İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EFFECT OF PARAMORPHOGENS ON THE MORPHOLOGY AND THE PRODUCTIVITY OF *ASPERGILLUS ORYZAE* CULTURES

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PARAMORFOJENLERİN *ASPERGİLLUS ORYZAE* KÜLTÜRLERİNİN MORFOLOJİSİ VE ÜRETKENLİĞİNE ETKİLERİ

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ABBREVIATIONS

PDA	:Potato dextrose agar
PEG 4000	:Polyethylene glycol 4000
NaDC	:Sodium deoxycholate
C	:Concentration
M	:Molar
VSC	:Vesicle supply center
MyS	: Mycelial solution
SpS	: Spore solution

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ABSTRACT

EFFECT OF PARAMORPHOGENS ON THE MORPHOLOGY AND THE PRODUCTIVITY OF *ASPERGILLUS ORYZAE*

In this study, the effect of paramorphogenic chemicals on the morphology of *Aspergillus oryzae* (CL01) was investigated. The studies on paramorphogens showed that these chemicals have the capability to enhance fungal branching. Certain studies showed that correletions between increased branching and protein secretion. In this work, the effect of different paramorphogens on linear extension of *Aspergillus oryzae* mycelia were investigated both on solid state and submerged fermentations. Within the solid media experiments, four paramorphogens and a total of 32 concentrations were employed. The organism almost responded all of the chemicals to some extent and its morphology was modified differently for each paramorphogen and concentration.Two paramorphogens with three concentrations, 0.01% and 0.02% sodium deoxycholate as the best two and 0.5 M L-sorbose, were observed to create the highest paramorphogenic effects. During the studies, more than 2000 microscope and densitometer images were employed. Different types of inoculations were also tested and mycelial inoculations were shown to be more effective than spore inoculations. Also upper limits for chemical concentrations that inhibit growth within solid cultures were found out. Taking these findings into consideration, the effect of these two chemicals on the lipase produtivity of *Aspergillus oryzae* (CL01) was investigated. L-Sorbose resulted in higher and earlier lipase production, with 6.3 fold increase achieved on the 4th day whereas Na salt of deoxycholic acid resulted in relatively lower production, 4.3 fold increase, but held over a longer period (4-6 days). Observing the increase on productivity, conditions for a sensitive assay system (pHstat) were optimized.

ÖZET

PARAMORFOJENLERİN *ASPERGİLLUS ORYZAE* KÜLTÜRLERİNİN MORFOLOJİSİ VE ÜRETKENLİĞİNE ETKİLERİ

Bu çalışmada, *Aspergillus oryzae* (CL01) suşunun morfolojisi üzerinde, paramorfojenik kimyasalların etkileri incelendi. Paramorfojenler üzerindeki çalışmalar, bu kimyasalların fungusun dallanmasınI arttırıcı etkisi olduğunu göstermiştir. Bazı araştırmalar, artan dallanma ile protein salgılanması arasında bir korrelasyon bulunduğuna işaret ediyor. Bu çalışmada, *Aspergillus oryzae* miselinin linear uzaması üzerinde paramorfojenlerin etkileri gerek katı ortam gerekse sıvı ortamlarda incelenmiştir. Katı kültür deneylerinde dört paramorfojenin 32 farklı derişimi denenmiştir. Organizma her kimyasala farklı tepkiler vermiş ve organizmanın morfolojisi her derişim ve kimyasaldan farklı biçimlenmiştir. 2 kimyasala ait 3 konsantrasyonun (0.01% ve 0.02% sodyum deoksikolat -en etkili iki derişim- ve 0.5 M L-sorbose) en belirgin paramorfojenik etkileri ortaya çıkardığı gözlenmiştir. Çalışmalar sırasında, 2000'den fazla mikroskop ve densitometre görüntüsü kullanılmıştır. Farklı ekim türleri de denenmiş, misel ekimlerinin spor ekimlerinden daha etkili olduğu gözlenmiştir. Ayrıca söz konusu kimyasalların katı kültürlerde büyümeyi engelleyen derişimleri için üst sınırlar saptanmıştır. Tüm bulgular göz önüne alınarak, daha etkili olan iki kimyasalın *Aspergillus oryzae* (CL01) lipazının üretimine etkileri incelenmiştir. L-Sorboz kullanımı, dördüncü günde 6.3 kat artışa yol açarak erken ve hızlı bir artış grafiği çizerken sodyum deoksikolat daha düşük (4.3 kat) bir artışı 4.-6. günler aralığında korumuştur. Sıvı kültür ortamında lipaz enzim ü*retkenligindeki* artış üzerine, duyarlılığı yüksek bir analiz sisteminin (pHstat) çalışma koşulları optimize edilmiştir.

1. INTRODUCTION

1.1 Fungi

Fungi are different from all other groups of organisms in their behaviour and cellular organization. They are accepted eukaryotic organisms as they have membrane-bound nuclei containing several chromosomes and a range of membrane-bound cytoplasmic organelles like mitochondria and vacuoles. They have a unique structure and organization, central to their mechanism of growth and to many of their activities.

Fungi have a typically branched filamentous, tubular structure which is called hyphae when it is a single branch and is essentially a tube with a rigid wall, containing a moving slug of protoplams and has an unlimited length depending on the species and the growth conditions. The rigid structure, hyphae is their basic cellular unit which contains nuclei, mitochondria, ribosomes, Golgi and membrane bound cytoplasm. A network of hyphae branching behind the tips, is called mycelium. Fungi are heterotrophs (chemo-organotrophs), therefore they need organic compounds as energy and carbon sources for cellular synthesis, in contrast to autotrophic plants and some bacteria. They do not have chrolophylls and perform photosynthesis but absorb nutrients through their cell membrane (Prescot, *et al*, 1993; Alexander and Glazer, 1995).

Vegetative growth of filamentous fungi involves hyphal extension and occurs at the hyphal tip. As a result of the development of new extension zones, branching occurs. Hyphal tip growth allows fungi to extend into new regions from a point of source which may be called as inoculum. Older parts of hypha are heavily vacuolated and separated from the younger areas within the mycelium by cross walls called septa. Septa occurs at fairly regular intervals like those of Ascomycota and Basidiomycota, but these septa are absent in hyphae of other classes such as most Oomycota and Zygomycota (Deacon, 1997). The roles of septa still are not completely understood, but have been debated for many years. They might help in providing structural support to hyphae, especially in relatively dry conditions, and in this concept it is

notable that the septate fungi generally are much more tolerant of water stress than are aseptated fungi (Alexopoulos *et al.*, 1996). Also they can help in defend against damage and provide interesting examples of localized wall growth behind the hyphal tips (Deacon, 1997).

They reproduce by both sexual and asexual means. The differences in their sexual reproduction mechanisms used as a basis for the division of four phyla within the fungi. The four major phyla of fungi are Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota. Also as a fifth phylum, Deuteromycota exist where asexual reproduction is seen. Within each phyla, there are several classes of fungi, indicated by names ending with –etes, eg. Basidomycetes, and within each of the classes, there are subclasses, indicated by –ales which are followed by genera and then by species. Some of microfungi are single celled and they are called yeast and reproduce by binary fission or budding.

Chemical analysis of the fungal walls indicates that polysaccharides are the dominant compounds, with less amount of proteins and still smaller amounts of lipids.Fungal walls are formed mainly from chitin microfibrils in addition to chitosan in Zygomycota while glucan in Deuteromycotina. They play an important role, because the interface between a fungus and its environment, shapes its structure. The wall protects against osmotic lysis and acts as a molecular sieve regulating the passage of large molecules through the wall pore space. Also can protect against ultraviolet radiation if it contains pigments and can have several physiological roles by having binding sites for enzymes (Neil and Geoffrey, 1995).

Nuclei are usually small with some exceptions. They are surrounded by a double nuclear membrane with pores, and they contain a ribonucleic acid (RNA)-rich nucleolus. Most fungi are haploid while some others can alternate between diploid and haploid generations (Alexopoulos *et al.*, 1996). The nuclear membrane and nucleolus usually remain intact during most stages of division, also most fungi do not have centrioles. The plasma membrane consists of phospholipid bilayer and associated proteins and sterols. The main role of the membrane is to regulate the uptake and release of materials. Also it can anchor some enzymes; mainly chitin synthase and glucan synthetase, besides it can relay signals from external environment to the cell interior in a process called signal transduction (Deacon,1997).

The secretory system of fungi consists of Golgi apparatus and membrane-bound vesicles. The secretory system is both involved and in tip growth and in the secretion of enzymes for degrading nutrients in the external environment. Also in addition to that they are important for the production of foreign (heterologous) gene products (Deacon,1997).

Fungi have a vacuolar system which has several functions, including the storage of compounds and recycling of cellular metabolites. Also they were found to be major sites for storage calcium (Neil and Geoffrey, 1995).

1.2 Fungal Growth :

1.2.1 Apical Growth and Branching

Apical growth accounts for the importance of fungi in natural environments. It can be observed using a compound microscope by placing a cover slip on the colony margin of a fast-growing fungus, for example *Neurospora crassa*, on agar. The tips are seen to extend across the field of view, and by focusing on a septum behind the apex the protoplasm is seen to move steadily towards the apex. Such a rapid rate is possible only if the protoplasm is supplied from behind. According to this fact apical growth is actually apical extension, the true rate of extension can be defined as the increase in biomass per unit of time, is much slower. Studies of apical growth mechanism in early experiments, carried out by Robertson (1958), shown that the normal process of apical growth involves two separate phenomenon (i) extension of plastic, deformable tip; (ii) rigidification of the wall behind the extending tip (Deacon, 1997).

The following experiment revealed that some enzymes synthesized at the fungal wall are involved in the branching and growth of hyphae, these include chitin synthase, glucan synthetase which is the major enzyme involved in wall growth because it catalyses the synthesis of β-1,3-glucans, and then wall-lytic enzymes (Neil and Geoffrey, 1995).

Over the last few years there has been much speculation concerning the driving force of the apical growth, that electrical field or ionic fields could be involved, especially calcium ions, but recent studies revealed that the electrical fields are intimately

involved in nutrient uptake rather than in growth. Instead cytoskeletal components are strongly involved in apical growth (Deacon, 1997).

Branches arise by the development of new apices as a colony grows and synthesizes new protoplasm. These apices can arise from any point along a hyphae but very rare at the tips. Most probably branching occur immediately behind the septa because septa interrupt the flow of the cytoplasm to some extent so that vesicles might accumulate over that point (Alexopoulos, *et al* 1996). Another possibility is that wall-lytic enzymes might be involved in the process (Deacon, 1997). But generally we can say that the pattern of branching is mostly ecologically relevant. When fungi are grown onto nutrient-poor medium they form sparsely branched colonies, whereas they branched densely in a rich medium (Deacon, 1997).

1.2.2 Models for hyphal growth

Advances in computer science lead mycologists collaborate with computer scientists in order to find out generalized mathematical rules especially for hyphal growth, branching or fungal morphogenesis. It is not possible to derive formulas for any state of mycelial organisms grown on suitable media or isolated from nature, but in an engineering point of view, considering a few variables as modelling parameters and neglecting some cellular mechanisms, many models were proposed by various authors. One of them modelled hyphal growth as a consequence of a VSC (vesicle supply center) that supplies and distributes vesicles in all directions of hyphae. Any vesicle was accepted to occupy a unit volume and existence of different kinds of vesicles and their physiological variations were neglected. Mathematical basis of the functions of VSC and fungal morphogenesis were investigated employing spherical or polar coordinates, numerical methods where possible. The model yielded the following equation:

$y = x \cdot \cot (V.x / N)$ (Equation 1.1),

where N: the amount of wall-destined vesicle release from the VSC per unit time,

V: rate of linear displacement of the VSC.

The VSC seems to be a hypotethical analogue of the fungal Spitzenkörper although all mycelial fungi do not have Spitzenkörpers. The model generates a tubular shaped hyphae from a tip growing cell via initial spherical growth and spreading vesicles and increasing asymmetry (Bartnicki-Garcia, *et al.* 1989).

Cellular mechanisms are rarely considered to be a part of mathematical models for hyphal growth and branching due to the requirements of simplification. It may be necessary to create a general outline of hypothetical intracellular subparticles and functions. In a work by Prosser and Trinci (1979), hyphae and tip production and flow were modelled. The model is based on the assumptions that as spores are units consisting of hyphal segments which produce vesicles, septation delimit intercalary compartment formation and rate of vesicle adsorption of tips yield in branching. The model was simulated on a 1906S ICL digital computer with results displayed graphically on a Calcomp Graph Plotter. Predictions made using the model were in good agreement with growth kinetics of *Aspergillus nidulans* and *Geotrichum candidum* (Prosser and Trinci, 1979).

As well as guessing the hyphal kinetics and branch initiation by means of mathematical modeling, continuous observation of fungal cultures on solid media is also applicable in elucidating the mechanisms mentioned. Time lapse photography is a method that figure hyphal development by continuous imaging. Growth of *Mucor hienalis*, *Geotrichum candidum*, *Aspergillus nidulans*, *Neurospora crassa* and *Penicillium crysogenum* was studied by time lapse photography and photos were taken every 15 minutes. Unlike bacteria and yeasts, mould growth is a consequence of hyphal growth. Hyphal branching is initiated by the random distribution of vesicles that probably carry some precursor substances. Random distribution causes initiation of new branches in some parts of the cytoplasm, whereas in some parts maximum extension rate/length is not achieved yet. Every branch was initiated over a critical mean volume of cytoplasm per tip volume (Trinci, 1974).

Fungal image analysis

As it is necessary to control and monitor fungal morphology in a process control view, morphological characterization of filamentous organisms had to be standardized. This requirement was met with various methods that eliminate subjectiveness and inconsistency caused by human observation. An algorithm for digital analysis of filamentous organisms was proposed. The software written in C programming language combined image processing and pattern recognition features and all operations used within the analysis of different morphological characteristics of various strains were performed under the control of a PC. The images were analyzed on-line and automatically. The system consisted of a microscope, a camera,

a PC-based image processing system and original images taken were turned into binary, identified and classified images. The organism, to which the system was applied, was *Streptomyces tendae* Tü 901/8c (Treskatis, *et al* 1997).

As well as the pellet and sparse mycelial states of fungal morphology, pellet size and concentration affects oxygen and nutrient distribution and viscosity within the broth. There might be an upper limit for pellet diameter in order to provide a better control. 400 micron was proposed as an upper pellet diameter limit for *Penicillium chrysogenum* pellets (Cox and Thomas, 1992). The hairy (annular) extensions around the central pellet cores become a function of stirrer rate as increased rates have the potential to chop them off. It was suggested that chopping off the hairiness might be useful providing a reduced viscosity and lead to a better oxygen transfer. An automated image analysis system was proposed. It has the capability to capture images, define pellets and hairiness separately and constitute a new image combining both (Cox and Thomas, 1992).

In microbiology, staining is a common method to identify and visualize both cells, organelles and their states. Unlike yeast, animal and plant cells, filamentous fungi consist of several cells some of which reproduce, remain alive or die and exhibit different types of metabolic activity. During cultivation of *Aspergillus awamori* (CBS 115-52), fluorescence staining combined with microscopic investigation was employed. In order to provide information on propagation and protein production, FDA (fluorescein diacetate), PI (propidium iodide), EB (ethidium bromide), FTIC (fluorescein isothiocyanate), PY (Pyronine Y) /MG (Methyl Green), AO (Acridine orange) were the dyes used. Observations yielded important results on spore germinations, pellets and age, mycelial zones and protein synthesis rates, state of mycelia and presence of different types of RNA (Freudenberg, *et al.* 1996).

Off-line quantifying of fungal morphology necessitates withdrawal of too many colonies from submerged cultures, which cannot be otherwise of similiar homogeneity, in order to provide useful information on morphology-biochemistry relationship. In a work by Spohr *et al* (1998), morphology of *Aspergillus oryzae* (A5160) was investigated using a flow-through cell. The images are taken at a periodical basis and fungal growth is observed. Up to now, flow-through cells were employed in experiments with *Streptomyces tendae* to determine the effect of temperature on growth and morphology of fermentation broth, with *Aspergillus niger*

to determine the influence of short-term salt stress on growth. Another on-line technique is called "surface growth". It was exploited at several studies with *Aspergillus niger*, *Aspergillus nidulans*, *Geotrichum lactis* (Spohr, *et al.* 1998).

1.3 Economic Importance of Fungi

Fungi have many traditional and novel roles in biotechnology, and there is a great interest in their future commercial development.

Concerning the food and food flavouring, about 1.5 million tonnes of edible mushrooms are produced every year, such as *Agaricus bisporus*. Also as a recent development, the mycelium of the fungus *Fusarium graminearum* has been grown in fermenter vessels and marketed as a novel food known as "Quorn" or mycoprotein (Moses, 1994).

Also fungi are used in production of several traditional foods and beverage, including alcoholic drinks, for producing ethanol from *Saccharomyces cerevisiae*, and in bread production where yeast produces carbon dioxide for raising the dough (Deacon, 1997). Some are used in the production of gourmet cheeses, such as *Penicillium roquefortii* used in production of blue-veined cheese, and others used in the production of soft cheeses. So fungi are set to play a new role as sources of natural flavour and odour components.

When fungi grown in a vessel they can produce metabolites, either primary which are end-product of the common metabolic pathway, or secondary metabolites, which are diverse range of compounds formed by specific pathways. Primary metabolites like ethanol and organic acids were produced from sugar when the growth is limited by low pH. Production of citric acid by *Aspergillus niger*, other primary metabolites like glycerol and mannitol are also some examples (Moses, 1994). Meanwhile, antibiotics such as penicillin from *P. chrysogenum*, grisefulvin from *P. griseofulvum* and cyclosporin from *Trichderma polysporum* are examples to secondary metabolites (Deacon, 1997).

Some fungi produce a range of extracellular enzymes with important commercial characteristics, like α-amylases and glucoamylases from *A. niger, A. oryzae* and sometimes *Rhizopus oryzae*, invertase from *S. cerevisiae*, and lipases from *A. niger* and *A. oryzae* and a wide variety of heterologous proteins. Moreover, fungi also

produce many internal enzymes and enzymic pathways that can be exploited for bioconversion of compounds such as pharmaceuticals (e.g. steroids). Genetic engineering of fungi, particularly *S. cerevisiae*, has developed to the stage where the cells can be used as factories to produce pharmaceutical products, by introduction of foreign (heterologous) genes (Deacon, 1997).

On the other hand fungi can be problematic by causing diseases to humans and plants. Most of the fungi, that infect humans, can infect through wounds or when air-born spores enter the lungs such *A. fumigatus* causes Aspergillosis, *Alternaria alternata* which causes allergy (Deacon, 1997). A different type of infection can be caused by *C. albicans* which infect mucosal membrane of the mouth, intestines and vagina. In plants, they cause more than 70% of all the major crop diseases. The most common fungi that infect wheat and sorghum crops are smut and rust fungi, for example *Erysiphe graminis,* causing powdery mildew of cereals (Alexopoulos *et al*, 1996) (Deacon, 1997).

1.4 Paramorphogens

In mycelial organisms, hyphal length and tip number increase exponentially at essentially the same specific rate, the ratio (hyphal length:tip number) between these parameters will approximate a constant. There have been many compounds identified which inhibit hyphal extension but apparently have no effect on specific rate. Such compounds are called paramorphogens (Trinci, *et al.* 1994; Olusfilsayo and Trinci, 1988).

1.4.1 Paramorphogenic effect:Their potentials

Paramorphogens are compounds capable of inducing a reversible morphological change in fungi (Olusfilsayo and Trinci, 1988) and because they alter the ratio (E/μ) they can reduce hyphal growth unit, i.e. cause increase in branching (Trinci, *et al* 1994).

Their effect can be formulated as:

$E = (H_t - H_0)/ (B_0 + B_t) /2$ (Equation 1.2),

where E: mean rate of extension,

H_o: total hyphal length of the mycelium at zero time,

H_t : the total length 1 hour later,

B_0 : the number of tips at zero time,

B_t : number of tips 1 h later.

Also E is defined as,

$E = G \mu = V_g \mu / \Pi r^2$ (Equation 1.3),

where G : the hyphal growth unit,

μ : specific growth rate,

V_g : hyphal growth unit volume,

r : hyphal radius

considering unrestricted growth where $\mu = \mu_{max}$ (Trinci, *et al.* 1994).

