, Harano, Y. (1986). Enhancement of enzymatic hydrolysis of cellulose by surfactant. Biotechnol. Bioeng., 28:1727-1734

Padmanabhan, S., Ramakrishna, M., Lonsane, B.K., Krishnaiah, M.M. (1992). Enhanced leaching of product at elevated temperatures: alpha-amylase produced by *Bacillus licheniformis* M27 in solid state fermentation system. Lett. Appl. Microbiol.,15:235-238

Padmanabhan, S., Ramana Murthy, M.V., Lonsane, B.K., (1993). Potential of *Aspergillus oryzae* CFTRI 1480 for producing proteinase in high titres by solid state fermentation. Appl. Microbiol. Biotechnol., 40:499-503

Pandey., A. (1990). Improvements in solid-state fermentation for glucoamylase production. Biol. Wastes, 34:11-19

Pandey, A. (1991a). Effect of particle size of substrate on enzyme production in solid-state fermentation. Bioresour. Technol., 37:169-172

Pandey , A. (1991b). Aspects of fermenter design for solid-state fermentations. Process Biochem., 26:355-361

Pandey, A. (1992). Recent process developments in solid-state fermentation. Process Biochem., 27:109-117

Pandey, A., Ashakumary, L., Selvakumar, P., Vijayalakshmi, K.S. (1994). Influence of water activity on growth and activity of *Aspergillus niger* for glycoamylase production in solid-state fermentation. World J. Microbiol. Biotechnol., 10:485-486

Pandey, A., Selvakumar, P., Ashakumary, L. (1996). Performance of a column bioreactor for glucoamylase synthesis by *Aspergillus niger* in SSF. Process Biochem., 31(1):43-46

Pardo, A.G. (1996). Effect of surfactants on cellulase production by *Nectria catalinensis*. Curr. Microbiol., 33:275-278

Paredes-López, O., Alpuche-Solís, A. (1991). In: Bioconversion of waste material to industrial products (Ed. Martin, A.M.), Elsevier Applied Science, p. 117

Paredes-López, O., Harry, G. I. (1988). Food biotechnology review: Traditional solid-state fermentations of plant raw materials - application, nutritional significance and future prospects. Crit. Rev. Food Sci. Nutr, 27(3):159-187

Parkinson, M. (1985). Bio-surfactants. Biotech. Adv., 3:65-83

Polman, K., Bala, G.A., St.Clair, P. (1995). Models for the biological production of glycerol and biosurfactants from potato-processing industry residuals. Appl. Biochem. Biotechnol., 51/52:511-518

Prior, B.A., Du Preez, J.C., Rein, P. W. (1992). In: Solid State Cultivation (Eds. Doelle, H.W., Mitchell, D.A., Rolz, C.E.). Elsevier Applied Science, p. 65

Pushalkar, S, Rao, K.K., Menon, K. (1995). Production of β-glucosidase by *Aspergillus terreus*. Curr. Microbiol., 30:255-258

Raghava Rao, K.S.M.S., Gowthaman, M.K., Ghildyal, N.P., Karanth, N.G. (1993) A mathematical model for solid state fermentation in tray bioreactor. Bioprocess. Eng., 8:255-262

Raimbault, M., Alazard, D. (1980). Culture method to study fungal growth in solid fermentation. Eur. J. Appl. Microbiol. Biotechnol., 9:199-209

Ramana Murthy, M.V., Karanth, N.G., Raghava Rao, K.S.M.S. (1993a). Biochemical aspects of solid-state fermentation. Adv. Appl. Microbiol., 38:99-147.