As the presence of the paramorphogens decrease the specific growth rate and keep the specific rate constant, E /μ ratio (also G) is decreased. This yields in decreased V_g values for constant hyphal radii values supporting the mycelium, so increased branching is observed. In many studies, the benefits of paramorphogen chemicals were exploited in enhancing mass transfer, handling medium rheology, inhibiting and/or localizing pathogenic growth. In a work, effects of L(-) sorbose on *Neurospora crassa* were investigated. Its addition to solid media containing another carbon source induced many fungi to form dense colonies which extended radially at much slower rates than the control colonies. It is also known that some fungi will metabolize certain carbon sources only after a period of growth on some other carbon source and many times L(-) sorbose could not be a sole carbon source. In batch liquid cultures L(-) sorbose did not effect *Neurospora crassa* spco 9 morphology, but concentration of pellets in batch spco 1 culture was frequently increased. Branch initiation was increased when spco 1 and spco 9 were grown on solid media (Trinci and Collinge, 1973).

The mechanism of inhibition or reduction could be by inhibition of membrane and/or wall biosynthesis. Extension of hyphae is limited by the rate at which membrane and wall precursors can be incorporated into the tip wall, but not limited by the rate of supply of these precursors (Trinci *et al* 1994). This could also be restricted on the surface of the hyphae by reducing, inactivating or inhibiting the synthesis of enzymes involved in the synthesis of cell walls such as β-1,3-glucan (Olufisayo A & Trinci 1988). These compounds could be sugars, like L-sorbose, fungicides like

validamycin used to control rice sheath blight (Olufisayo A & Trinci 1988), and other compounds like lysocellin (Trinci *et al* 1994), which inhibits the phosphoinositides turn over (Sarah L. *et al*, 1995).

The idea that branch initiation might be regulated by volume was studied by another work on different *Neurospora crassa* mutants. The experiments concentrated on employment of different temperatures and paramorphogens both in solid and liquid media. As colony radial growth rate and specific growth rate ratio approximated a constant value, colony radial growth rate was a reliable parameter to determine the optimum growth temperature. Mean length of growth units were unaffected by the temperature. Deoxycholate, cycloheximide and triphenyl tin acetate were also not so much effective on hyphal growth unit of spco 1 hyphae. In liquid cultures, spco 9 and SYR-17-3A strains formed a narrower main (initial) hyphae then leading mature hyphae, so branch initiation was proposed to be regulated by volume as well as cell division in unicellular organisms as it was valid to correlate hyphal length with hyphal mass (Trinci, 1973). This demonstrates that paramorphogens do not have to be effective on all type of mycelial organisms, but volume : area ratio may be effective in both paramorphogenic and normal cases.

Another posibility to make use of paramorphogens is inhibition of pathogenic growth. Validamycin A was used in Japan and China to control *Rhizoctonia solani*, the causal agent of rice sheath blight. Mode of action of Validamycin A (antibiotic) does not effect protein or nucleic acid synthesis in that organism, but a significant amount of reduction in inositol content is observed. As inositol contributes to both structural elements like phospholipids and pathogenicity, its reduction limits the radial growth and spreading of pathogenic hyphae. The effect of validamycin was completely a paramorphogenic one, as specific growth rate was not limited and branching frequency was increased. Inositol is synthesized from glucose-6-phosphate, first with the formation of inositol 1-phosphate which is then dephosphorylated to form inositol, so phospholipid biosynthesis was proved to be an appropriate target for fungicides that display selective toxicity. The paramorphogenic effect was lost when some inositol was added to the medium, whereas inositol levels in *Neurospora crassa* and *Aspergillus nidulans* were unaffected (Robson, *et al.* 1989).

If pathogens are treated with paramorphogens which could not be exploited as carbon sources, their partial or complete termination might be possible. L (-) sorbose, which normally has paramorphogenic effects on *V. faba,* could not inhibit its growth, unlike 3-O-methyl-$_D$-glucose and glucosamine which cannot be used as sole carbon sources for growth inhibition (Jejelowo, *et al.* 1988).

Another mechanism claimed to play a role in hyphal extension and branching in *Neurospora crassa* was phosphoinositide turnover. Lysocellin, piericidin B_1-N-oxide and the inositol analogue, all of which are phosphoinositide inhibitors, behave as paramorphogens. The mechanism of inhibition could not be elucidated, but according to the experiments phosphoinositides were involved in the regulation of mycelial morphology (Hosking, *et al.* 1995).

It was very interesting that choline causes anti-paramorphogenic effect in a wild-type (A3/5) and a highly branched variant (C106) of *Fusarium graminaerum.* Although specific growth rate was constant, more sparsely branching has been observed up to addition of 1.5 mM choline. Experiments yielded similiar results with betaine, ethanolamine, monomethylethanolamine and dimethylethanolamine (Wiebe, *et al.* 1989).

1.4.2 Paramorphogenic effect: Rheology and mass transfer

In liquid fermentations, two extreme types of morphology for fungal cultures can be mentioned: Free filaments and pellets. Even though both of them are employed in various types of industrial fermentations, cultures containing filamentous mycelia are known to be free of pellets whereas cultures containing pellets also always contain different amounts of free mycelia (Cui, *et al.* 1997). The possibility of exploitation of paramorphogenic chemicals have been possible where pellet morphology is more favourable such as in itaconic acid production by *Aspergillus terreus,* citric acid production by *Aspergillus niger* and many cell and enzyme immobilization processes. Pellets of *Aspergillus niger* species have been used as an effective immobilized support for glucose oxidase and aminoacrylase. Also in some cases, cultures with few pellets combined with free mycelia may be favourable such as in penicillin production (Pazouki and Panda, 2000). Both cases can be achieved by employment of optimized amounts of paramorphogens.

As mentioned earlier, paramorphogenic chemicals enhance branching in mycelial cultures as a consequence of inhibited extension rate and constant specific growth rate. All microbial growth elements and metabolites synthesized, bioconverted or secreted affect liquid culture systems in terms of rheology and mass transfer. The harshness of mechanical effects, homogeneous or heterogeneous distribution of nutrients, oxygen and microorganisms are determined by processing conditions in return. The idea that microorganisms effect bioprocesses and influenced by them create an outline of exploitation of paramorphogens.

Low solubility and limited volume of oxygen within the bioreactors made oxygen become the limiting component for fungal growth many times (Cui, *et al.* 1998). This problem and requirement of homogeneity necessitate continuous stirring of liquid media. In these systems, three different damaging mechanisms might exist: Effects of eddies caused by stirring on particles, interactions between particles and impellers or baffles and collision between particles and particles (Cui, *et al.* 1997). Advantages of stirring were contribution to fungal growth by enhancing availability of oxygen and even distribution of nutrients, but mechanical impacts listed above that may inhibit fungal growth, and fractionate mycelia/pellets are the drawbacks. Mean hyphal length was related to the specific energy dissipiation rate by a power function
(–0.25 ± 0.08) (Cui, *et al.* 1997). The work by Cui, *et al.* 1997 explained effects of mechanical equipments were mainly shaving (chopping of the mycelia) instead of pellet break-up. Length of hyphal/pellet growth was considered to be a function of geometry of the reactor, its subunits and energy dissipiation. Different energy dissipiation rates and their capacity to "shave" the moulds were investigated.

Energy costs is one of the most important issues in all types of processes including bioprocesses. Very high stirring rates may not be applicable always since both microbial growth will be inhibited and energy costs will increase. The optimal bioreactor design includes appropriate impeller, stirrer and stirring rate selection as changes in rheological properties of fermentation broths are very common due to microbial growth and product formation such as in the case of *Leuconostoc mesenteronides* in which dextran synthesis caused non-newtonian flow behaviour characterizable by a power-law type of equation (Veljkovic and Lazić, 1988). First, considering all types of impacts, appropriate selection of agitated vessels with

appropriate stirrers is made. These effects are functions of impeller speeds and physical properties of particles and it is possible to scale them up (Takahashi, *et al.* 1993). Then, as fermentation advances, microbial growth and metabolites formed usually make handling of medium rheology and oxygen/nutrient distribution harder and harder generally by means of non-newtonian flow characteristics' formation. Therefore controlling oxygen concentrations, stirrer rate increase and energy cost increase, handling rheology deficiency, cell deaths, reduced efficiencies in bioconversion and biomass production are observed in a cycling sequence. A controllable morphology yielding a controllable rheology making moderate mixing sufficient for optimizing efficiency and reducing energy costs may again be possible by means of utilizing paramorphogens. That claim may be supported by the work of Karsheva, *et al.* 1996, demonstrating that biomass concentrations and morphological changes of mycelia may be observed by means of viscosity. In another work, filamentous forms of *Aureobasidium pullulans* and production of extracellular polysaccharide (pullulan) were found responsible for non-newtonian behviour of the fermentation broth (Roukas, 1999).

Another factor that may harden controllability of liquid media is the compactness and volume of fungi. As free dispersed hyphal elements have very high surface areas when compared to pellets of the same volume, their probability of being chopped off or harmed is much more higher. That again results in harder controlled rheologies, higher energy costs and decreased efficiencies. It was possible to model agitation-induced, off-line agitation of *Aspergillus oryzae* grown in chemostat culture making use of the data and formulas from a former study. Break-up frequency depended upon power input, impeller diameter and circulation frequency (Amanullah, *et al.* 2000). However, in some strains hyphal fragmentation may not affect productivity, therefore it is altogether possible to obtain shorter hyphae, reduced viscosity, improved mixing and increased mass transfer capacity (Li, *et al.* 2002). During the fermentation of filamentous bacterium *Streptomyces clavuligerus*, increasing the stirrer speed accelerated fragmentation, but productivity did not seem to be affected by the morphology (Belmar-Beiny and Thomas, 1991).

Being sure about the effects of hyphae fragmentation on fungal fermentations, hyphal tensile strength should be well understood as well as hydrodynamic stresses. Even if the filamentous fungi grow very slowly or do not grow at all, the

composition of cell wall and other components, hence the hyphal tensile strength varies continuously during fermentation periods. Hyphal tensile strength of *Aspergillus oryzae* was shown to be varying (Li, *et al.* 2002) and this state necessitates elucidation of hyphal strength and fragmentation characteristics of the organism in order to relate fragmented or unfragmented morphologies and product formation.

A critical pellet diameter estimation was done for batch-grown *Aspergillus oryzae* (A1560) pellets. Pellets with greater diameters might yield oxygen limitation and this state might favour ethanol production. Pellet formation was a function of hyphal tip extension rate defined with a saturation type kinetics and pellet fragmentation during batch cultivation was generally negligible (Carlsen, *et al.* 1996).

Generally, filamentous fungi are appropriate candidates to substitute bacteria, yeast and mammalian expression systems for production of heterologous gene products, but many fungal fermentations have the disadvantage of high broth viscosity, oxygen and nutrient transfer limitations. Exposure to nutrient gradients within submerged cultures both affect fungal morphology and product expression. Shorter branches and enhanced oxygen transfer are typical results of enhanced agitation rates. In a work concentrating on fragmentation behaviour of fed-batch *Aspergillus oryzae* fermentations, two different fragmentation mechanisms were observed: Fermentation of clumps (shaving off) and fragmentation of clumps (even shorter fragments). In all batches, fragmentation was found to dominate fungal growth and branching (Li, *et al.* 2000).

Different carbon sources might affect both morphology of and biopolymer production by filamentous fungi. Maltose was found to be more efficient than sucrose as carbon source in submerged cultures of *Paecilomyces japonica* during biopolymer production studies, but it was not favourable by terms of cost (Bae, *et al.* 2001). Maltose colonies were shown to be more compact and branched when compared to sucrose colonies. Therefore it has an effect as that of a paramorphogen. Similiar results were obtained for red pigment production characteristics of *Paecilomyces sinclarii*. Starch was advantageous when production was concerned, whereas sucrose was a better choice when cost was taken into account (Cho, *et al.* 2002).

Fungal morphology was influenced by a variety of factors such as medium composition, agitation intensity and oxygen tension, but it was still difficult to relate the broth rheology to every aspect of microbial morphology. As well as morphology and rheology relations, the effect of dissolved oxygen tension on these parameters might become significant. It was shown by various authors on many strains that different dilution rates might have a great impact on fungal morphology (Olsvik *et al*, 1988).

Not all the times do fungal micromorphology determine the extent of product formation. Extracellular lipase and cutinase production by a recombinant *Aspergillus awamori* strain was not limited by fragmentation and cell damage. The morphology impacted product formation indirectly by means of viscosity increase, mass transfer decrease and therefore inefficient mixing. The authors claimed that hyphal length and number of tips was not very important (Johansen, *et al.* 1998).

1.5 Aspergillus Species: *Aspergillus oryzae*

Aspergillus oryzae is a filamentous fungi from the class ascomycetes. Ascomycota, sometimes called sac fungi, are monophyletic and accounts for about 70-80% of all fungi. Most of the fungi that can form lichenes with algae are included in this class. It also includes the majority of fungal species that lack morphological evidence of sexual reproduction. The most common and known fungi among this class are: *Penicillium chrysogenum* which produces penicillin, *Candida albicans* which cause thrush and vaginitis (Prescot *et al*,1993), *A. flavus* that produces aflatoxin, fungal contaminant of nuts and stored grains which is toxic and carcinogenic (Alexopoulos *et al.*, 1996), *Neurospora crassa* which is known as the "one-gene-one enzyme" organism, and *Saccharomyces cerevisiae*, the yeast being widely used in baking and brewing.

The distinctive character shared by all members of Ascomycota is the formation of ascus, within which nuclear fusion and meiosis takes place (Alexopoulos *et al.*, 1996). In the ascus, one round of mitosis takes place followed by meiosis results in formation of eight nuclei (most probably), which forms eight ascospores. Ascospores in ascus are surrounded by an enveloping membrane system, which packages each nucleus with its adjacent cytoplasm leading to the formation of ascospore walls that

protects ascospores against adverse conditions when released (Alexopoulos *et al.*, 1996).

The body of Ascomycota consists of a typical eukaryotic cell surrounded by cell wall. It could be single cells or a long tubular septated filament or hyphae. Both structures contain a cell wall made of different amounts of chitin and β glucans. Genera of this class could be found on all the continents (Alexopoulos *et al.*, 1996).

The life cycle of most *Aspergillus* species can be divided into sexual, asexual and parasexual life cycle. The aggergate of hyphae, which form a vegetative mycelium, are multinucleate and they grow by apical extension and sub-apical branching (Powell, *et al* 1993). If the nuclei inside are genetically identical or different, the mycelium is called homokaryotic or heterokaryotic respectively. Sexual reproduction is by the formation of ascospores carried in a fruiting body (cleistothecium), which contain a large number of spores. Asexual reproduction or sporulation involves formation of conidia, which is derived by mitotic division and carried on a conidiophore (Alexopoulos, *et al.*, 1996).

The mode of growth results in time-dependent hyphal cellular differentiation due to the flow of cytoplasm toward the tips, which leaves the older part of the hyphae vacuolated and inactive (Teit Agger *et al*, 1998).

A. oryzae has been widely used for the production of high levels of heterologous proteins such as aspartic proteinase, α-amylase, lipase, and recently, active human proteins; for example lactoferrin and lysozyme have been produced in recombinant strains of *A. oryzae* (Morten Carlsen et al, 1996). However, little is known about the morphology, mechanism of growth, and the product formation.

The safety of a microorganism is generally accepted as harmlessness of the genetically modified and wild-type strains and their products. When this state can be assured, industrial production of antibiotics, amino acids, enzymes and various other useful compound becomes possible. In that respect, *Aspergillus oryzae* is considered a safe organism, which is exploited both in solid-state fermentation for koji production, a complex enzyme preparation for the production of soy-sauce, *miso* and *sake* in the East and in liquid fermentations in the production of ezymes, initially α-amylase, then lipase, proteases and tannase in the West. Both *Aspergillus oryzae* and

its enzymes were accepted as constituents of food by Joint FAO/WHO Commitee in 1987 (Barbesgaard, *et al.* 1992). According to JECFA (Joint Expert Committee on Food Additives), there was no reason to be suspicious about the potential carcinogenicity of the *oryzae* metabolite, β- nitropropionic acid, as claimed earlier; and employment of α- amylase, proteases and lipase were agreed as food components. It was also stated that the widespread use of *A. oryzae* in food production was a fact (Anonymous, 1987).

Actually *A. oryzae* is a "domesticated variety" of aflatoxin-producing *A. flavus*. The organism is thought to have been modified in morphology and loss of certain metabolic activities as a consequence of thousand years of cultivation (Barbesgaard, *et al* 1992). Aflatoxins are potent carcinogenic and mutagenic secondary metabolites produced mainly by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. These species can contaminate several food commodities including cereals, peanuts and spices (Mayer, *et al.*, 2002).

In the past, there have been discusssions on the pathogenicity of an *A.oryzae* strain (NRRL strain 1988) due to the aflatoxin production. It was claimed by El-Hag and Morse that NRRL strain 1988 approved for use in food processing produced aflatoxins B_1, B_2, G_1, G_2 on cowpeas (*Vigna sinensis*) expressing the aflatoxin yield depended on the substrates employed as known earlier (El-Hag and Morse, 1976). That claim was denied by Fennell (1976) who told aflatoxin tests on corn, wheat, rice, millet, cowpeas, soy beans and liquid medium was negative for hundreds of strains of *A. oryzae* sampling food and enzyme industries. Fennell also claimed *A. oryzae* strain should have been replaced by *A. parasiticus* through mite infestation in Morse& El-Hag laboratory. Morse (1976) further claimed that the question still remained open and this paper was published within the same article. This was replied by other authors who classified the mold as *A. parasiticus* and assured the safety of *A.oryzae* NRRL strain. It was reported that, no variant of aflotoxin producer *A. oryzae* existed (Stoloff, 1976). The further explanation by Morgan-Jones (1976) stressed the fact that mycological taxonomy involved delimitation of species as well as diversity and variability. Nowadays, the discussions on the safety of *A. oryzae* is over and both organism and its enzymes are accepted as food constituents as told earlier.

A. oryzae is an aerobic, filamentous fungus whose olonies are initially white, later yellowish-greenish. Older colonies appear darker. The second stage of reproduction is not known, but conidia (asexual spores) are easily formed and released into the air. Sporulation is sparse and conidia have diameters ≥ 7.0 μm. Optimal growth temperature is 32-36°C. So far, *A. oryzae* has been isolated in soil or on decaying plant material in India, former USSR, former Czechoslovakia, Tahiti, Peru, Syria, Italy and from the air in the USA and the British Isles. As the fungus has a relatively high optimal growth temperature, very few reports claim its isolation in the continental Europe (Barbesgaard, *et al.* 1992).

1. 6 Industrially important enzymes: lipases

As lipases (triacylglycerol acylhydrolysases, E.C. 3.1.1.3) are responsible for development of hydrolytic rancidity of various food products, they have been extensively studied (Petrovic, *et al.* 1990). Lipases are excreted by microorganisms to assist in digestion of lipid materials and catalyze the hydrolysis of triacylglycerols and glycerol. They also catalyze acyl exchange reactions in non-polar medium in the presence of a small amount of water (Pabai, *et al.* 1995). The microbial sequence of lipases from both bacterial and fungal origins are known to vary between 258 to 544 amino acid residues. Two fungal sequences have been found in *Geotrichum candidum*, named I and II. *Geotrichum candidum* lipase II is 544 residues long, the longest of fungal lipases. Other known fungal lipase sequences were from the phycomycete fungus *Rhizomucor miehei*, previously classified as *Mucor miehei*, from *Humicola lanuginosa* and from *Penicillium camemberti* U-150 (Wooley and Petersen, 1994). Another *Penicillium* strain (*Penicillium roqueforti*) synthesizes lipases that induce the production of the characteristic taste of roqueforti and blue cheese. It is specific for lysis of triacylglycerols of small molecular mass and lower fatty acids liberated are used as precursors for production of methyl ketones and secondary alcohols (Petrovic, *et al.* 1990).

Lipases cleave ester bonds in glyceride and related molecules using a nucleophilic mechanism via an activated serine. They are inactive under aqueous conditions and their catalytic activation occurs within the lipid-water interface (Wooley and Petersen, 1994). Lipase activity is closely related to the quality of the formed emulsion (Vordewülbecke, *et al.* 1992). The unique characteristics of being stable in

both aqueous and non-aqueous media make lipases suitable for synthesis processes, which would otherwise require very harsh processing conditions like high temperatures/pressures and pH extremes. Lipases are common in having the catalytic triad (Ser, Asp, His) as serine proteases. Both enzyme families catalyze a fundamentally similiar hydrolytic reaction, cleaving an ester bond (Wooley and Petersen, 1994).

In the dairy industry, *Mucor miehei* lipase contributes to romano, domiati, feta, fontina, ros and rami cheeses production, whereas *Aspergillus oryzae* and *Aspergillus niger* lipases contribute to cheddar, manchego and blue cheese in cheese-making and accelerated cheese-ripening. Current applications include flavor enhancement of cheese-like products and lipolysis of butterfat and cream. Lipases also are applicable to imitation cheese production and EMC (enzyme-modified cheeses). In the household detergents industry, the applicability of lipases are decreased when higher laundering temperatures and highly alkaline conditions are desired. *Humicola* lipase expressed in *Aspergillus oryzae* ("Lipolase" of Novo Nordisk) has resulted in the introduction of several suitable preparations (Wooley and Petersen, 1994).

Another possible application area of lipases is in the oleochemical industry where hydrolysis, glycerolysis and alcoholysis are common processes. Lipases bring specificity to transesterification processes of natural oils, unlike chemical catalysts that randomise the fatty acids in triglyceride mixtures and warrant the formation of products with the required physico-chemical characteristics (Wooley and Petersen, 1994). Due to the trends in food industry, triglycerides possessing valuable dietetic and nutritional properties are favored in order to be used as milk-fat substitutes and they are obtainable by acidolysis of palm-oil (Wooley and Petersen, 1994).

Due to the environmental concerns, the enzymatic synthesis processes are more advantageous than chemical ones. That is the case for solvent-free esterification of simple alkyl-glycosides using molten fatty acid and immobilised *Candida antartica* lipase. The advances in lipase-based technology helps reducing energy costs and preventing formation of undesirable by-products. Mono- and di-esters of monosaccharides, (poly)glycerol-based surfactants, lysopphospholipids may be produced enzymically in the presence of lipases. There are also some personal-care products, pharmaceuticals, agrochemicals, polymers where various types of lipases are employed in their fabrication (Wooley and Petersen, 1994).

Lipases achieve ester production by synthesis from free acid and hydroxyl groups or by ester exchange or transesterification. Transesterification reactions include alcoholysis, acidolysis and interesterification all of which proceed through the formation of the acyl-enzyme intermediate followed by nucleophilic attack (Lortie, 1997).

The two main methods to determine lipase activity are pH-stat and spectrometric assays. pH-stat assays are employed in constant temperature reaction vessels in the presence of emulsified triacylglycerols treated with lipases/lipase solutions. The fatty acids liberated decreases pH and this decrease is compensated by automatic titration of a basic solution, maintaining the original pH value. There are a few different procedures to determine lipase activity by means of pH-stat assays. One of them employs Triolein (1 g) and Triton – X (30 ml) and NaCl (0.9 %, 200 ml) as emulsification solution (pH 7.5 with 1 M. NaOH) and so the solution is heated to 55°C, cooled and treated with enzyme sample. The mixture in the vessel is held at 30 °C and titrated with 10 mM NaOH. One unit of enzyme liberates μmol fatty acid min^{-1}, which is equivalent to 0.1 ml of NaOH consumed. Triolein can be replaced by tributyrin (Vorderwulbecke, *et al.* 1992). Another pH-stat assay using tributyrin as substrate employs gum arabic, sodium taurocholate, $CaCl_2$ and NaCl. The enzyme sample is filtrated and then purified from its salts using a Sephadex G-50 column before the assay (Petrovic, *et al.* 1990). The activity of an *Aspergillus niger* lipase purified to a specific activity of 729 U/mg (approximate molecular weight of 40 kDa on SDS PAGE) was determined by emulsifying 10% (v/v) olive oil and 1% (m/v) gum arabic in distilled water, in a sonicator for 2 mins and mixing with diluted enzyme and assay buffer (1 mM Tris-glycine, 0.1 M NaCl and 5 mM CaCl2, pH 5.5). The reaction was stopped by addition of Cu^{2+} reagent (2.5 ml) and chloroform (5.0 ml). The pH stat assay was carried out in the absence of bile salts and BSA, as BSA was shown to be a strong inhibitor of lipolysis in spite of its role as a complexing agent for fatty acids released on hydrolysis of the oil (van Heerden, *et al.* 2002). A final pH-stat procedure proposes using triolein as substrate and emulsifying it via sonication again in gum arabic with deoxycholic acid and NaCl, adjusting pH to 8.0. Titration with 0.02 M. NaOH is to be continued for 2-10 minutes (Woolley and Petersen, 1994).

Spectrophotometric methods of lipase activity determination include several methods like ρ-nitrophenol release from ρ-nitrophenol palmitate and reactions with artificial substrates. 10 mM p-nitrophenyl palmitate dissolved in acetone is emulsified in the presence of sodium deoxycholate, gum arabic and Tris-HCl, treated with lipase sample and the reaction is stopped by Na_2CO_3. This causes ρ-nitrophenol release from ρ-nitrophenol palmitate and assayed at 410 nm spectrophotometrically (Tamerler *et al.* 1999). pNPP (90 ml) in propane-2-ol is an artificial substrate employed in determination of lipase activity. The solution mentioned is mixed with Triton-X-100 (2 g), gum arabic (0.5 g) dissolved in 450 ml buffer (Tris-HCl 50 mM, pH 8.0). Measurement of liberated ρ-nitrophenol at 410 nm denotes the lipase activity (Vorderwulbecke, *et al.* 1992). TBTG assay (at 412 nm) and DGGR assay (at 571 nm) are other ways of determining lipase activity (Vorderwulbecke, *et al.* 1992). In order to determine the activity of *Aspergillus niger* lipases, 1ml. enzyme solution is mixed with 200 mM triglyceride, 0.4 g gum arabic, 1 ml. of 100 mM $CaCl_2$ solution, 4 ml of 100 mM Na-phosphate buffer (pH 7.0) and incubated at 40°C for 2 h with constant agitation (200 rpm) in conical flasks. The reaction was stopped by addition of 1 N H_2SO_4 (1 ml) and the released fatty acids extracted with petroleum ether (40-70°C) and titrated with 0.01 M KOH to pH 7.0. (Pokorny, *et al*).

1. 7 Targets of the work

Extracellular secretion of proteins in filamentous fungi is a question of appropriate porosity as well as capability of synthesis. Apical pores have a capacity of secreting proteins with M_r values smaller than 15000-20000, so it was thought that proteins could diffuse from the most stretched and porous outer region of the wall. As a consequence of growth in sandwiched solid cultures and localization of growth, glucoamylase secretion of *Aspergillus niger* was shown to be demonstrated at the tips of growing leading hyphae. It was also shown by various authors that *Neurospora crassa* strains defective in wall synthesis oversecrete enzymes, so the claim suggesting the wall to be a barrier in protein secretion is valid. It is never so easy to exactly determine the protein secretion distribution throughout the hyphae in liquid cultures as filamentous fungi simultaneously extend and secrete proteins (Wösten, *et al.* 1991).

Staining of free *Aspergillus awamori* CBS 115-52 mycelia has shown that subapical regions of hyphae have higher RNA concentrations indicating higher protein synthesis rates. There was a weak color gradient from pellet center to perimeter, so increase of hyphal tips were observed. DNA-replication investigation by sandwich immunofluorescence method also determined that growth in pellet center stopped, whereas hyphae kept on growing at the expanding zone (periphery). The highest protein synthesis rates were not observed at the very end of hyphal tip but in the subapical domain. Also the vacuoles were formed in the oldest part of hyphae (Freudenberg, *et al.* 1996), so one can claim that sparse mycelia has lower rates of protein synthesis than pellet forms due to vacuole formation and aging within increasing length. Even if pellet corpus lacks in reproduction and synthesis as a consequence of quicker branching, it yields in formation of shorter branches with protein synthesis rates increasing total proteins synthesized in long-term. The densest pellets reached zero reproduction and protein yield after highest rates of branching and protein synthesis of longest periods possible, however the peripheral mycelia exhibited the fastest synthesis rates and pellet formation. That is one of the reasons for why pelleted growth might be more preferable when industrial-scale enzyme production is targeted.

As it is thought that the total area of extending cell wall increases with increasing number of hyphal tips, it is assumed that the protein secretion in filamentous fungi occurred at the fungal tip. In a study with highly branched mutants of *Aspergillus oryzae* obtained by UV or nitrous acid treatment has shown very little correlation between increased branching and protein secretion (glucoamylase, α-amylase). Earlier it was found that branching and secretory pathways could be affected simultaneously by certain types of mutations, such as those observed in glucose-6-P dehydrogenase and phosphoglucomutase mutants of *Neurospora crassa.* This is a pleiotropic response causing an increase in both branching and enzyme production. It was certain that strains with high branching mycelia decreased the viscosity of cultures as well as energy input required for adequate mixing which was quite favorable in terms of simplifying the process and cost reduction (Bocking, *et al.* 1999).

Glucose oxidase synthesis by a recombinant strain of *Aspergillus niger* was studied in submerged cultures. The fungal pellets were characterized and the outermost

region was found to be the densest and has done the biggest contribution to synthesis, whereas the innermost region the least dense and could be a hollow region when pellets were more than or equal to 2.5-3 cm in diameter. Decreased pellet size was an indicator of increased mycelial density (El-Enshasy, *et al.* 1999).

On the other hand, agitation shocks due to high and inequal distribution of agitation pressures had influences on mycelia even if no fractionation was observed. Pellets of *Aspergillus niger* ATCC 26036 was fractionated at a shear rate of 5 Pa and branching of free mycelia was induced in order to resist mechanical damage (Mitard and Riba, 1988).

Consequently, it is possible to claim that fungal morphology might effect protein synthesis, especially commercial enzyme production, and may be affected by processing conditions. If any kind of manipulation that was advantageous for both production and processing had existed, it would probably have been correlated to fungal morphology. This work concentrates on modifying fungal morphology both in solid and liquid cultures. The agents employed within the work in order to provide the effect mentioned above are a group of chemicals called paramorphogens. Table-1 summarizes the mode of action of some paramorphogens, although it was stated that these modes have not been elucidated yet for all paramorphogens such as in the case of cellobiose, glucosamine, 3-*O*-methylglucose, sclareol (Trinci, *et al.* 1994):

Table 1.1: The mode of action of some paramorphogens in the experimental works.

Paramorphogens	**Mode of action of paramorphogens**
L- sorbose	Inhibition of β-1-3 glucan synthetase
Edifenphos	Inhibition of phosphotidylcholine and chitin biosynthesis
Validamycin A	Inhibition of phosphotidylinositol biosynthesis
Cilofungin, Echinocandin B, Tetrahydroechinocandin B	Inhibition of β-1-3 glucan synthetase
Cyclosporin A	Inhibition of calmodulin-dependent protein phosphotase
Lysocellin	Inhibition of phosphotidylinositol turnover

(Ref: Trinci, *et al.* 1994)

As listed earlier on different fungal cultures, these chemicals seemed to have capability to enhance fungal branching tested. Here, the effects of different paramorphogens on linear extension of *Aspergillus oryzae* mycelium were investigated both on solid state and submerged fermentations. The effect of paramorphogens was first tested on solid environment using various paramorphogens concentrations. Also the effect of inoculum was investigated in terms of the spore or

mycelial inoculation. The promising paramorphogens were examined in liquid cultures in detail. The morphological differences as well as the enzyme production was followed in these cultures. Possible correlations between lipase production and mycelial extension characteristics of the same organism together with morphological changes were investigated. Both steps included fungal image analysis utilizing a densitometer, a petri microscope and a fluoresence microscope with a digital camera attachment. Samples taken from liquid cultures were treated with an offline pH stat system in order to determine lipase activity.

2. MATERIALS and METHODS

2.1 Fungal Strain and its maintenance

The filamentous fungi used in this study was *Aspergillus oryzae* (CL01), which was naturally, isolated soil samples. It has kindly provided from Professor Tajalli Keshavarz, University of Westminster, School of Bioscience, Department of Biotechnology, London, UK.

A. oryzae was preserved as slants or slopes. Spores maintained on potato dextrose agar (PDA) slants were scrapped off using sterile glass beads. The sterilization duration was 30 minutes at 121 °C for glass beads and 15 minutes at 121 °C for all media and glassware excluding glass petri plates. The glass beads were placed in 100 ml shake flasks and autoclaved. After cooling, sufficient number of glass beads were poured onto the PDA slants and were moved up and down the surface of the slants. The spores gaining mobility or sticking the beads were collected adding ~ 10 ml of sterile water and transferred to another sterile shake flask. This stock was used as the *initial spore solution* to prepare spore and mycelial solutions. The *initial spore solution* was inoculated into PDA containing petri plates. Slopes were prepared by pouring 25mL of autoclaved medium (PDA) into sterile petris aseptically. The PDA containing petri plates were inoculated spreading ~ 2-3 of the *initial spore solution* The PDA containing petri plates were inoculated spreading ~ 2-3 ml. of the *initial spore solution.* Inoculum was incubated at 26°C for 5-6 days then kept in the fridge (4°C) to be used when needed.

2.2 Media

Potato Dextrose Agar (PDA) per liter

Potato Extract	4.0g
Glucose	20.0g
Agar	15.0g

Liquid Medium for Growth and Production per liter

Mycological Peptone	10 g
Dextrin	20 g
KH_2PO_4	5 g
$MgSO_4.\ 7H_2O$	0.5 g

The pH of the medium was adjusted to 5.5 with 0.1 M NaOH before being autoclaved at 10 psi for 15 min.

2.3 Buffers

Tris-glycine buffer, 25mM, pH 7 kept at room temperature.

Lipase Emulsifier

Gum Arabic	1 %
Tributyrin	10%

Tributyrin was emulsified in the presence of gum arabic getting use of ultrasonication.

3 ml of the emulsified substrate was added to 45 ml of buffer placed in the reaction chamber of the pHstat system.

2.4 Method for Growth on Solid Medium

2.4.1 Materials, equipments and solutions used for solid media experiments

Sterile petri plates, glass beads (2 mm), sterile glassware, water and falcon tubes, potato dextrose agar (PDA) (Merck), tween 20 (Merck), tube mixer (Heidolph), blender (Waring), magnetic stirrer (Labworld), incubator (28 °C) (Nüve), orbital shaker (Biolab and B. Brown), densitometer (Biolab), Fluoresence microscope (Olympus BX60) supported with a digital camera and PC adaptable software (Spot RT Software v3.2), disecting microscope (SOIF), mycelial solution, spore solution, D (+) Glucose (RdH), sodium deoxycholate (Fluka), polyethylene glycol 4000 (Merck), L (-) Sorbose (Fluka).

2.4.2. Preparation of spore solution

The plate stocks were exploited in order to prepare spore solutions as explained in strain maintenance section. The spores transferred to the 0.1% sterile Tween 20 solution was prepared in order to avoid aggregation were used as the *spore solution* solid media experiments within the next 24 hours to ensure standard metabolic activity. Spore solution was mixed about a minute at the tube mixer gently to make sure the separation of spores achieved..

2.4.3 Preparation of the mycelial solution

The plate stocks were exploited in order to prepare mycelial solutions. Again, 0.1% sterile Tween 20 solution was prepared in order to avoid aggregation. 10-15 ml. of the solution was taken into a sterile falcon tube and mixed with the colonies on one of the stock plates. The colonies spread plate-wide were collected using an inoculation loop or sterile spatula. After gentle mixing about a minute at the tube mixer, the mycelia - Tween 20 solution become ready for inoculation. The contents of the falcon were added to 100 ml *liquid growth media* containing dextrin (2%), mycological peptone (1%), KH_2PO_4 (0.5%), and $MgSO_4$. $7H_2O$ (0.05%). pH of the liquid growth media was adjusted to 5.5 before sterilization. After addition, the culture was grown for 96 hours at 30 °C and 120 rpm in an orbital shaker.

At the last step of preparation of the mycelial solution, culture broth was placed in a sterilized blender and blended for 1 minute at intermediate speeds. The blended culture was employed as *mycelial solution* within the solid media experiments and the next 48 hours to ensure standard metabolic activity.

2.4.4 Inoculation on the solid media

The paramorphogens employed were sodium deoxycholate, polyethylene glycol 4000, L(-) sorbose and D (+) glucose. PDA plates were prepared as explained earlier, paramorphogenic plates contained as much PDA as control plates and the defined amount of the paramorphogenic chemical.

In the first step, 2 different (1 low, 1 high) concentrations of the paramorphogens were added to the plates and both *mycelial* and *spore solutions* inoculated. Observing the effects of the chemicals and solutions, the ones with the most desired responses were selected and 5 different concentrations of the "best" two chemicals were inoculated with the "better" solution. The concentrations were extended until growth inhibition was observed. Below inhibitive concentrations, "best" responses of two chemicals were countered. The results were compared to that of control plates.

After the preparation, the solid media wer held at 4 °C overnight to ensure structural integrity. Both mycelial solutions and spore solutions were used for the inoculations. Inoculation of media consisted of micropippetting 3 μL of one of the solutions in 3 different places on the petri plate surface (Figure-1). The inoculated petri plates were held at a 28 °C incubator for 48 hours. All inoculations were done in quadriplicate at least and considering three colonies were formed on each plates, at least 12 colonies were observed for any single data. Each experiment was controlled getting use of PDA plates.

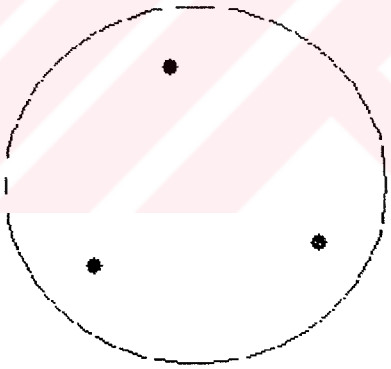

Figure 2.1: Inoculation of solid media.

2.5 Method for Growth and Production in Liquid Medium

2.5.1. Materials, equipments and solutions used for liquid media experiments

Olive oil (Fluka), gum arabic (J.T.Baker), tributyrin (Merck), starch (RdH), mycological peptone (Acumedia), NaH_2PO_4 (RdH), KH_2PO_4 (RdH), $FeSO_4$. $7H_2O$ (RdH), $MgSO_4$. $7H_2O$ (BDH), $MnSO_4$. $7H_2O$ (RdH), $CaCl_2$ (Merck), $ZnSO_4$. $7H_2O$ (RdH), also all the materials used within the solid culture experiments, micro

centrifuge (Beckman Coulter), cell disruptor (B. Braun Labsonic U), pHstat system (Metrohm 18 Stat Titrino), water reservoir equipped with heating and cooling units (Heto), ethyl alcohol (J.T.Baker), diethyl ether (Fluka), mono and di basic phosphate salts (RdH), KOH (Merck), tris-glycine (Merck), NaCl (BDH) methyl orange, appropriate NaOh (RdH) and HCl (Merck) solutions, dark colored glass bottles, buchner funnel, filter paper (Whatman No:4 equivalent), mycelial solution for liquid culture, spore solution for liquid culture.

2.5.2. Preparation and inoculation of standard liquid media

Standard liquid media consisted of 20 g. mycological peptone, 12 g. NaH_2PO_4, 2 g. KH_2PO_4, 0.005 g. $FeSO_4$. $7H_2O$, 0.3g. $MgSO_4$. $7H_2O$, 0.25 g. $CaCl_2$, 0.03 g. $ZnSO_4$. $7H_2O$, 0.015 g. $MnSO_4$. $7H_2O$, 10 g. starch (slowly hydrolyzed carbon source), 15 ml. olive oil (lipase inducer) per liter. pH was adjusted to 6.

Mycelial solution for liquid culture was prepared in the same way as *mycelial solution* used for solid media. *Spore solution for liquid culture* was prepared on PDA slants. In order to prepare PDA slants, 30-40 ml sterilized PDA solution was poured into sterilized dark colored glass bottles and bottlenecks were placed on a few centimeters' height to provide inclined surface within the bottle after solidification of potato dextrose agar (PDA). Then PDA slants were held at 4 °C overnight to ensure structural integrity. Later slants were inoculated rubbing an inoculation loop throughout the slant surface that was previously rubbed through a *stock plate* surface. Inoculated slants were incubated at 28 °C for 5-10 days until sufficient spore formation was observed. The spores formed were collected getting use of glass beads just in the same way as the one employed prior to the solid culture experiments. The spore number per milimeter was determined using a haemacytometer. Spore solution was held 4 °C and used within the following 7 days.