Ramana Murthy, M.V., Thakur, M.S., Karanth, N.G. (1993b). Monitoring of biomass in solid state fermentation using light reflectance. Biosens. Bioelectron., 8:59-63

Ramesh, M.V., Lonsane, B.K. (1987a). A novel bacterial thermostable alpha-amylase system produced under solid state fermentation. Biotechnol. Lett., 9(7):501-504

Ramesh, M.V., Lonsane, B.K. (1987b). Solid state fermentation for production of α-amylase by *Bacillus megaterium* 16M. Biotechnol. Lett., 9(5):323-328

Ramesh, M.V. (1988). Factors affecting recovery of thermostable alpha-amylase from bacterial bran produced under solid state fermentation. Chem. Mikrobiol. Technol. Lebensm., 11:155-159

Ramesh, M.V., Lonsane, B.K. (1989). Solid state fermentation for production of higher titres of thermostable alpha-amylase with two peaks for pH optima by *Bacillus licheniformis* M27. Biotechnol. Lett., **11**(1):49-52

Ramesh, M.V., Lonsane, B.K. (1990). Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system. Appl. Microbiol. Biotechnol., **33**:501-505

Ramesh, M.V., Lonsane, B.K. (1991). Ability of a solid state fermentation technique to significantly minimize catabolic repression of α-amylase production by *Bacillus licheniformis* M27, Appl. Microbiol. Biotechnol., **35**:591-593

Reese, E.T., Maguire, A. (1969). Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol., **17**(2):242-245

Reinbold, R.S., Takemoto, J. (1988). Use of swiss cheese whey permeate by *Kluyveromyces fragilis* and mixed culture of *Rhodopseudomonas sphaeroides* and *Bacillus megaterium*. J. Dairy Sci., **71**:1799-1802

Reu, C., Zwietering, M.H., Rombouts, F.M., Nout, M.J.R. (1993). Temperature control in solid substrate fermenation through discontinuous rotation. Appl. Microbiol. Biotechnol., **40**:261-265

Ride, J.P., Drysdale, R.B. (1972). A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol., **2**:7-15

Rodriguez de Sotillo, D., Hadley, M., Holm, E.T. (1994). Potato peel waste: stability and antioxidant activity of a freeze-dried extract. J. Food Sci., **59**(5):1031-1033

Rosenberg, E. (1986). Microbial surfactants. Crit. Rev. Biotechnol., 3(2):109-132

Sargantis, J., Karim, M.N., Murphy, V.G., Ryoo, D., Tengerdy, R.P. (1993). Effect of operating conditions on solid substrate fermenation. Biotechnol. Bioeng., **42**:149-158

Sato, K., Nakamura, K., Sato, S. (1985). Solid-state ethanol fermentation by means of inert gas circulation. Biotechnol. Bioeng., **27**: 1312-1319

Selvakumar, P., Ashakumary, L., Pandey, A. (1996). Microbial synthesis of starch saccharifying enzyme in solid cultures. J. Sci. Ind. Res., **55**:443-449

Sheppard, J.D., Cooper, D.G. (1991). The response of *Bacillus subtilis* ATCC 21332 to manganese during continuous-phased growth. Appl. Microbiol. Biotechnol., 35:72-76

Sheppard, J.D., Jumarie, C., Cooper, D.G., Laprade, R. (1991). Ionic channels induced by surfactin in planar lipid bilayer membranes. Biochim. Biophys. Acta, **1064**:13-23

Sheppard, J.D. (1989). Ph.D. Thesis: Feedback control and the continuous phasing of microbial cultures, McGill University, Montreal, Quebec

Silva, E.M., Yang, S. (1998). Production of amylases from rice by solid-state fermentation in a gas-solid spouted bed bioreactor. Biotechnol. Prog., 14:580-587

Siqueira, E.M. A., Mizuta, K., Giglio, J.R. (1997). *Pycnoporus sanguineus*: a novel source of α-amylase. Mycol. Research, **101**(Part 2):188-190

Smits, J.P., Rinzema, A., Tramper, J., Schlösser, E.E., Knol, W. (1996). Accurate determination of process variables in solid-state fermentation system. Process Biochem., 31(7): 669-678

Spohr, A., Carlsen, M, Nielsen, J, Villadsen, J. (1998). α-Amylase production in recombinant *Aspergillus oryzae* during fed-batch and continuous cultivation. J. Ferment. Bioeng., 86(1):49-56

Srilatha, H.R., Nand, K., Sudhakar Babu, K., Madhukara, K. (1995). Fungal pretreatment of orange processing waste by solid-state fermentation for improved production of methane. Process Biochem., 30(4):327-331