2.6 Analysis Techniques

2.6.1 Determination of hyphal extension and pellet formation on solid state

The hyphal extension and pellet formation were observed getting use of a densitometer (Biolab), a light microscope (Olympus BX60) supported with a digital

camera and a PC adaptable software (Spot RT Software v3.2) and a petri microscope (SOIF). Just after the inoculation moment (0^{th} hour), 5^{th} hour, 10^{th} hour, 15^{th} hour, 25^{th} hour, 30^{th} hour, 35^{th} hour, 48^{th} hour, the colony lengths were measured getting use of Olympus BX60 (4x objective) and Spot RT Software v3.2. At the 48^{th} hour, densitometer images were also taken to compare macro morphologies. The petri microscope was exploited where colonies are too large to be observed by a 4x objective.

2.6.2 Lipase Assays

Proving the presence of lipase in fermented liquid media

Prior to determining liquid culture strategy and lipase assay to be applied, presence of lipase in the broth was checked. The experiment scheme within TS 894 was roughly employed to the 96 hours fermented, but not blended *mycelial solution* of solid media. The procedure was simplified as sampling each day until 168 hours of fermentation, incubation for 30 minutes at 30 °C and 200 rpm, stopping reaction (lipase activity) with the addition of ethyl alcohol:diethyl ether (1:3) mixture and titration with 0.1 N KOH in the presence of a few drops of an appropriate indicator (1% methyl orange). The experiment was controlled incubating olive oil without the addition of sample, but adding alcohol-ether solution and titration just in the case of samples. Comparison of titration results for samples and control clearly demonstrated the presence of lipase activity within the samples.

Lipase assay by pHstat system

A pHstat system is an automatic titrator that adjusts pH of a reaction chamber to a set value. The reaction chamber is initially prepared at the set value and the reaction is started for a defined period of time and how much ever pH change does a reaction yield is measured by terms of the titrated acid/base in order to catch again back the set pH. This system is quite beneficial considering lipase activity determination. When the reaction chamber is a pH and temperature controlled system, in the presence of an appropriate pH buffer, emulsified triacylglycerols can be fractionated into its fatty acids after addition of lipase (in this case samples from fermented liquid media), thus pH of the system decreased. The reaction is lasted independently at the chamber for a previously determined period of time. Then automatic titrator is started

and does titration until the pH before reaction is caught. This operation calculates the volume of a standardized NaOH solution that eliminates the effect of added lipase sample. As lipase activity is defined by means of titrated base volume many authors, the pHstat system is probably the most precise method.

The preciseness limit of base (0.01 M NaOH) volume within the pHstat equipment used was 1 µl.

The installation system of the pHstat system

The dark colored base tank (carrying 0.01 M NaOH) was placed on the base provided on the digital unit.

The stirrer and reaction chamber were placed on the magnetic stirrer unit.

The appropriate electrodes were placed in the reaction chamber to ensure pH and temperature control.

The reaction chamber was connected to digital unit, base tank and water reservoir equipped with heating and cooling units (in order to ensure water temperature accuracy).

The reaction chamber was a jacketed one, so heat transfer between the reservoir and chamber was provided by means of the continuously flowing water.

Remote control keyboard was connected to the digital unit.

All units were connected to electric current.

pH electrodes were calibrated and getting use of keyboard set values were specified.

After the steps mentioned, system was ready to be used. The optimum pH of lipase of *Aspergillus oryzae* was determined as 6.5 after reaction and titration operations were carried out for a 7 day liquid culture filtrate in the 4.5 to 9 pH interval. The each experimented value was tried as the set pH of the system. The biomass was seperated by a predried Whatman No:4 equivalent filter paper placed on a Buchner funnel. The filtrate was sampled several times for the values experimented within the 4.5 to 9 pH range. The titrated amounts were recorded each time. When the experimented values were compared, the highest titration amount determined pH optimum. The amounts of buffer and substrate were also optimized using 1 ml lipase sample in each case.

Determination of lipase production in liquid media

Samples for observing lipase production were taken on a daily basis. The lipase activities were measured in terms of 0.01 M NaOH titrated after one minute of reaction at set pH 6.5 and constant temperature (30 °C). The reaction chamber contained 45 ml. Tris-glycine buffer [1 mM Tris-glycine (pH 6.5), 0.1 M NaCl and 5 mM $CaCl_2$] and 1.5 ml emulsified substrate [10% tributyrin (the triacylglycerol to be emulsified), 1% gum arabic (emulsifier) solution in distilled water]. The above mentioned substrate solution was emulsified by the help of a cell disruptor. After the addition of emulsified substrate onto the buffer solution, the pH was set to the 6.5, the pH optimum. 1 ml sample taken from different liquid media was added each time to the system and titration with NaOH was started 1 minute later. The buffer and substrate was renewed each time to ensure that accumulation of samples or fatty acids did not create inhibition of enzyme activity. Phosphate buffer with appropriate pH was clearly shown to inhibit the determination of lipase activity. Buffer contained mono and dibasic phosphate salts and its pH was adjusted to 6.5 prior to reaction in reaction chamber of pHstat system.

3. RESULTS and DISCUSSION

In the scope of this work, the effect of paramorphogens on fungal morphology was studied mainly on solid cultures. The methods explained in detail in the materials and methods part of the thesis, were used to study the effects of four different paramorphogens: NaDC, PEG 4000, L(-) sorbose and D (+) glucose on growth, morphology and productivity of the filamentous fungi *Aspergillus oryzae.* Different methods were carried out to study the effect of different paramorphogens with different concentrations. The fungus was first grown on solid medium, potato dextrose agar (PDA) for activation, preparation of slopes and to study the effect on morphology and colony radial growth for certain paramorphogens. PDA plates were exploited as control plates and this medium was also incorporated into paramorphogen containing media. *A.oryzae* was grown in liquid medium (shown below), to study the effect of sodium deoxycholate and L(-) sorbose on hyphal extension and production of an extracellular enzyme, lipase, after both chemicals showed a significant effect on solid medium. In solid culture experiments the best paramorphogenic effect was exhibited by sodium deoxycholate at 0.01% and 0.02% concentrations, whereas L(-) sorbose was the second most effective at 0.5 M. Higher concentrations resulted in growth inhibition. Data were supported by hyphal extension measurements, microscope and densitometer images. Using TS (Turkish Standard) 894, measurements have shown the presence of lipase activity in liquid cultures. In the presence of L (-)sorbose, the maximum lipase activity was reached at the 4th day and a sharp decrease was observed following days. Sodium deoxycholate caused initially lower activities, but the activity was maintained for the following two days.

3.1 Evaluation of paramorphogenic compounds used in solid media.

As a first step in our studies, D (+) glucose, L (-) sorbose, sodium deoxycholate and polyethylene glycol 4000 (PEG 4000) as paramorphogenic chemicals, were tested on *Aspergillus oryzae*. The organism was grown on potato dextrose agar (PDA). Inititally, a minimum and a maximum concentration, for four of the chemicals and two different inoculation types were studied (mycelial solution and spore solution) hence, 16 different variables and their effects on the organism's morphology, branching and pellet formation properties were evaluated. The results were compared with the control plates that contained no paramorphogenic compounds. After the preparation, the solid media were held at 4 °C overnight to ensure rigidity of gel structure. The inoculated petri plates were held at 28 °C for 48 hours. All inoculations were done at least quadruplicate and considering three colonies were formed on each plates, at least 12 to 24 colonies were observed for any single data shown on graphs. The colony lengths shorter than 0.8 cm were measured using the light microscope. The sensitivity of the measurements with a 4x objective was within 1 nanometer. Longer colonies were measured using a ruler with a sensitivity of 0.05 cm. The densitometer was employed in the comparison of control plates and plates with paramorphogens, but no measurements were taken from these images. Table 3.1 shows all different concentrations of all paramorphogens employed.

Table 3.1: Concentrations of the paramorphogens and inoculation type within the solid media.

First step	**Mycelial solution, Low concentration**	**Mycelial solution, High concentration**	**Spore solution, Low concentration**	**Spore solution, High concentration**
D (+) Glucose	10 mg/ml	50 mg/ml	10 mg/ml	50 mg/ml
NaDC	0.005%	0.01%	0.005%	0.01%
PEG 4000	0.005 M	0.0125 M	0.005 M.	0.0125 M.
L (-) Sorbose	0.1 M	0.5 M	0.1 M	0.5 M.

Second step *(All inoculations are mycelial)*	**C1**	**C2**	**C3**	**C4**	**C5**
NaDC	0.005 %	0.010 %	0.020 %	0.050 %	0.100 %
L (-) Sorbose	0.1 M	0.3 M	0.5 M	1 M	2.5 M

Third step *(All inoculations are mycelial)*	**C 1**	**C2**
NaDC	0.01 %	0.02 %
L (-) Sorbose	0.5 M	-
Cross-Related (Both NaDC and L (-) Sorbose)	*NaDC* : 0.01 % + *L (-) Sorbose* : 0.5 M	*NaDC* : 0.02 % + *L (-) Sorbose* : 0.5 M

Figure 3.1 and Figure 3.2 summarize the effects of chemicals, their concentrations and inoculation type:

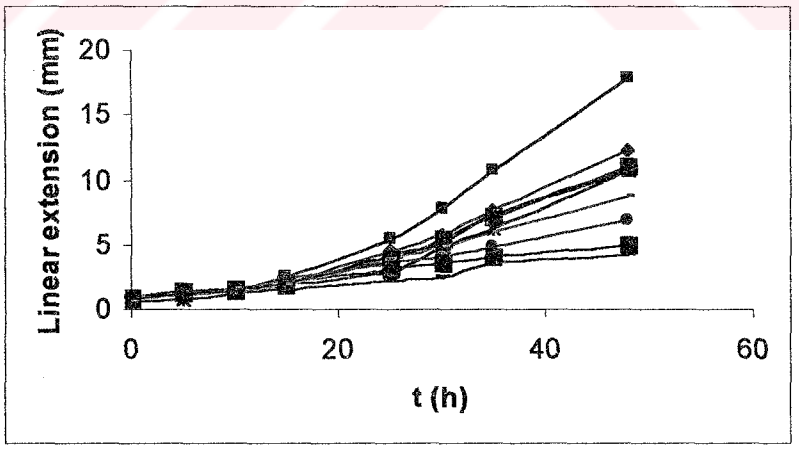

Figure 3.1: The effects of different paramorphogens with minimum and maximum concentrations on linear extension of fungal colonies. Inoculum is mycelial solution. (PDA:♦, PEG 4000, 0.005 M: ■, PEG 4000: 0.025:▲, NaDC, 0.005% M: ×, NaDC, 0.010%: *, L-sorbose, 0.1 M: ●, L-sorbose, 0.5 M: +, Glucose 10 mg/ml: -, Glucose 50 mg/ml: —).

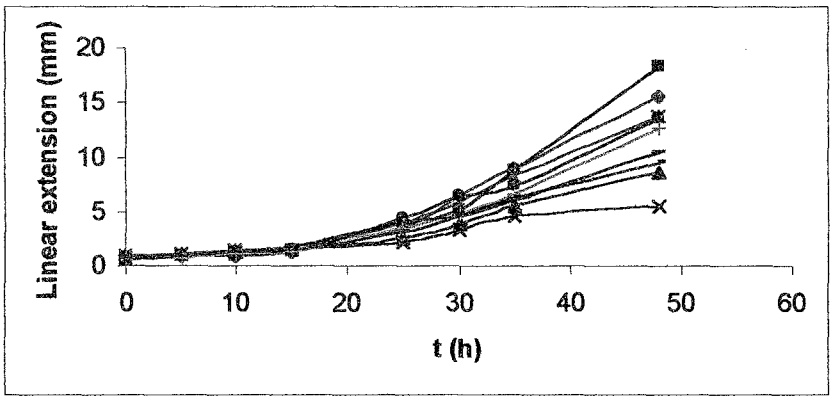

Figure 3.2: The effects of different paramorphogens with minimum and maximum concentrations on linear extension of fungal colonies. Inoculum is spore solution. (PDA: ♦, PEG 4000, 0.005 M: ■, PEG 4000: 0.025: ▲, NaDC, 0.005% M: ×, NaDC, 0.010%: *, L-sorbose, 0.1 M: ●, L-sorbose, 0.5 M: +, Glucose 10 mg/ml: -, Glucose 50 mg/ml: —).

As it may be inferred from both graphs, following tables and densitometer images, spore inoculations resulted in less branching with mostly longer mycelial extensions, sometimes growth inhibition. Even in the control plates inoculated with spores, the average final lengths were 30% higher than those inoculated with mycelia suspension. Spore solutions and mycelial solutions were prepared as detailed in Section 2.4.2 and Section 2.4.3. In plates inoculated with spores, a higher variation was observed in linear extension values, paramorphogenic effect could not be claimed in all cases. Considering growth inhibition, variations, colonies with longer extensions and branching level, spore inoculation was discontinued in the following steps. Plates inoculated with mycelial solution had higher tendencies to reproduce branched, shorter (linear extension limited, but not the specific growth rate) colonies. Paramorphogens were defined as chemicals causing highly branched colonies without affecting specific growth rate and paramorphogenic effect was assumed to be more successful by enhanced branching, so the most successful paramorphogenic effect was provided by sodium deoxycholate and L (-) sorbose was the second. The effect of different compounds and concentrations on average linear extension of colonies in all kinds of petri plates and their statistical evaluation including standard deviation, absolute error and F and T test results were summarized on tables between Table 3.2 and Table 3.18. Also densitometer photos of colonies, which were grown on control (PDA) and paramorphogen containing plates, were added (Figures from

3.3 to 3.14 and figures in Appendix 1). Any single point on tables represent 12 to 24 different colonies observed and any figure represent more than or equal to 4 different petri plates. The figures compare control plates and paramorphogen containing plates and also different concentrations of paramophogens in paramorphogen containing plates.

Control Colonies -PDA (Mycelial inoculation): When the organism was cultivated from mycelial inoculum, the growth rate was higher in comparison to when it was cultivated from spore inoculum. The organism had a faster growth rate when mycelia were inoculated compared to the growth rate with spores. Pellet form was not observed. Finally colonies were 1.07 cm. long.

Table 3.2: Average linear extension of colonies on control (PDA) plates inoculated with mycelial solution and their statistical evaluation.

	time (h)							
PDA (control), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value (μ)	920,66	1343,47	1611,6	1898,98	4119,14	5125	7343,75	10687,5
Absolute Error (%)	24,79	8,89	9,35	4,33	5,26	12,74	9,35	15,67

Control plates inoculated with mycelial solutions formed colonies that are slightly longer than 1 cm after a period of 48 hours. This value is lower than that of spore inoculated control plates. Linear extension values for PEG 4000 containing plates inoculated with mycelial solution were slightly above that value, highly above for glucose containing plates. Each inoculation consisted of 3 microliters of mycelial solution.

Glucose (Plates containing the low concentration and inoculated with mycelial solution): Colonies on these plates were than the ones grown within the spore solutions were formed. Mostly their intensity was as in the center, but not at all could be called as pellets. Average final length after 48 hours of incubation was 1.225 cm. This value was 21.6% lower than the corresponding result when the inoculum was sore solution, but this concentration did not yield an enhanced branching and average final extension was 12.76% higher than that of the mycelial control.

Table 3.3: Average linear extension of colonies on minimum glucose (10 mg/ml) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
Glucose (10 mg/ml), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value (μ)	884,01	1460,43	1502,56	2081,16	4437,5	5687,5	7562,5	12250
Absolute Error (%)	23,10	30,84	6,85	9,19	5,39	9,75	1,65	10,27
T-test	0,305	0,174	0,227	0,269	0,315	0,185	0,406	0,081
F-test	0,859	0,933	0,797	0,115	0,021	0,222	0,001	0,386

Glucose (Plates containing the high concentration and inoculated with mycelial solution): Compared to plates inoculated with spore solutions as well as the plates containing lower concentration of glucose, longer mycelial extensions were observed and the hyphae spread in a large area. Average final extension was approximately 1.79 cm. The longest extensions among all the control plates and paramorphogen containing plates were formed on these plates. The final extension value was 31.5% higher than that of the plates containing lower concentration of, so no increase in paramophogenic effect due to the increase in glucose level could be seen.

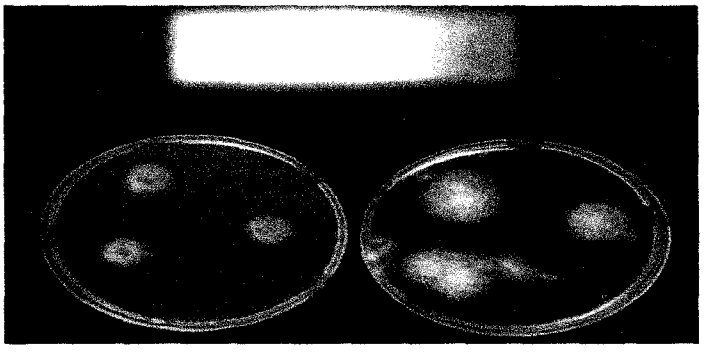

Figure 3.3: The comparison of mycelial conrol (PDA) plate with a plate containing 50 mg/ml glucose.

PEG 4000 (Plates containing the low concentration and inoculated with mycelial solution): The colonies on plates containing PEG 4000 plates extended more than the ones on control plates, and similiar in terms of branching and limited hyphal

extension. Colonies were similiar to that of the plates containing glucose, but the final length was about 1.1 cm. The final colony length was almost the same as the colonies on mycelial control plates and high concentration PEG 4000, whereas it was 18.76% lower than that of corresponding plate inoculated with spore solution.

Table 3.4: Average linear extension of colonies on minimum PEG (0.005 M) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
PEG 4000, (0,005 M), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value (μ)	780,84	1244,02	1452,99	1898,05	3859,38	5375	7093,75	11000
Absolute Error (%)	17,76	12,94	26,54	13,84	12,85	8,32	12,32	7,42
T-test	0,199	0,105	0,078	0,499	0,384	0,411	0,423	0,374
F-test	0,435	0,940	0,977	0,253	0,154	0,903	0,281	0,139

PEG 4000 (Plates containing the low concentration and inoculated with mycelial solution): Colonies were larger than the control plates. Linear extension and branhing poperties were similiar to that of glucose plates. 1.06 cm was the final extension after 48 hours. That was almost the same as that of mycelial control plates and 16.4% lower than the corresponding PEG 4000 containing plates inoculated with spore solution.

Sodium deoxycholate (Plates containing the low concentration and inoculated with mycelial solution): Almost no free hyphal elements was observed. The pellet was very dense possiby causing an internal mass transfer limitation. The pellet intensity prevented taking pictures using a 4x objective even when colony lengths were smaller than 0.3 cm. The final mycelium extension was measured as 0.675 cm after 48 hours of incubation at 28°C. This concentration caused shorter mycelial extensions by 18.7% when compared to that of mycelial control plates and the colonies were much more branched. No growth inhibition was observed in contrast with the inhibition found in plates inoculated with spore solution. Especially the

difference in sodium deoxycholate plates suggested the use of mycelial inoculation instead of spore inoculation.

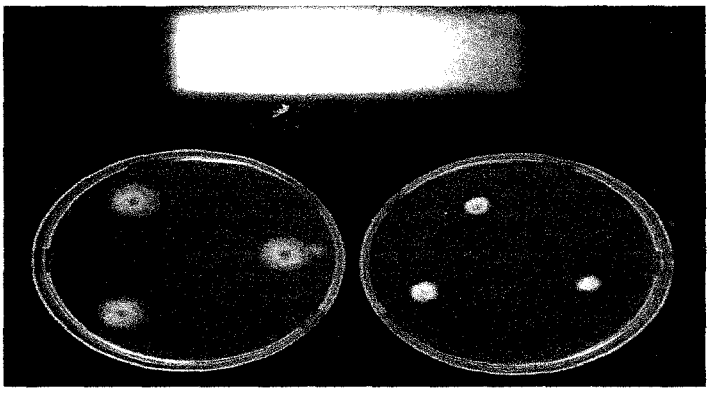

Figure 3.4: The comparison of mycelial conrol (PDA) plate with a plate containing 0.005% sodium deoxycholate.

Table 3.5: Average linear extension of colonies on minimum sodium deoxycholate (0.005 %) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
NaDC (0,005 %), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	885,11	1110,51	1354,51	2335,19	3490,48	3968,75	4781,25	6750
Absolute Error (%)	16,68	26,62	13,92	19,18	25,80	9,05	13,56	15,71
T-test	0,336	0,062	0,013	0,281	0,132	0,068	0,017	0,007
F-test	0,493	0,457	0,930	0,516	0,580	0,072	0,135	0,264

Sodium deoxycholate (Plates containing the high concentration and inoculated with mycelial solution): It was observed that this concentration of NaDC produced the best paramorphogenic effect. Almost no free hyphal elements were observed. The pellet intensity prevented taking clear pictures using a 4x objective even when mycelial extensions were shorter than 0.3 cm. The paramorphogenic effect increased with increasing amounts of sodium deoxycholate. The average length was 0.497 cm. The increase in the concentration of NaDC stimulated branching and decreased linear extension by 43%, so paramorphogenic effect was significant.

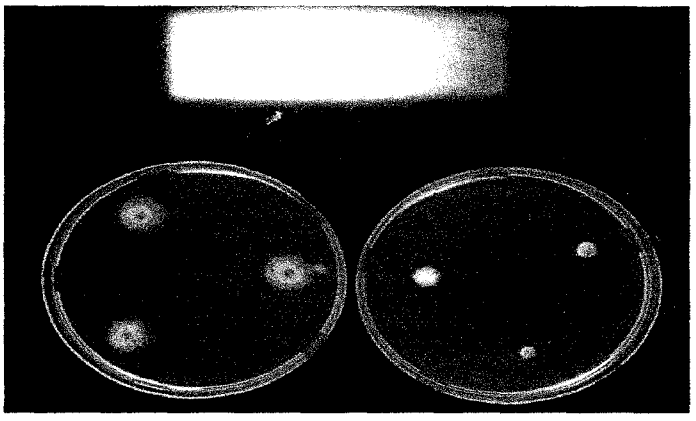

Figure 3.5: The comparison of mycelial conrol (PDA) plate with a plate containing 0.005% sodium deoxycholate.

Table 3.6: Average linear extension of colonies on maximum sodium deoxycholate (0.01 %) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
NaDC (0,010 %), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	941,25	1409,21	1595,01	1992,39	3031,4	3500	4000	4968,75
Absolute Error (%)	26,14	25,75	37,59	26,66	25,71	19,34	13,50	15,42
T-test	0,429	0,315	0,451	0,192	0,034	0,012	0,009	0,003
F-test	0,904	0,669	0,606	0,950	0,438	0,351	0,083	0,118

L(-)-Sorbose (Plates containing the low concentration and inoculated with mycelial solution): The organism in these plates exhibited higher growth rates and pellet formation than all plates inoculated with spore solutions and also contained some free hyphal elements, especially towards the outside of colony center. The avarage mycelial extension was around 0.87 cm. This is much higher than that of the plates inoculated with spores, as growth inhibition was observed within corresponding plates inoculated spores. Therefore in the following experiments, concentration of the chemical was increased until growth inhibition and branching/extension characteristics were then compared.

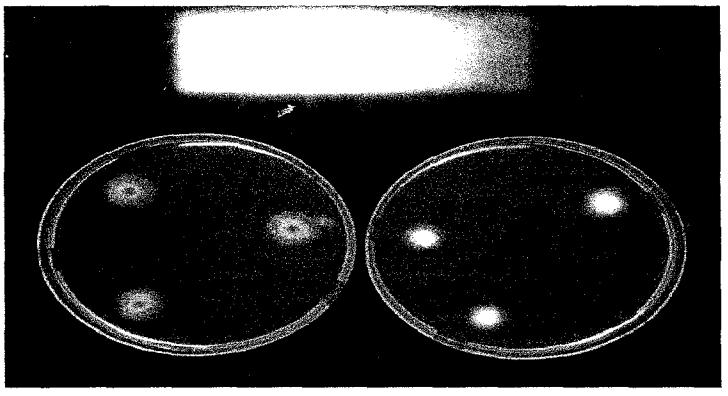

Figure 3.6: The comparison of mycelial conrol (PDA) plate with a plate containing 0.1 M L-sorbose.

Table 3.7: Average linear extension of colonies on minimum L (-) sorbose (0.1M) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
L (-) sorbose (0.1 M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	637,72	1230,64	1499,18	1870,77	3937,5	4843,75	5937,5	8687,5
Absolute Error (%)	23,35	8,91	8,21	12,58	13,09	8,26	2,11	4,32
T-test	0,061	0,161	0,054	0,370	0,385	0,373	0,093	0,094
F-test	0,501	0,828	0,789	0,960	0,170	0,097	0,001	0,016

L(-)-Sorbose (Plates containing the high concentration and inoculated with mycelial solution): Higher sorbose concentrations yielded smaller colonies. The fragmentation tendency and/or few small pellet formation was observed instead of one pellet/colony formation, but the paramorphogenic effect of sorbose was also evident, since 0.42 cm was the average mycelia length, which was the shortest length among all plates. To decide which chemical (L-sorbose or sodium deoxycholate) displayed the best paramorphogenic effect, experiments were conducted. Linear extension observed on the plates containing this concentration of L-sorbose was one of the lowest in all experiments and corresponds to 39.5% of that of the control plate.

Table 3.8: Average linear extension of colonies on maximum L (-) sorbose (0.5M) containing petri plates inoculated with mycelial solution, and their statistical evaluation.

	time (h)							
L (-) sorbose (0.5M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	828,78	1210,3	1384,21	1586,62	2182,81	2539,94	3526,03	4218,75
Absolute Error (%)	23,04	8,70	21,84	4,33	5,26	12,74	9,35	21,96
T-test	0,011	0,031	0,121	0,171	0,016	0,011	0,002	0,003
F-test	0,776	0,790	0,728	0,723	0,278	0,467	0,337	0,191

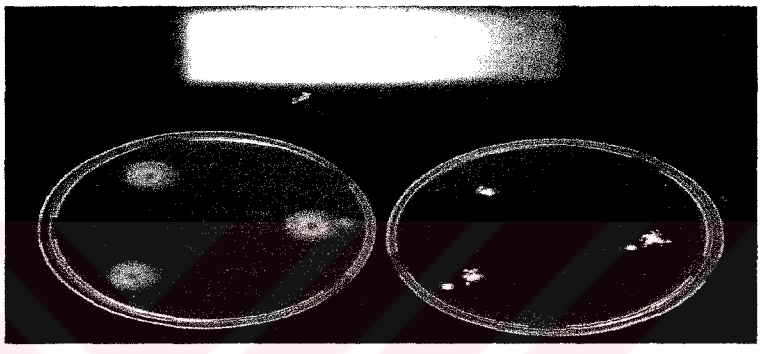

Figure 3.7: The comparison of mycelial conrol (PDA) plate with a plate containing 0.5 M L-sorbose.

PDA (Inoculated with spore solution): Control plates were similiar to other spore inoculated plates by means of low growing rates. Very loose hyphal elements were observed, even if the hyphal density had a tendency to increase towards the colony center. The colonies were 1.37 cm. long at the end of 48 hours of incubation.

Table 3.9: Average linear extension of colonies on control (PDA) petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
PDA (control) Spore solution	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	856,39	1125,78	1406,04	1531,37	4042,55	5919,42	8166,67	13708,33
Absolute Error (%)	24,23	17,30	2,37	4,39	20,00	14,81	18,19	23,33

Glucose (Plates containing the low concentration and inoculated with spore solution): Separate hyphal elements were observed instead of pellets. Their density was increasing towards the center of colony. Average linear extension was 1.56 cm. That concentration yielded colonies that had 14% higher extension rates than control plates inoculated with spore solutions. Also glucose containing plates inoculated with mycelia had lower extension rates by 21.6%.

Glucose (Plates containing the high concentration and inoculated with spore solution): Linear extension rates were higher than that of low concentration plates. The paramorphogenic effect of glucose did not appear to be increasing for increasing amounts of the chemical on plates inoculated with spore solution. The average extension was 1.84 cm after 2 days. That implied a 15.2% increase in linear extension rate of colonies. These colonies were the longest among all colonies grown on all petri plates and were the most sparsely branched. The increase in average linear extension compared to that of control plate was 34.3%.

Table 3.10: Average linear extension of colonies on maximum glucose (50 mg/ml) containing petri plates inoculated with spore solution, and their statistical evaluation.

	time (h)							
Glucose (50 mg/ml), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	751,61	883,02	1006,81	1246,02	3923,38	5141,28	8750	18416,67
Absolute Error (%)	34,66	13,54	10,48	7,84	28,34	18,22	12,86	17,68
T-test	0,327	0,190	0,136	0,174	0,455	0,292	0,328	0,010
F-test	0,355	0,258	0,173	0,269	0,967	0,786	0,523	0,713

Sodium deoxycholate (Plates containing the low concentration and inoculated with spore solution): Highly branched and pelleted colonies were observed when compared to that the colonies observed on glucose and PEG 4000 containing plates. Paramorphogenic effect was less pronounced than *mycelial inoculations* and high concentration sodium deoxycholate containing plates. The observed effect was a combination of both slow growth observed in most plates inoculated with spore solution (possibly decrease in specific growth rate) and paramorphogenic effect of the compound. The average length after 48 hours of incubation at 28 °C was 0.86 cm. The linear extension was decreased by 37.3% when compared to that of the colonies grown on control plates. The paramorphogenic effect was significant.

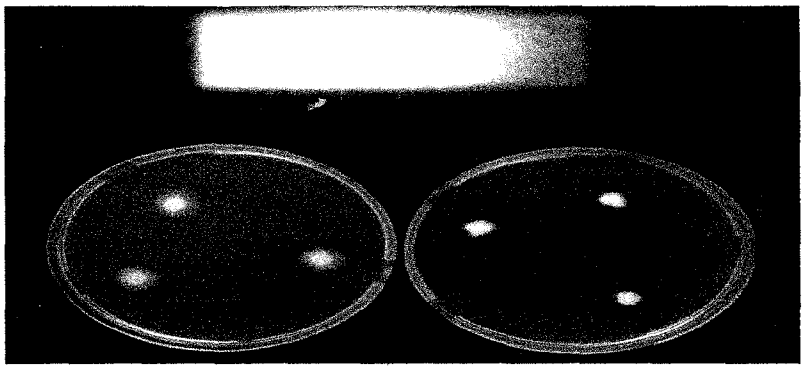

Figure 3.8: The comparison of spore conrol (PDA) plate with a plate containing 0.005% sodium deoxycholate.

Table 3.11: Average linear extension of colonies on minimum sodium deoxycholate (0.005 %) containing petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
NaDC (0,005%), SpS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	807,33	1067,57	1256,67	1478,73	2617,07	3857,15	5462,07	8583,33
Absolute Error (%)	38,40	6,47	19,73	22,59	21,65	5,39	15,23	22,30
T-test	0,409	0,353	0,212	0,348	0,191	0,106	0,041	0,009
F-test	0,508	0,363	0,541	0,851	0,343	0,732	0,539	0,586

Sodium deoxycholate (Plates containing the high concentration and inoculated with spore solution): Colonies observed were more efficiently branched and pelleted colonies than that observed in plates with glucose and PEG 4000, but they were less branched than the colonies on plates with same composition but inoculated with mycelial solutions. The effect was due to a combination of both slow growth observed in most plates inoculated with spore solution and paramorphogenic effect of the compound. The paramorphogenic effect of sodium deoxycholate was more notable with increasing concentrations. Very little freely dispersed hyphal elements were observed. Average length was approximately 0.56 cm after incubation. This value was the smallest encountered in plates inoculated with spore solutions. The

linear extension was only 40.5% of that obtained in control plates inoculated with spore solution and 35.2% to the low concentration sodium deoxycholate.

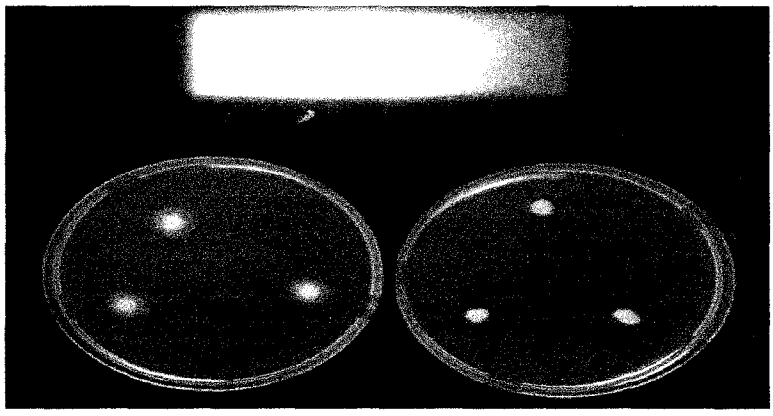

Figure 3.9: The comparison of spore conrol (PDA) plate with a plate containing 0.01% sodium deoxycholate.

Table 3.12: Average linear extension of colonies on maximum sodium deoxycholate (0.01 %) containing petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
NaDC (0,010%), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	661,11	1043,94	1237,69	1414,41	2247,53	3309,71	4562,08	5562,5
Absolute Error (%)	23,00	9,75	36,63	30,01	24,70	15,71	18,18	16,88
T-test	0,142	0,304	0,253	0,210	0,036	0,008	0,001	0,002
F-test	0,294	0,947	0,922	0,968	0,611	0,597	0,742	0,237

PEG 4000 (Plates containing the low concentration and inoculated with spore solution): Growth rate was relatively low. Many hyphal elements existed on the plates. Average linear extension was 1.35 cm after 48 hours. Following glucose containing plates, the second longest extension rates were observed on PEG 4000 containing plates.

PEG 4000 (Plates containing the high concentration and inoculated with spore solution): Paramorphogenic effect was significant and the branching increased, but also the free hyphae existed especially towards the outside of the colonies. No pellets were observed. The linear extension was measured as 1.27 cm after 2 days of

incubastion. Colonies observed had slightly lower linear extension rates than that of both low concentration PEG 4000 containing plates and control (spore inoculated) plates (6.2% and 7.3%).

L(-)-Sorbose (Plates containing the low concentration and inoculated with spore solution): Colonies observed on these plates were more efficiently branched and pelleted than that of the plates containing glucose and PEG 4000, but the colonies formed were less branched than *mycelial inoculations* of L(-)-sorbose and NaDC. Colonies usually fragmented into a few little fractions, but their form could be thought to be a pellet precursor. The linear extension was approximately 0.28 cm. Linear extension rate was the shortest among all inoculations, but the most branched colonies were not observed neither as growth inhibition was observed. Colony length of the colonies observed on these plates corresponded only to 20.5% of that of the control plates.

Table 3.13: Average linear extension of colonies on minimum L (-) sorbose (0.1 M) containing petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
L (-) sorbose (0,1 M), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	180,48	431,40	672,12	723,14	1709,99	2494,78	1920,68	2809,95
Absolute Error (%)	660,47	899,65	1316,13	1501,91	3123,14	4553,16	6269,84	9625,00
T-test	0,151	0,147	0,211	0,383	0,091	0,030	0,008	0,008
F-test	0,149	0,569	0,952	0,989	0,754	0,819	0,668	0,837

L(-) Sorbose (Spore inoculated high concentration): Colonies observed were much better branched and pelleted than that of colonies grown on glucose and PEG 4000 containing plates, but were less effective in increased branching when compared to L(-) sorbose and sodium deoxycholate containing plates inoculated with mycelial solutions. Final linear extension was 1.05 cm after 48 hours. The decrease in the final extension was 23.3% when linear extension of both concentrations of L-sorbose were compared. Unlike mycelial inoculations, increasing concentration of the chemical increased linear extension. The final linear extension was much greater than that of corresponding mycelial plates and the colonies were much less branched.

Table 3.14: Average linear extension of colonies on maximum L (-) sorbose (0.1 M) containing petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
L (-) sorbose (0,5 M), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	668,55	881,67	1405,38	1915,31	4125	4500	6000	10500
Absolute Error (%)	10,24	11,38	1,45	5,07	12,86	15,71	23,57	20,20
T-test	0,034	0,109	0,006	0,168	0,180	0,029	0,148	0,180
F-test	0,712	0,368	0,955	0,498	0,330	0,459	0,819	0,698

3.2 Evaluation of different concentrations of the selected paramorphogenic compounds used in solid media.

After testing two concentrations of paramorphogenic compounds and two different inoculation types (spore and mycelial solutions); two compounds (sodium deoxycholate and L- sorbose) and one inoculation type (mycelial solution) were selected. Both chemicals' paramorphogenic effect increased within increased concentrations, so in the second step it was decided to increase the concentration of these two paramorphogens until inhibition of growth was observed inoculating the plates with mycelial solutions. In addition to increasing paramorphogenic effects within increasing concentrations, sodium deoxycholate and L (-) sorbose containing plates were shown to yield more efficiently branched colonies when compared to that of glucose and PEG 4000 containing plates. Higher branching resulted in formation of colonies that might be expected to face oxygen limitation. The solid media experiments were employed in order to determine role of paramorphogens in branching. The issues considering effect of oxygen limitation on cultures or enzyme synthesis rate and capability were targeted merely by liquid cultures to some extent. The selected chemicals were continued on experiments carried out on solid cultures. As the aim of the second step of the solid media experiments was observing growth inhibition, concentrations of sodium deoxycholate and L (-) sorbose should be increased until inhibition of growth was observed, so five different concentrations of these two paramorphogens were used in the second step. Two of the earlier applied concentrations from previous run, were tested with higher concentrations of NaDC, whereas for L-sorbose, one medium level and two higher level concentrations were

applied together with the two previous sets concentrations. Inhibition of growth was observed for high concentrations especially the highest two concentrations of both chemicals. All inoculations were mycelial. Graph 3.3 and Graph 3.4 show the effects of different concentrations of both chemicals. Tables between Table 3.16 and Table 3.26 concentrate on average linear extension of colonies on all kinds of petri plates and their statistical evaluations. Again densitometer photos and some of the linear extension data were added to Appendix-I and II.

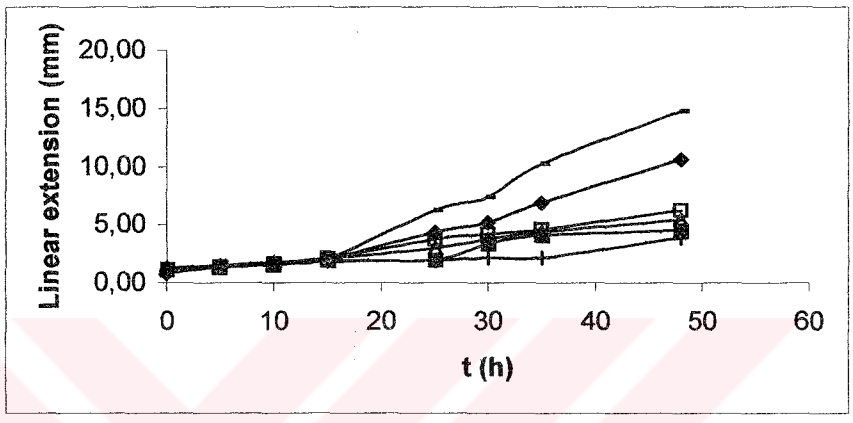

Figure 3.10: The effects of different L (-) sorbose concentrations on the linear extension of colonies inoculated from mycelial solutions (L-sorbose, 0.1 M :♦, L-sorbose, 0.3 M:■, L -sorbose, 0.5 M:▲, L -sorbose, 1 M: ×, L-sorbose, 2.5 M: +, PDA:-).