Srinivasulu, S., Appu Rao, A.G. (1993). Kinetic and structural studies on the interaction of surfactants with lipoxygenase L1 from soybeans (*Glycine max*). J. Agric. Food Chem., 41:366-371

Stephenson, K., Harwood, C.R. (1998). Influence of a cell-wall-associated protease on production of α-amylase by *Bacillus subtilis*. Appl. Environ. Microbiol., 64(8):2875-2881

Sudo, S., Ishikawa, T., Sato, K., Oba, T. (1994). Comparison of acid-stable α-amylase production by *Aspergillus kawachii* in solid-state culture and submerged cultures. J. Ferment. Bioeng., 77(5):483-489

Sukan, S.S., Güray, A.., Vardar-Sukan, F. (1989). Effect of natural oils and surfactants on cellulase production and activity. J. Chem. Tech. Biotechnol., 46:179-187

Torrado, A., González, M.P., Murado, M.A. (1998). pH regulation in solid state culture through the initial ratio between oxidized and reduced sources of nitrogen. A model applicable to the amylase production by *Aspergillus oryzae*. Biotechnol. Tech., 12(5):411-415

Viesturs, U.E., Apsite, A.F., Laukevics, J.J., Ose, V.P., Bekers, M.J., Tengerdy, R.P. (1981). Solid-state fermentation of wheat straw with *Chaetomium cellulolyticum* and *Trichoderma lignorum*. Biotechnol. Bioeng. Symp., 11:359-369

Virolle, M., Morris, V.J., Bibb, M.J. (1990). A simple and reliable turbidimetric and kinetic assay for alpha-amylase that is readly applied to culture supernatants and cell extracts. J. Ind. Microbiol., 5:295-302

Whistler, R.L., Daniel, J.R. (1985). In: Food Chemistry (Ed. Fennema, O.R.), Marcel Bekker, New York, p. 69

Wirtanen, G., Alanko, T., Mattila-Sandholm, T. (1996). Evaluation of epifluorescence image analysis of biofilm growth on stainless steel surfaces. Colloids Surf., 5:319-326

Wirtanen, G., Mattila-Sandhom, T. (1993). Epifluorescence image analysis and cultivation of foodborne biofilm bacteria grown on stainless steel surfaces. J. Food Prot., 56(8):678-683

Xavier, S., Lonsane, B.K. (1994). Sugar-cane pressmud as a novel and inexpensive substrate for production of lactic acid in a solid-state fermentation system. Appl. Microbil. Biotechnol, 41:291-295

Xavier, S., Karanth., N.G. (1992). A convenient method to measure water activity in solid state fermentation systems. Lett. Appl. Microbiol., 15:53-55

Yang, S., Ling, M. (1989). Tetracycline production with sweet potato residue by solid state fermentation. Biotechnol. Bioeng., 33:1021-1028

Zheng, Z., Shetty, K. (1998). Cranberry processing waste for solid state fungal inoculant production. Process Biochem., 33(3):323-329

Zottola, E.A., Sasahara, K.C. (1994). Microbial biofilms in the food processing industry - should they be a concern? Int. J. Food Microbiol., 23:125-148

APPENDICES

Appendix 1 - Experimental Data

1. The effect of synthetic surfactants on α-amylase produced by *A. oryzae* ATCC 1011 (units/g solid)

Time (hours)	SDS (0.05%)		Tween 20 (0.05%)		Tween 80 (0.05%)		No surfactant	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0	0	0	0	0	0	0	0
12	0	0	74.6	3.4	56.0	0.6	9.9	1.0
24	58.3	0.8	113.9	1.8	93.0	4.1	22.3	1.3
36	70.8	1.9	130.4	2.2	189.1	3.2	29.0	1.7
48	91.4	3.0	171.1	5.2	314.0	5.1	57.1	3.6
60	100.7	3.7	233.2	3.1	385.3	5.6	66.9	4.1
72	118.3	0.8	134.8	1.8	389.4	5.7	34.5	2.7
84	-	-	-	-	-	-	33.6	0.8