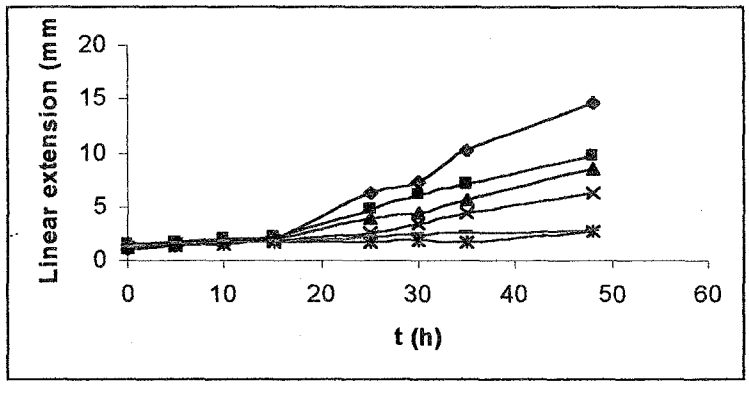

Figure 3.11: The effects of different sodium deoxycholate concentrations on the linear extension of colonies inoculated from mycelial solutions (PDA :♦, NaDC, 0.005%: ■, NaDC, 0.010%: ▲, NaDC, 0.020%: ×, NaDC, 0.050%: *, NaDC, 0.100% :-).

PDA(control), Mycelial solution:

Table 3.15: Average linear extension of colonies on PDA containing petri plates inoculated with spore solution and their statistical evaluation.

	time (h)							
PDA (control)	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1074,93	1351,58	1649,35	2078,92	6250	7375	10250	14750
Absolute Error (%)	3,22	9,21	16,71	4,84	15,32	12,83	9,34	3,39

L(-)-Sorbose (0.1 M) containing plates inoculated with mycelial solution: Loose pellets were observed. Their linear extension rates were slightly below the control colonies. Branching increased towards the colony center. Few hyphal elements, attached to the main colony were observed. The linear extension after 48 hours of observation was 1.06 cm. The final length of colonies was 72% of that of colonies grown on control plates.

Table 3.16: Average linear extension of colonies on L (-) sorbose (0.1 M) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
L (-) sorbose (0,1 M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	771,15	1375,31	1701,26	2112,96	4375	5187,5	6875	10625
Absolute Error (%)	8,85	7,69	16,31	6,15	5,71	4,61	3,64	4,51
T-test	0,001	0,412	0,426	0,300	0,008	0,007	0,004	0,001
F-test	0,294	0,024	0,112	0,685	0,054	0,049	0,084	0,004

L(-)-Sorbose (0.3 M) containing plates inoculated with mycelial solution: The morphology could not be interpreted as pellet, but was much more branched than free mycelia. Smaller colonies were observed to form around the main colony. The final linear extension was 42.3% of that of control plates after 48 hours.

Table 3.17: Average linear extension of colonies on L (-) sorbose (0.3 M) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
L (-) sorbose (0.3M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1055,98	1401,67	1645,42	2084,37	3750	4187,5	4562,5	6250
Absolute Error (%)	16,46	11,56	11,27	5,98	7,70	5,72	6,89	8,00
T-test	0,415	0,296	0,491	0,484	0,009	0,005	0,001	0,000
F-test	0,025	0,022	0,130	0,732	0,080	0,049	0,239	0,017

L(-)-Sorbose (0.5 M) containing plates inoculated with mycelial solution: This was the most suitable concentration of L (-) sorbose to be called as a pellet. These colonies could be taken as the best candidate for checking for any kind of metabolite production. The final linear extension of colonies was 36.44% of that of colonies on control plates after 48 hours.

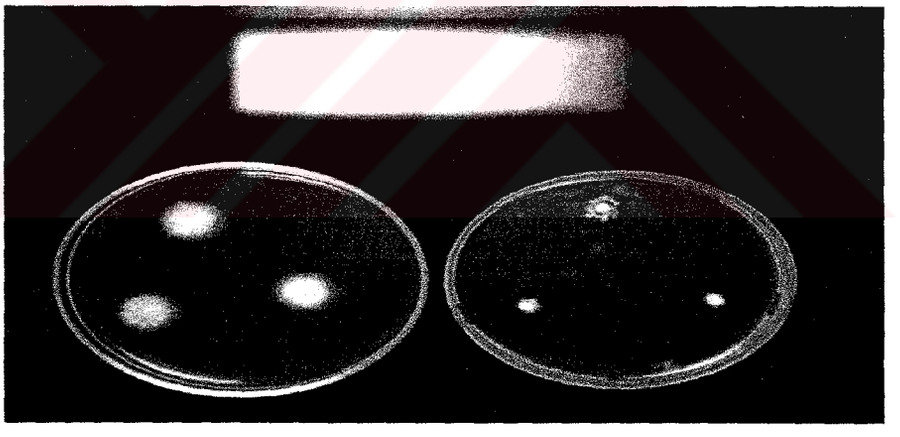

Figure 3.10: The comparison of mycelial conrol (PDA) plate with a plate containing 0.5 M L-sorbose.

The figure above shows the most highly branched L-sorbose plate and its comparison to control plate. Still no inhibition was observed and there was a correlation between increased branching and increased concentration of the chemical.

Table 3.18: Average linear extension of colonies on L (-) sorbose (0.5 M) containing petri plates inoculated with mycelium solution and their statistical evaluation.

time (h)								
L (-) sorbose (0,5 M), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	1256,68	1499,51	1742	2101,93	3000	3750	4375	5375
Absolute Error (%)	13,67	5,06	14,27	3,86	13,61	7,70	3,30	8,91
T-test	0,087	0,039	0,263	0,414	0,006	0,002	0,001	0,000
F-test	0,026	0,019	0,108	0,733	0,195	0,082	0,269	0,026

L(-)-Sorbose (1 M) containing plates inoculated with mycelial solution: Growth inhibition was observed at this concentration of L-sorbose, colonies could not be claimed to be a pellet. In addition to small colony formation, point-like growth was observed. The linear extension of colonies was 30.75% of that of control plates after 48 hours.

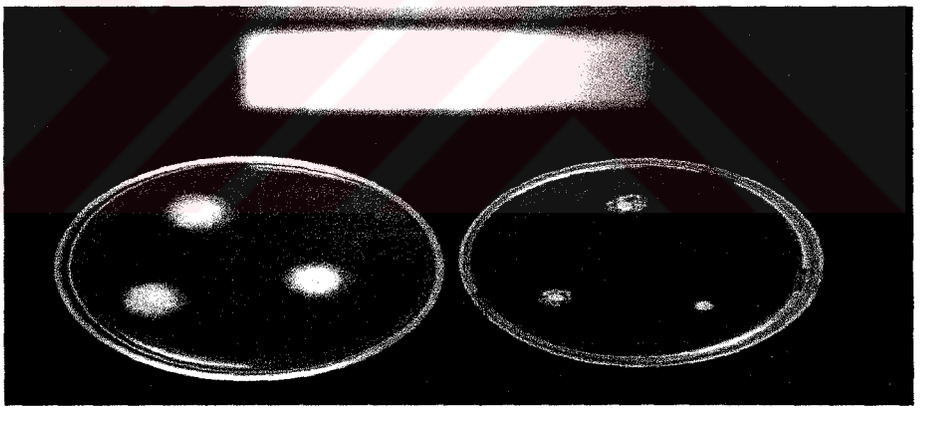

Figure 3.11: The comparison of mycelial conrol (PDA) plate with a plate containing 1 M L-sorbose.

The figure above shows that after a certain concentration of the chemical, growth inhibition would be observed for the organism. This is an important finding in terms of the concentration limits. There is no more a correlation between increasing concentrations of the chemical and enhanced branching.

Table 3.19: Average linear extension of colonies on L (-) sorbose (1 M) containing petri plates inoculated with mycelium solution and their statistical evaluation.

time (h)								
L (-) Sorbose (1 M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1161,1	1310,58	1551,35	1928,93	1972,37	3375	4083,33	4500
Absolute Error (%)	6,82	10,11	13,20	1,87	18,39	14,18	3,53	7,86
T-test	0,032	0,346	0,339	0,071	0,018	0,001	0,005	0,000
F-test	0,207	0,027	0,149	0,126	0,256	0,292	0,244	0,033

L(-)-Sorbose (1 M) containing plates inoculated with mycelial solution: Growth was almost completely inhibited by this concentration of the chemical. Colonies observed were a little larger than the colonies grown on plates containing the highest concentration of NaDC. The final linear extension of colonies was 26.3% of that of control plates after 48 hours.

Table 3.20: Average linear extension of colonies on L (-) sorbose (2.5 M) containing petri plates inoculated with mycelium solution and their statistical evaluation.

time (h)								
L (-) sorbose (2,5 M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1152,83	1429,99	1529,09	1816,68	1918,56	2151,83	4083,33	3875
Absolute Error (%)	3,86	2,25	12,17	4,31	15,07	9,74	3,53	6,45
T-test	0,042	0,187	0,281	0,012	0,001	0,001	0,000	0,000
F-test	0,690	0,023	0,156	0,692	0,080	0,034	0,997	0,067

Na-deoxycholate (0.005%) containing plates inoculated with mycelial solution: Loose pellets were observed. Their linear extension rates were slightly below that of control colonies. Branching increased towards the colony center. Few hyphal elements were observed outside, but they were attached to the pellet. Larger but similar (in terms of linear extension and branching) colonies formed when compared to the lowest concentration of L-(-)sorbose. The linear extension of colonies was 66.1% of that of control plates after 48 hours.

Table 3.21: Average linear extension of colonies on sodium deoxycholate (0.005 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	Time (h)							
NaDC (0,005 %), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	1575,68	1766,92	1972,07	2166,32	4750	6125	7125	9750
Absolute Error (%)	10,35	9,37	8,57	1,30	10,53	7,82	3,51	5,13
T-test	0,007	0,009	0,005	0,074	0,029	0,078	0,005	0,001
F-test	0,030	0,012	0,082	0,065	0,314	0,292	0,076	0,005

Sodium deoxycholate (0.010%) containing plates inoculated with mycelial solution: More efficient pellets were observed than the ones observed with the plates containing the previous concentration of NaDC. Branching increased and colony radii decreased. There were almost no hyphal elements around the pellet. Probably the best successful results that should be evaluated during the following experiments. The final length of colonies was 57.6% of that of control plates after 48 hours.

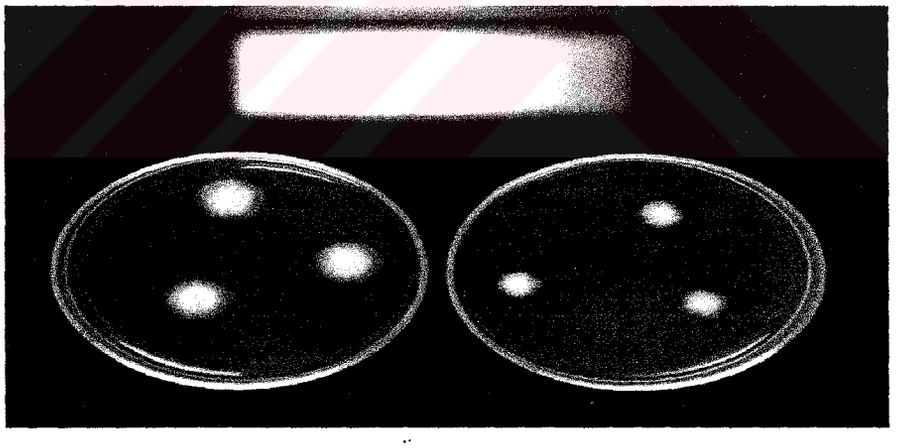

Figure 3.12: The comparison of mycelial conrol (PDA) plate with a 0.01% sodium deoxycholate containing plate.

Table 3.22: Average linear extension of colonies on sodium deoxycholate (0.010 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
NaDC (0,010 %), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1159,17	1502,74	1877,58	2043,24	3875	4437,5	5625	8500
Absolute Error (%)	12,50	11,62	11,08	5,59	16,24	11,61	8,51	5,88
T-test	0,136	0,088	0,201	0,372	0,016	0,005	0,002	0,007
F-test	0,042	0,018	0,092	0,839	0,508	0,345	0,139	0,005

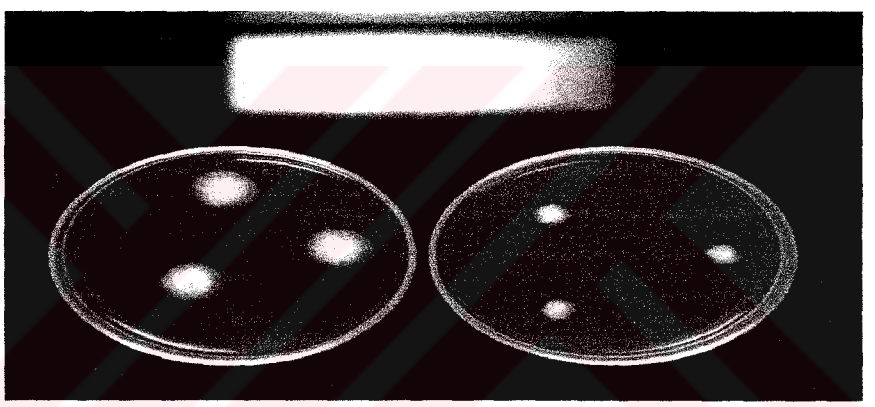

Figure 3.13: The comparison of mycelial conrol (PDA) plate with a 0.01% sodium deoxycholate containing plate.

Sodium deoxycholate (0.020%) containing plates inoculated with mycelial solution: Similar morphologies were observed as with the first two concentrations of sodium deoxycholate. Colony radius decreased again. Effects on secondary metabolites have to be compared with NaDC *(0.010%)*. These two concentrations were selected for the following experiments. The linear extension rate of colonies was 42.2% of that of control plates after 48 hours.

Table 3.23: Average linear extension of colonies on sodium deoxycholate (0.020 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
NaDC (0,020 %), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	1245,02	1466,24	1553,88	1950	2584,45	3500	4500	6250
Absolute Error (%)	14,72	6,19	5,73	4,76	13,60	11,66	9,07	8,00
T-test	0,104	0,130	0,270	0,109	0,001	0,001	0,000	0,003
F-test	0,021	0,021	0,158	0,899	0,134	0,201	0,243	0,017

Sodium deoxycholate (0.050%) containing plates inoculated with mycelial solution: Increased concentration of sodium deoxycholate caused growth inhibition. Both colony radii and branching were reduced. This implied a neat decrease in specific growth rate of the organism. The linear extension rate of colonies was only 19% of that of the colonies grown on control plates.

Table 3.24: Average linear extension of colonies on sodium deoxycholate (0.050 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
NaDC (0,050 %), MyS	***0***	***5***	***10***	***15***	***25***	***30***	***35***	***48***
Average Value	1182,99	1326,23	1486,98	1680,74	1746,06	1958,83	1677,39	2793,62
Absolute Error (%)	12,46	7,99	12,09	13,49	10,34	12,88	9,08	21,63
T-test	0,142	0,394	0,208	0,045	0,001	0,001	0,002	0,000
F-test	0,040	0,027	0,168	0,215	0,021	0,057	0,797	0,133

Sodium deoxycholate (0.010%) containing plates inoculated with mycelial solution: Level of inhibition increased with increasing amounts of sodium deoxycholate. The colonies were as small as single dots. Neither pellets nor free

hyphal elements were observed. The linear extension rate of colonies was as low as 19.11% of that of colonies on control plates at 48th hour.

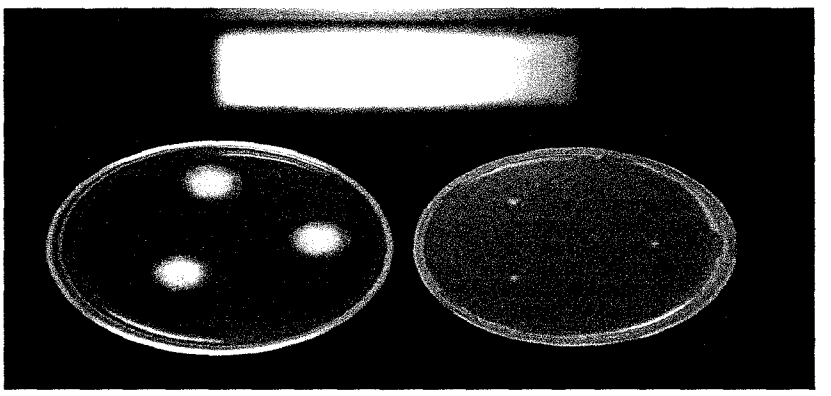

Figure 3.14: The comparison of mycelial conrol (PDA) plate with a 0.1% sodium deoxycholate containing plate.

Table 3.25: Average linear extension of colonies on sodium deoxycholate (0.100 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
NaDC (0,100 %), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1182,37	1526,3	1719,37	1711,74	2187,53	2367,47	2612,57	2819,15
Absolute Error (%)	13,60	4,67	7,83	9,81	6,29	11,29	8,90	13,45
T-test	0,116	0,026	0,335	0,019	0,001	0,001	0,000	0,000
F-test	0,031	0,018	0,120	0,422	0,010	0,067	0,756	0,148

The last set of experiments carried out within the solid media targeted cross-relating the effects of the selected concentrations of the selected paramorphogens, so the best performanced concentrations of the second step of the solid media (2 sodium deoxycholate: N1, N2 and 1 L (-) sorbose: S1 concentrations) were added into different solid media solely (N1, N2, S1) and together (N1 and S1, N2 and S1). As shown in following tables, graphs and figures, the two paramorphogens inhibited growth when added together, so their sole use was more preferable. Figure 3.15 shows the effects of different concentration of both sole and cross-related chemicals on linear extension. As it can be seen on the figure, all the chemicals used resulted in a decrease in the linear extension. The effect of different concentrations is detailed on Table 3.26 and Table 3.27. Again densitometer photos are added to Appendix-I.

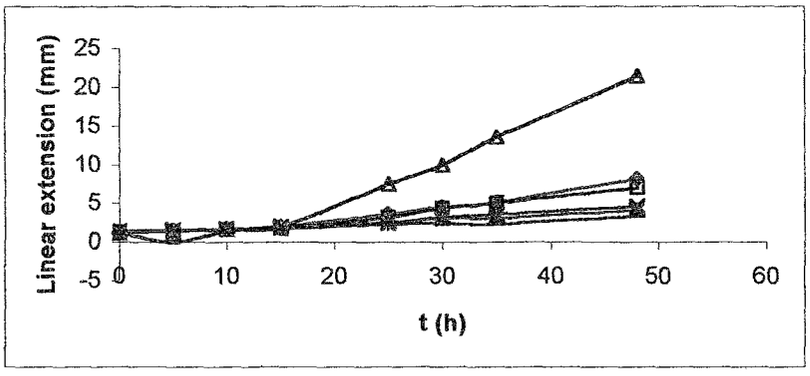

Figure 3.15: The effects of both sole and cross-related concentrations of selected chemicals on linear extension inoculated from mycelial solutions into solid media (NaDC, 0.010% :♦, NaDC, 0.020%: ■, PDA, 0.5 M: ▲, L-sorbose, 0.5 M: ×, L-sorbose, 0.5 M & NaDC, 0.010%: *, L-sorbose, 0.5 M & NaDC, 0.020%:-).

The results of mycelial solution inoculations of Na-deoxycholate (0.010%), Na-deoxycholate (0.020%), L-(-) sorbose (0.5 M) and PDA (control) were found to be similar when compared with the previous experiments given in Table 3.15,Table 3.18, Table 3.22 and Table 3.23.

L(-)-Sorbose (0.5 M) & Sodium deoxycholate (0.010%) containing plates inoculated with mycelial solution: Again growth inhibition was observed. Colony size was slightly smaller than colonies grown on sorbose containing plates, but their pellet structures were less organized. Average linear extension after 48 hours was about 4.1 mm.

Table 3.26: Average linear extension of colonies on L (-) sorbose (0.5 M) and sodium deoxycholate (0.01 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	time (h)							
L (-) sorbose (0,5 M) and NaCD (0,010 %), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1212,15	1537,25	1717,75	1770,48	2422,96	3048,77	3020,38	4125
Absolute Error (%)	7,17	14,81	7,02	12,83	18,21	17,57	28,71	11,61
T-test	0,425	0,386	0,264	0,033	0,001	0,000	0,000	0
F-test	0,268	0,830	0,834	0,310	0,670	0,507	0,521	0,766

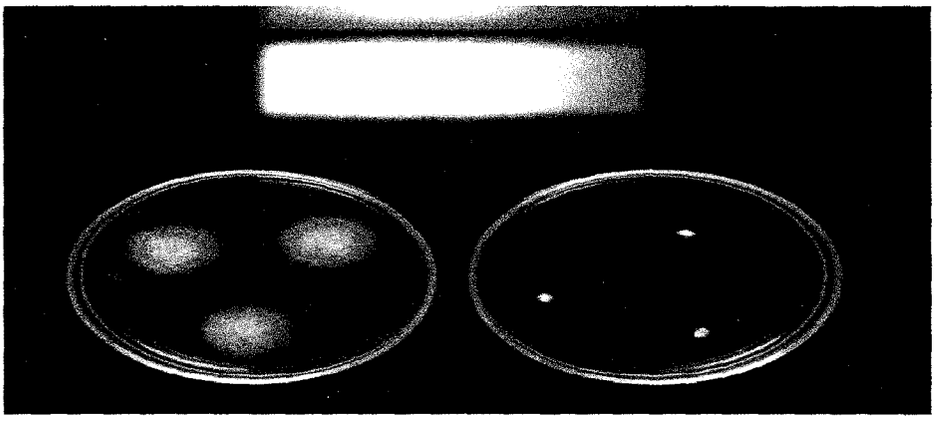

Figure 3.13: The comparison of mycelial conrol (PDA) plate with a L(-) sorbose (0.5 M) & sodium deoxycholate (0.010%) containing plate.

L(-)-Sorbose (0.5 M) & Sodium deoxycholate (0.020%) containing plates inoculated with mycelial solution: In this experiment, we have not observed any effect on growth parameters such as linear extension and branching. It seems, the growth deceised at this concentration after around 35th hour. Colony size decreased and branching was strongly inhibited. The average linear extension was about 0.34 cm after 48 hours of incubation.

Table 3.27: Average linear extension of colonies on L (-) sorbose (0.5 M) and sodium deoxycholate (0.020 %) containing petri plates inoculated with mycelium solution and their statistical evaluation.

	Time (h)							
L (-) sorbose (0,5 M) and NaCD (0,02 %), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	1176,53	1467,7	1414,81	1741,25	2317,6	2420,85	2291,7	3375
Absolute Error (%)	14,32	15,69	15,45	6,55	23,28	16,41	9,89	22,22
T-test	0,449	0,007	0,026	0,013	0,001	0,000	0,000	0,000
F-test	0,927	0,568	0,467	0,959	0,914	0,267	0,159	0,678

Within the solid media experiments, four paramorphogens and totally 32 different concentrations of them were employed. The organism almost responded all of the chemicals to some extent and its morphology was modified differently for each paramorphogen and concentration. The chemicals providing the highest branching were mainly sodium deoxycholate and also L (-) sorbose. The colonies formed on these plates could enhance primary and especially secondary metabolite production

or could decrease its rate because of oxygen limitations and dead zones formed within the dense pellet structures. Pellet structures might be advantageous within liquid cultures by means of enhanced controllability and the metabolite production. These issues were experimented within liquid culture experiments. Increased concentrations of both chemicals and their mixture resulted in inhibition of growth within the solid culture; so upper limits for paramorphogenic effects were determined. The paramorphogenic effects of both chemicals up to some extent enhanced branching, concentrations higher than upper limits caused inhibition. Correlation between upper limit and lower concentrations of sodium deoxycholate, L (-) sorbose and lipase production might be sought if any for liquid media. There might be no correlation found as in the case of *Neurospora crassa* (Trinci and Collinge, 1973) where the effect was limited to solid media, whereas liquid media was unaffected. L(-) sorbose plays its role via inhibition of β-1-3 glucan synthetase (Trinci, *et al.* 1994), but may not be functional when L(-) sorbose can be used as a sole carbon source by the organism. The same probabilities also exist for sodium deoxycholate. This chemical is an anionic surface wetting agent with emulsifying properties and may affect the permeability of cell membrane, without causing mutagenesis (Skone, 1982), so our findings were important to clear these possibilities for *A.oryzae* cultures.

Effects of PEG 4000 were not desirable by means of enhanced branching and pellet morphology, glucose added plates have not shown increased branching with increased concentrations, but were more branched than freely dispersed mycelial elements.

The mycelial inoculums of the organism had a much stronger tendency to survive on most plates, whereas adaptation ability, extension and growth rates of spores were lower. After the incubation period, survival tendency of the organism increased for most plates again. Even storage at 4 °C did not prevent progressive growth. Figure 3.17 shows a high concentration sodium deoxycholate plate inoculated with mycelial solution, which continued growing at cold room (4 °C) during a 7-day storage after incubation. It was very interesting that paramorphogenic effects were preserved and fungal growth went on.

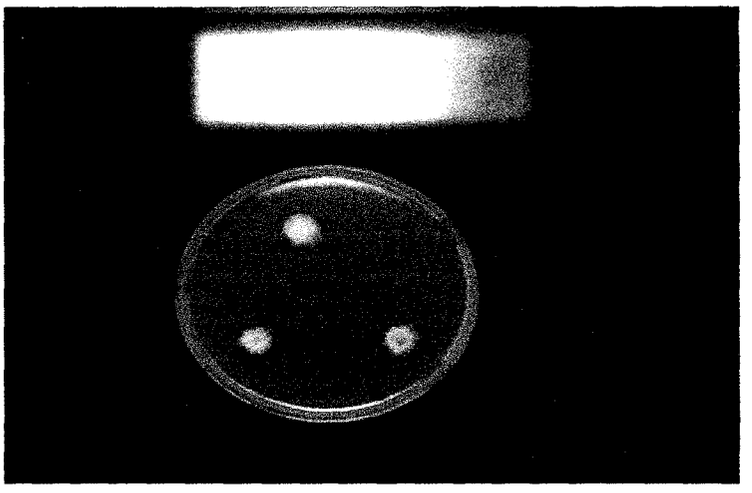

Figure 3.17: Progressive growth on a plate containing high concentration sodium deoxycholate after 48 hours of incubation and storage at cold room for a 7-day storage.

3.3 Liquid media strategy

After carrying out experiments on all 32 different compositions, the effects of 0.010% and 0.020% sodium deoxycholate was found to be the best while 0.5 M L-sorbose was also effective. Their combinations inhibited the growth, so they should be added solely to the media. To figure out, if any, the variation in protein synthesis (lipase) depending on the variations within the paramorphogenic effect, presence of lipase was verified getting use of TS (Turkish Standard) 894. The increases in lipase activity was found to be as seen on Table 3.28:

Table 3.28: Increases in lipase activity in the presence of paramorphogens compared to contol cultures.

Days of Production	*Increase in Lipase Activities Compared to Control Cultures*	
	Sodium Deoxycholate	*L-Sorbose*
4th day	4.3	6.33
5th day	4	0.225
6th day	4.25	2.75
7th day	1.67	1.67

That table basically summarizes that in 20% cultures, up to one week lipase production/activity might be observed. On the 4th day, lipase activity for L(-) sorbose containing liquid culture reached a peak value and then started going down.

In the case of sodium deoxycholate, relatively lower concentrations were reached but maintained for a longer time. This implies the possibilities of exploiting either the peak of L-sorbose or the longer period of production for sodium deoxycholate which may correspond to a higher productivity. Of course, these values need to be evaluated by detailed studies to determine the specific productivities of the enzyme and the yield values. Then a precise optimization can be completed.

Lipase activities can be determined by different methods. One of the sensitive methods for the enzyme assay is the use of pHstat system. To be employed in the future optimization studies, a pHstat system was installed as explained in detail in the material & methods part of this thesis (Section 2.6.2). Tributyrin was used as the substrate for *A.oryzae* lipase and the system was optimized. The optimum assay conditions are defined as follows:

Table 3.29: Optimized pHstat conditions for determination of lipase activity.

pHstat Conditions	**Optimum values**
pHstat buffer	Constant composition and should be replaced each time
Reaction temperature	Constant (30°C)
Initial pH	6.5
Reaction time	1 minute
Amount of substrate	10% Tributyrin
Emulsification	1% Gum Arabic as emulsifier and ultrasonication

Thus effects of paramorphogenic chemicals were investigated, optimum concentration and inhibition thresholds were found out, presence of lipase within liquid cultures was verified. The determination of lipase activity for the samples withdrawn from liquid cultures of *Aspergillus oryzae* was standardized using a pHstat system in Tris-glycine buffer environment. It was interesting to find out activity determination could not be measured in the phosphate buffer environment of same pH. The consequences of study seem to be promising to optimize lipase production within bioreactor environment.

4. CONCLUSION & RECOMMENDATIONS

As a result of the study, the morphogenesis of *Aspergillus oryzae* (CL01) was thoroughly investigated and reported in detail. The majority of the study contained investigations of the organism on solid culture conditions in the presence of chemicals defined as paramorphogenic chemicals (paramorphogens), and their effect on the branching and apical growth characteristics of the microorganism. The experiments were designed by applying thirty-two different combinations of four different chemicals incorporated in the solid media (potato dextrose agar).

First step of solid culture experiments aimed for selection of the most effective inoculation type and paramorphogens efficient on increasing the branching of *A.oryzae*. The fungal growth on solid cultures was observed using a PC supported light microscope, a densitometer and a petri microscope. Linear hyphae growth was measured for all colonies grown on all different media compositions and measurements were statistically evaluated. As a result of the first step, two paramorphogens (sodium deoxycholate and L-sorbose) and an inoculation type (mycelium inoculation) were selected and following experiments were carried out using these conditions, as they were observed to result in an enhanced fungal branching, which might be expected to favor increased primary and secondary metabolite production.

In the second step of solid culture experiments, five different concentrations of previously selected chemicals were incorporated into solid media using mycelial type inoculum to investigate the effects of various paramorphogen concentrations, if any exists. Paramorphogens are known to limit linear hyphal extension without affecting the specific growth rate. Here, some concentrations were shown to carry out a paramorphogenic effect (specific growth rate not affected), whereas some did not (both linear extension and specific growth rate) was limited. The latter case might be evaluated as growth inhibition; therefore some concentrations were shown to cause inhibition (0.05% and 0.1% sodium deoxycholate and 1 M and 2.5 M L-sorbose).

Addition of three different concentrations to the solid media (0.01% and 0.02% sodium deoxycholate together with 0.5 M L-sorbose) were shown to result in highest paramorphogenic effects forming dense and highly branched colonies, which would not be observed under normal conditions.

Finally, a solid media experiment was carried out for cross-relating the three concentrations mentioned above to observe increases in fungal branching, if any. Growth inhibition was observed (Figure-3.14 and 3-16) and it was decided that each chemical should be added to the solid media individually to observe both normal fungal growth and enhanced branching of the organism.

The effect of paramorphogens was also studied on lipase production to select the chemicals with maximum induction, appropriate concentration and inoculum type. Using TS (Turkish Standard) 894, the microorganism's productivity was shown to increase via the addition of sodium deoxycholate and L-sorbose. L-sorbose containing media yielded a sharp and fast increase in lipase productivity on the 4^{th} day of incubation, whereas media containing sodium deoxycholate yielded a relatively lower production, while maintaining productivity for a longer duration (relatively high between 4^{th} and 7^{th} day). A sharp decrease was detected at 7^{th} day (end of productivity). In both cases, productivity was higher than that of standard media.

Finally observing the paramorphogenic effect and verifying lipase synthesis and increased productivity, a highly sensitive (as low as 1 microliter) assay system was installed and assay conditions for the lipase of *A.oryzae* were optimized. Optimized conditions may be employed within future studies for samples withdrawn from liquid cultures.

In general, paramorphogens found to be effective on modifying fungal morphology and capable of increasing lipase production in liquid cultures. Using paramorphogens for the production of lipase by *Aspergillus oryzae* should carry out more detailed liquid culture experiments. One of the tools that might be employed is detailed morphological image analysis in submerged cultures in bioreactor environments. These promising compounds need to be investigated in detail for better metabolite production in different fungal culture systems.

5. REFERENCES

[1] **Amanullah, A., Jüsten, P., Davies, A., Paul, G.C., Nienow, A.W. and Thomas, C.R.**, 2000. Agitation induced mycelial fragmentation of *Aspergillus oryzae* and P*enicillium chrysogenum*, *Biochemical Engineering Journal*, **5**, 109-114.

[2] **Anonymous**. 1987. Evaluation of certain food additives and contaminants, Thirty-first Report of the Joint FAO/WHO Expert Copmmittee on Food Additives, Geneva, Italy.

[3] **Bae, J.T., Park, J.P., Song, C. H., Yu, C. B., Park, M.K. and Yun, J.W.**, 2001. Effect of the carbon source on the mycelial growth and exo-biopolymer production by submerged culture of *Paecilomyces japonica*, *Journal of Bioscience and Bioengineering*, **91**, 52-524.

[4] **Barbesgaard, P., Heldt-Hansen, H.P. and Diderichsen B.**, 1992. On the safety of Aspergillus oryzae: a review, *Applied Microbiology and Biotechnology*. **36**, 569-572.

[5] **Bartnicki-Garcia, S., Hergert, F. and Gierz, G.**, 1989. Computer simulation of fungal morphogenesis and the mathematical basis for hyphal (tip) growth, *Protoplasma*, **153**, 46-57.

[6] **Belmar-Beiny, M.T. and Thomas, C.R.**, 1991. Morphology and clavulanic acid production of *Streptomyces clavuligerus*: Effect of stirrer speed in batch fermentations, *Biotechnology and Bioengineering*, **37**, 456-462.

[7] **Bocking, S.P., Wiebe, M.G., Robson, G.D., Hansen, K., Christiansen, L.H. and Trinci, A.P.J.**, 1999. Effect of branch frequency in *Aspergillus oryzae* on protein secretion and culture viscosity, *Biotechnology and Bioengineering*, **65**, 638-648.

[8] **Carlsen, M., Spohr, A.B., Nielsen, J. and Villadsen, J.**, 1996. Morphology and physiology of an α- amylase producing strain of *Aspergillus oryzae* during batch cultivations, *Biotechnology and Bioengineering*, **49**, 266-276.

[9] **Cho, Y.J., Hwang, H.J. and Kim, S.W.**, 2002. Effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by *Paecilomyces sinclairii* in a batch bioreactor, *Journal of Biotechnology*, **95**, 13-23.

[10] **Cox, P.W. and Thomas, C.R.**, 1992. Classification and measurement of fungal pellets by automated image analysis, *Biotechnology and Bioengineering*, **39**, 945-952.

[11] **Cui, Y. Q., van der Lans, R.G.J.M. and Luyben, K.Ch. A. M.**, 1998. Effects of dissolved oxygen tension and mechanical forces in submerged fermentation, *Biotechnology and Bioengineering*, **57**, 409-419.

[12] **Cui, Y. Q., van der Lans, R.G.J.M. and Luyben, K.Ch. A. M.**, 1997. Effect of agitation intensities on fungal morphology of submerged fermentation, *Biotechnology and Bioengineering*, **55**, 715-726.

[13] **El-Enshasy, H., Hellmuth, K. and Rinas, U.**, 1999. Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger*, *Applied Biochemistry and Biotechnology*, **81**, 1-11.

[14] **El-Hag, N. and Morse, R.E.**, 1976. Aflatoxin production by a variant of *Aspergillus oryzae* (NRRL Strain 1988) on cowpeas (*Vigna sinensis*), *Science*, **192**, 1345-46.

[15] **Fennell, D. I.**, 1976. *Aspergillus oryzae* (NRRL Strain 1988): A clarification, *Science*, **194**, 1188.

[16] **Freudenberg, S., Fasold, K., Müller, S.R., Siedenberg, D., Kretzmer, G., Schügerl, K. and Giuseppin, M.** 1996. Fluorescence microscopic investigation of Aspergillus awamori growing on synthetic and complex media and producing xylanase, *Journal of Biotechnology*, **46**, 265-273.

[17] **Hosking, S.L., Robson, G.D. and Trinci, A.P.J.**, 1995. Phosphoinositides play a role in hyphal extension and branching in *Neurospora crassa*, *Experimental Mycology*, **19**, 71-80.

[18] **Karsheva, M., Hristov, J., Penchev, I. and Lossev, V.**, 1997. Rheological behaviour of fermentation broths in antibiotic industry, Applied Biochemistry and Biotechnology, **68**, 187-206.

[19] **Jejelowo, O.A., Epton, H.A.S. and Trinci, A.P.J.**, 1988. Effects of paramorphogens and $_2$-deoxy-$_D$-glucose on development of lesions of *Botyrytis fabae* on *Vicia faba*, *Transactions of the British Mycologial Society*, **91**, 661-669.

[20] **Johansen, C.L., Coolen, L. and Hunik, J.H.**, 1998. Influence of morphology on product formation in *Aspergillus awamori* during submerged fermentations, *Biotechnol. Prog.*, **14**, 233-240.

[21] **Li, Z.J., Shukla, V., Wenger, K., Fordyce, A, Pedersen, A.G. and Marten, M.**, 2002. Estimation of hyphal tensile strength in production-scale *Aspergillus oryzae* fungal fermentations, *Biotechnology and Bioengineering*, **77**, 601-613.

[22] **Li, Z.J., Shukla, V., Fordyce, A.P., Pedersen, A.G., Wenger, K.S and Marten, M.R.**, 2000. Fungal morphology and fragmentation in a fed-batch *Aspergillus oryzae* fermentation at the production scale, *Biotechnology and Bioengineering*, **70**, 300-312.

[23] **Lortie, R.**, 1997. Enzyme catalyzed esterification, *Biotechnology Advances*, **15**, 1-15.

[24] **Mayer Z., Bagnara A., Färber, P. and Geisen, R.**, 2002. Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods, *International Journal of Food Microbiology*, Article in press.

[25] **Mitard, A. and Riba, J.P.**, Morphology and growth of *Aspergillus niger* ATCC 26036 cultivated at several shear rates, *Biotechnology and Bioengineering*, **32**, 835-840.

[26] **Morse, R.E.**, 1976. *Aspergillus oryzae* (NRRL Strain 1988): A clarification – Reply, *Science*, **194**, 1188.

[27] **Olsvik, B.S. and Kristiansen, B.**, 1992. Influence of oxygen tension, biomass concentration, and specific growth rate on the rheological properties of a filamentous fermentation broth, *Biotechnology and Bioengineering*, **40**, 1293-99.

[28] **Pabai, F., Kermasha, S. and Morin, A.**, Interesterification of butter fat by

partially purified extracellular lipases from *Pseudomonas putida*, *Aspergillus niger* and *Rhizopus oryzae*, *World Journal of Microbiology and Biotechnology*, **11**, 669-677.

[29] **Pazouki, M. and Panda, T.**, Understanding the morphology of fungi, **22**, 127-143.

[30] **Petrović, S.E., Škrinjar, M., Bećarević, A., Vujičić, I.F. and Banka, L.**, 1990. Effect of various carbon sources on microbial lipases biosynthesis, *Biotechnology Letters*, **12**, 299-304.

[31] **Prosser, J.I. and Trinci, A.P.J.**, 1979. A model for hyphal growth and branching, *Journal of General Microbiology*, **111**, 153-164.

[32] **Robson, G.D., Kuhn, P.J. and Trinci, A.P.J.**, 1989. Effect of Validamycin A on the inositol content and branching of *Rhizoctonia cerealis* and other fungi, Journal of General Microbiology, **135**, 739-750.

[33] **Roukas, T.**, 1999. Rheological properties of pullulan fermentation broth in a stirred tank fermentor, *Food Biotechnology*, **13**, 255-266.

[34] **Skone, E.J.**, 1982. Effect of paramorphogens sorbose, polyethylene glycol 200 and sodium deoxycholate on conidial germination of *Ceratocystis adiposa*, *Microbios*, **33**, 193-208.

[35] **Spohr, A., Dam-Mikkelsen, C., Carlsen, M., Nielsen, J. and Villadsen, J.**, 1998. On-line study of fungal morphology during submerged growth in a small flow-through cell, *Biotechnology and Bioengineering*, **58**, 541-553.

[36] **Stoloff, L., Misivec, P. and Schindler, A.F.**, 1977. *Aspergillus oryzae* (NRRL Strain 1988), *Science*, **196**, 1353-54.

[37] **Takahashi, K., Nakano, Y., Yokota, T. and Nomura, T.**, 1993. Effect of scale on particle-impeller impact in an agitated vessel equipped with a rushton turbine, *Journal of Chemical Engineering of Japan*, **26**, 100-102.

[38] **Treskatis, S.K., Orgeldinger, V., Wolf, H. and Gilles E.D.**, 1997. Morphological characterization of filamentous microorganisms in submerged cultures by on-line digital image analysis and pattern recognition, *Biotechnology and Bioengineering*, **53**, 191-201.