Time (hours)	SDS (0.10%)		Tween 20 (0.10%)		Tween 80 (0.10%)	
	Mean	SD	Mean	SD	Mean	SD
0	0	0	0	0	0	0
12	0	0	77.0	1.6	59.0	3.6
24	39.4	1.1	116.2	3.9	91.4	2.8
36	69.2	2.9	144.8	3.9	188.9	2.8
48	80.3	1.3	137.5	3.3	309.8	5.9
60	92.7	3.5	149.8	1.5	350.9	3.4
72	110.5	3.1	110.1	3.3	378.7	5.8
84	-	-	-	-	-	-

2. The effect of synthetic surfactants on α-amylase produced by *B. subtilis* ATCC 21556 (units/g solid)

Time (hours)	SDS (0.05%)		Tween 20 (0.05%)		Tween 80 (0.05%)		No surfactant	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	2.8	0.1	20.9	1.6	18.4	1.1	3.4	0.2
24	25.9	0.8	77.0	1.4	82.9	1.4	9.5	0.4
36	47.6	2.2	92.0	1.6	98.9	3.7	14.7	0.6
48	55.2	2.2	106.8	2.9	135.9	5.3	21.3	0.6
60	97.4	0.8	118.4	1.4	137.5	2.4	22.8	0.8
72	120.5	2.4	163.8	3.3	169.9	2.2	30.2	1.6
84	107.1	2.9	125.0	2.2	147.7	5.7	23.1	1.4

Time (hours)	SDS (0.10%)		Tween 20 (0.10%)		Tween 80 (0.10%)	
	Mean	SD	Mean	SD	Mean	SD
0	16.0	0	29.5	0.8	16.1	0.8
24	34.4	2.9	100.4	3.6	90.0	2.9
36	45.3	3.7	108.6	2.4	113.3	4.2
48	88.2	7.0	112.6	5.1	116.9	2.2
60	81.7	2.2	143.4	5.9	174.2	1.6
72	119.8	1.4	121.0	2.4	134.0	3.7
84	100.0	2.2	120.3	2.2	136.0	3.7

3. The effect of surfactin concentration on α-amylase produced by *B. subtilis* ATCC 21556 (units/g solid)

Time (hours)	0.005 µg/g		0.011 µg/g		0.020 µg/g		0.054 µg/g	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	29.9	2.2	36.2	3.7	18.9	1.6	25.9	3.6
24	43.9	2.8	90.9	2.9	45.1	2.9	70.0	2.2
36	91.5	4.5	139.0	2.4	117.6	2.9	100.4	2.8
48	183.4	5.9	204.1	4.3	144.0	3.7	168.0	4.9
60	205.0	4.5	220.3	5.7	257.6	2.8	261.1	3.3
72	234.3	4.5	252.9	5.7	306.7	6.2	266.4	4.3
84	261.4	5.9	260.4	4.5	298.6	1.6	293.8	6.4

4. The effect of surfactin concentration on α-amylase produced by *A. oryzae* ATCC 1011 (units/g solid)

Time (hours)	0.005 µg/g		0.011 µg/g		0.020 µg/g		0.054 µg/g	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	7.2	0.3	6.8	0.3	4.0	0.2	5.1	0.3
24	27.5	1.3	53.9	1.6	66.6	1.1	50.3	0.6
36	57.6	1.3	83.5	1.8	142.3	1.1	100.7	1.4
48	98.9	0.5	196.1	0.8	147.8	2.2	190.5	3.6
60	146.9	2.7	171.6	1.2	174.2	2.2	117.7	2.5
72	82.7	1.5	144.7	2.2	127.3	1.7	123.1	1.1
84	74.7	1.3	90.6	0.7	90.6	1.6	49.5	0.6

5. The effect of mixed culture on the production of α-amylase (units/g solid)

Time (hours)	Mixed bacterial culture	
	Mean	SD
0	14.3	0.7
12	76.4	3.2
24	103.4	2.5
36	166.7	6.3
48	231.1	3.2
60	258.5	1.7
72	288.2	4.6
84	223.4	2.8

6. α-amylase produced by *B. subtilis* ATCC 21332 (units/g solid)

Time (hours)	α-amylase by *B. subtilis* 21332	
	Mean	SD
0	2.7	0.5
12	3.5	0.3
24	5.5	0.4
36	6.3	0.3
48	7.6	0.6
60	9.8	0.2
72	12.7	0.6
84	9.9	0.6

7. α-amylase concentration resulting from the leaching process with *A. oryzae* ATCC 1011 and *B. subtilis* ATCC 21556 (units/ g solid)

Time (hours)	*A. oryzae* ATCC 1011			*B. subtilis* ATCC 21556		
	NaCl 0.5%	NaCl 1.0%	Phosphate buffer	NaCl 0.5%	NaCl 1.0%	Phosphate buffer
12	6.4	9.9	6.2	3.6	2.9	1.8
24	13.3	22.3	8.3	9.6	9.5	7.2
36	14.2	29.0	12.0	14.6	12.5	8.4
48	30.4	57.0	29.3	22.3	18.0	15.8
60	37.9	66.9	36.8	25.6	22.0	18.5
72	17.8	34.5	15.5	28.5	27.6	20.4
84	13.9	33.6	12.3	26.6	26.1	18.2

8. Recovery of surfactin from a mixed bacterial culture and from a pure culture of *B. subtilis* ATCC 21332 (mg/g substrate)

(*)	Mixed bacterial culture	*B. subtilis* ATCC 21332
1	0.06	0.05
2	0.24	0.12
5	0.32	0.38
10	0.48	0.65
20	0.78	0.74

* mL water/ g substrate

Appendix 2 - Scores given to the interactions (x_1 to x_{12}) components on the matrices

1. 10% Starch, Tween 80

Starch/surfactant/water			Starch/enzyme/water		
	100°	30°C		100°C	30°C
x_1	3	2	x_3	1	1
x_2	2	0	x_6	1	0
x_4	2	3	x_9	1	2
x_5	1	2	x_{10}	0	1
x_7	2	1	x_{11}	1	2
x_8	2	2	x_{12}	2	2
Total	**12**	**10**	**Total**	**6**	**8**

2. 1.0% Starch, Surfactin

Starch/surfactant/water			Starch/enzyme/water		
	100°C	30°C		100°C	30°C
x_1	1	2	x_3	2	3
x_2	2	2	x_6	1	1
x_4	1	2	x_9	1	1
x_5	2	3	x_{10}	2	2
x_7	1	2	x_{11}	2	2
x_8	3	2	x_{12}	2	2
Total	**10**	**13**	**Total**	**10**	**11**

3. 10% Starch, Surfactin

Starch/surfactant/water			Starch/enzyme/water		
	100°	30°C		100°C	30°C
x_1	2	1	x_3	1	2
x_2	2	3	x_6	2	1
x_4	1	2	x_9	2	1
x_5	2	2	x_{10}	2	2
x_7	1	1	x_{11}	1	1
x_8	1	1	x_{12}	1	2
Total	**9**	**10**	**Total**	**9**	**9**

4. Comparison between heating and no heating, Tween 80

Pictures compared	1.0% starch (Fig. 4.37/4.38)	10% starch (Fig. 4.39/4.40)
a	0	0
b	2	3
c	3	3
d	3	1
e	3	2
f	2	3
g	3	2
h	3	3
i	3	3
Total	**22/27**	**20/27**

5. Comparison between heating and no heating, surfactin

Pictures compared	1.0% starch (Fig. 4.41/4.42)	10% starch (Fig. 4.43 /4.44)
a	1	2
b	2	2
c	2	3
d	2	3
e	2	3
f	3	2
g	2	2
h	2	2
i	3	2
Total	19/27	21/27

6. Comparison between 1.0% starch and 10% starch, surfactin

Pictures compared	Heating (Fig. 4.41/4.43)	30°C (Fig. 4.42/4.44)
a	2	1
b	1	1
c	2	2
d	2	3
e	2	2
f	3	2
g	3	3
h	3	2
i	3	1
Total	**21/27**	**17/27**