[39] **Trinci, A. P. J., Wiebe, M.G. and Robson, G.D.**, 1994. The mycelium as an

integrated entity, in The Mycota 1: *Growth, differentiation and sexuality*, pp. 175-194, eds. Wessels and Meinhardt, Springer-Verlag Berlin Heidelberg.

[40] **Trinci, A.P.J.**, 1974. A study of hyphal extension and branch initiation of fungal mycelia, *Journal of General Microbiology*, **81**, 225-236.

[41] **Trinci, A.P.J.**, 1973. The hyphal growth unit of wild type and spreading colonial mutants of *Neurospora crassa*, *Archive für Mikrobiologie*, **91**, 127-136.

[42] **Trinci, A.P.J. and Collinge, A.**, 1973. Influence of L-sorbose on the growth and morphology of *Neurospora crassa*, *Journal of General Microbiology*, **78**, 179-192.

[43] **TS-894**, 1989. Yemeklik bitkisel yağlar muayene metodları, *Türk Standartları Enstitüsü*, Ankara.

[44] **van Heerden, E., Litthauera, D. and Vergerb, R.**, 2002. Biochemical characterisation and kinetic properties of a purified lipase from in bulk phase and monomolecular films, *Enzyme and Microbial Technology*, **30**, 902–909.

[45] **Veljkovic, V. B., Lazić, M.L. and Skala, D.U.**, 1988. Studies on dextran fermentation broth rheology, *Enzyme and Microbial Technology*, **10**, 686-688.

[46] **Vorderwülbecke, T., Kieslich, K. and Erdmann, H.**, 1992. Comparison of lipases by different assays, *Enzyme and Microbial Technology*, **14**, 631-639.

[47] **Wiebe, M. G., Robson, G.D and Trinci, A.P.J.**, 1989. Effect of choline on the morphology, growth and phospholipid composition of *Fusarium graminearum*, *Journal of General Microbiology*, **135**, 2155-62.

[48] **Woolley, P. and Petersen, S.B.**, 1994. Lipases -their structure, biochemistry and applications, Cambridge University Press, UK.

[49] **Wösten, H. A. B., Moukha, S. M., Sietsma, J.H. and Wessels, J.G.H.**, 1991. Localization of growth and secretion of proteins in *Aspergillus niger*, *Journal of General Microbiology*, **137**, 2017-23.

6. APPENDICES

6.1 Densitometer images of solid media experiments.

The following densitometer images are added to provide further proof on the effect of paramorphogens within solid cultures. The petri plates on the left represent the control plate, whereas the ones on the right represent the plates that contain indicated concentration of the paramorphogens under indicated inoculation conditions.

STEP –1

1. **Comparison of mycelium inoculated paramorphogenic plates with control plates:**

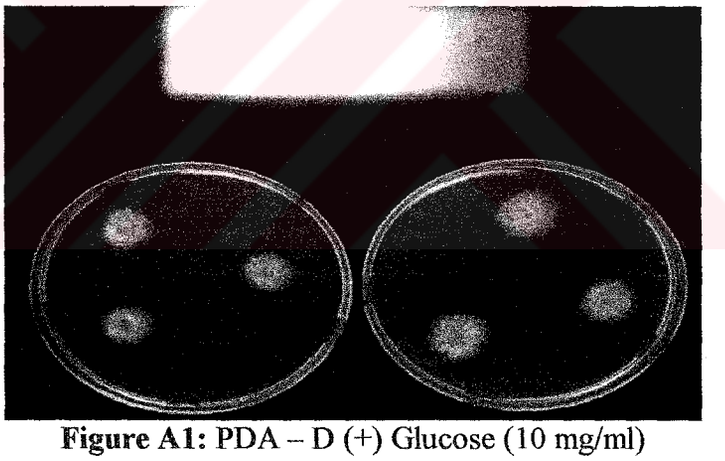

Figure A1: PDA – D (+) Glucose (10 mg/ml)

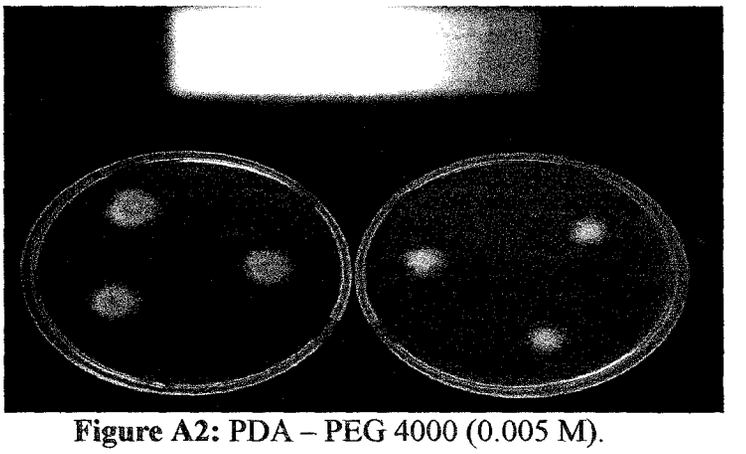

Figure A2: PDA – PEG 4000 (0.005 M).

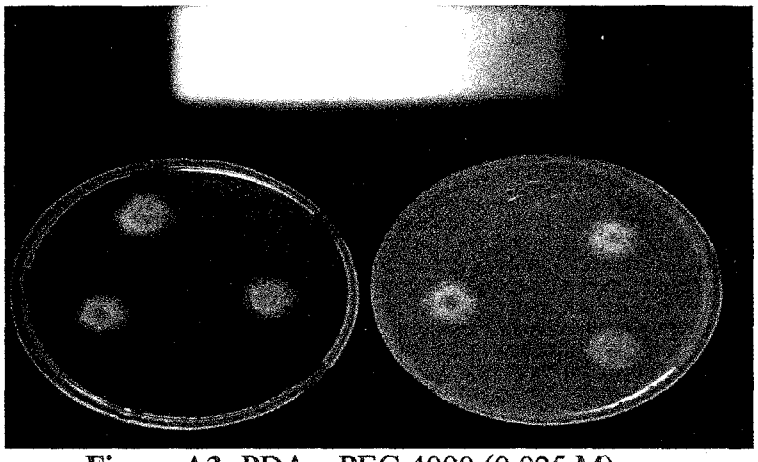

Figure A3: PDA – PEG 4000 (0.025 M).

2. Comparison of spore inoculated paramorphogenic plates with control plates:

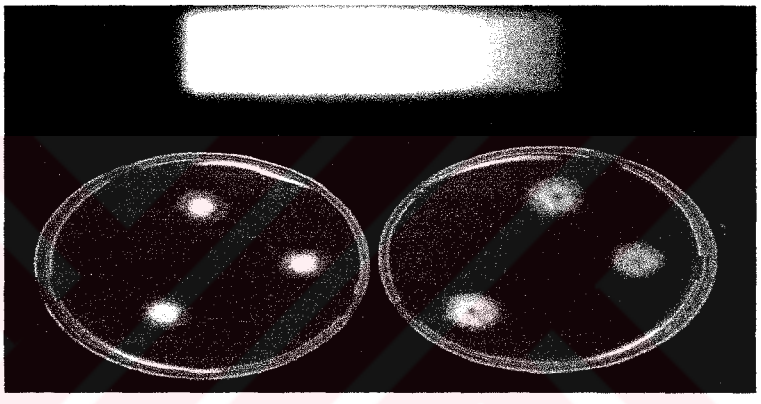

Figure A4: PDA – D (+) Glucose (10 mg/ml)

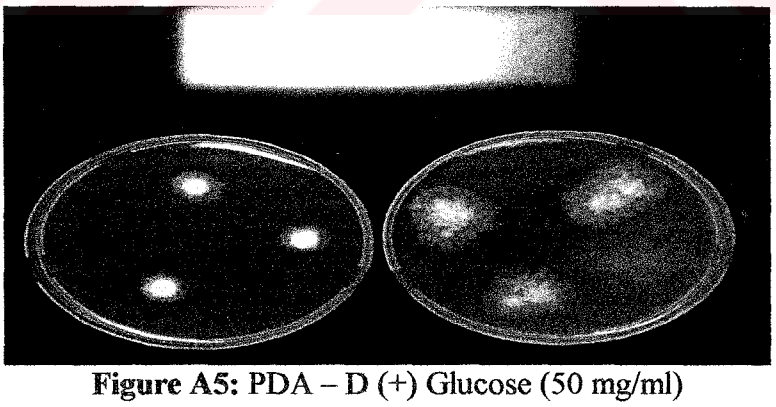

Figure A5: PDA – D (+) Glucose (50 mg/ml)

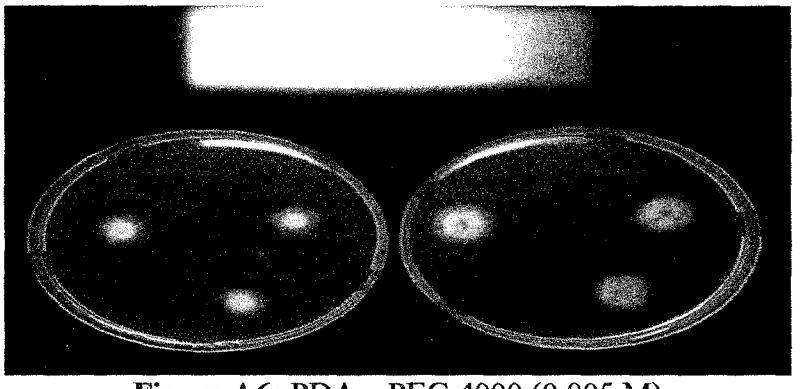

Figure A6: PDA – PEG 4000 (0.005 M)

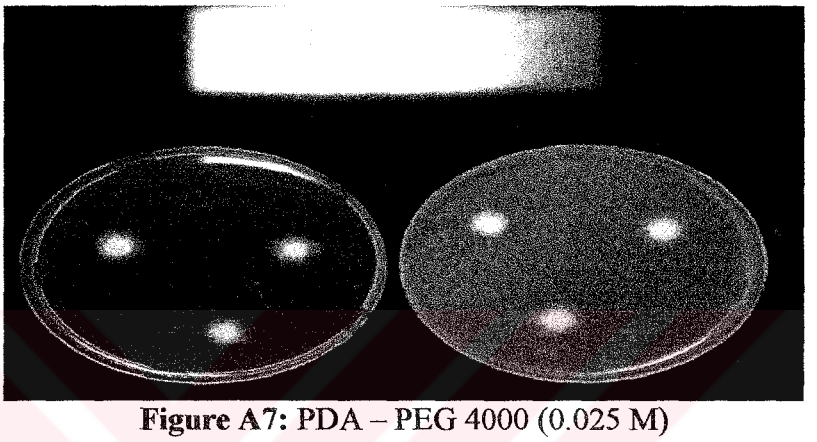

Figure A7: PDA – PEG 4000 (0.025 M)

STEP – 2

1. **Comparison of mycelium inoculated paramorphogenic plates with control plates:**

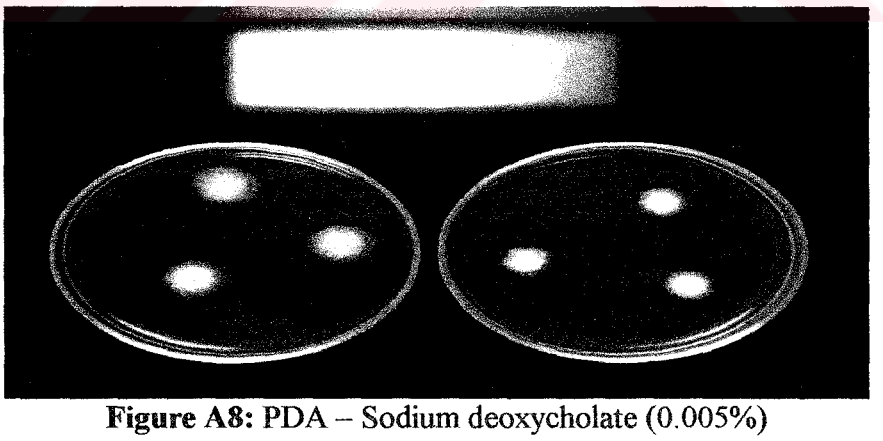

Figure A8: PDA – Sodium deoxycholate (0.005%)

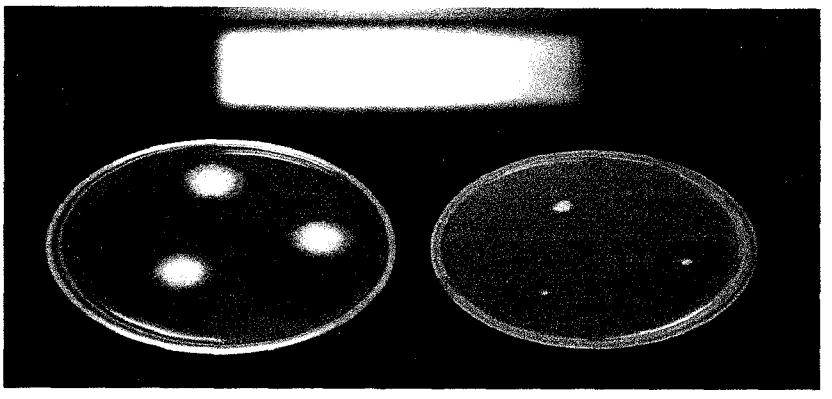

Figure A9: PDA – Sodium deoxycholate (0.050%)

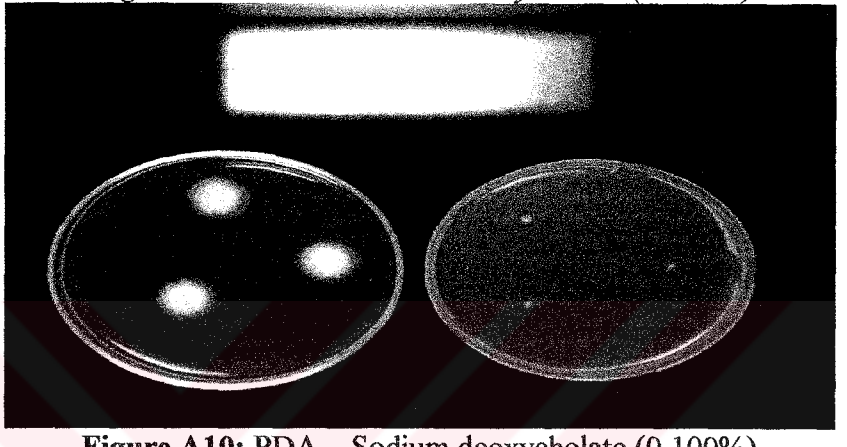

Figure A10: PDA – Sodium deoxycholate (0.100%)

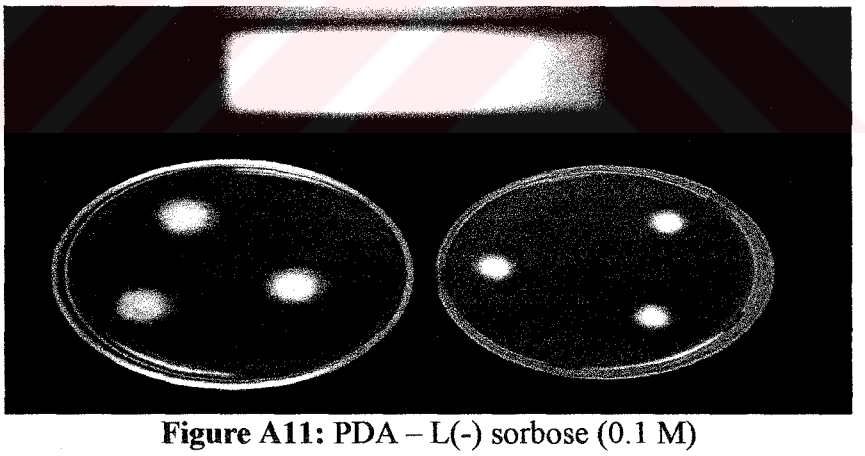

Figure A11: PDA – L(-) sorbose (0.1 M)

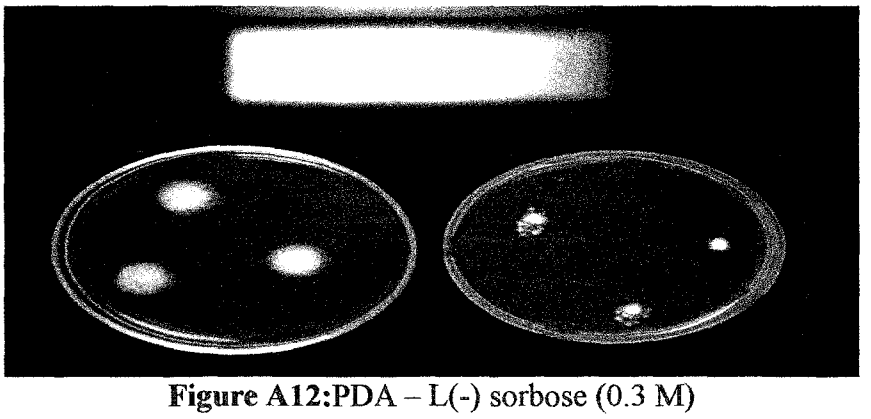

Figure A12:PDA – L(-) sorbose (0.3 M)

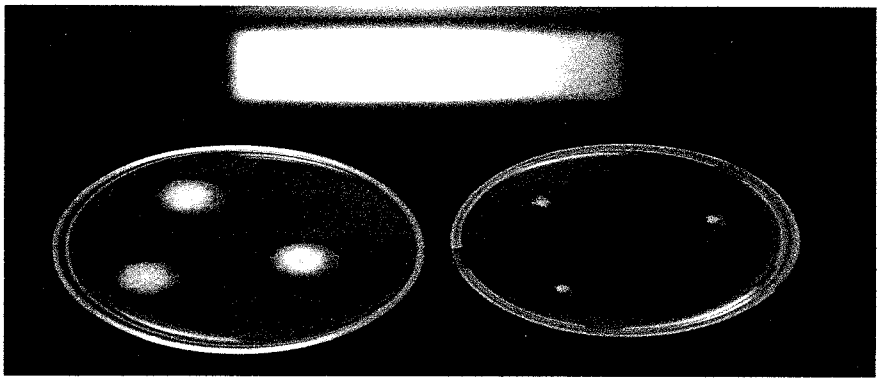

Figure A13: PDA – L(-) sorbose (2.5 M)

STEP –3: Cross relation

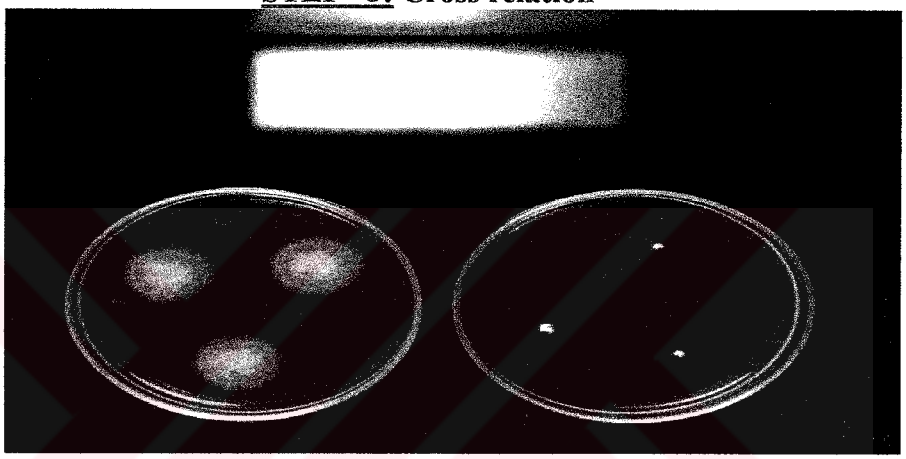

Figure A14: PDA – L(-) sorbose (0.5 M) & Na DC (0.010%).

6.2 Additional linear extension data for paramorphogen containing plates.

The following linear extension data are added to provide further proof on the effect of paramorphogens within solid cultures. The extension values were observed for 48 hours in regular intervals. More than 2000 microscope images were investigated and each single datum on tables is an average of 12 to 24 colonies.

STEP -1

Table B1: Average linear extension of colonies in mycelial solution inoculated of maximum glucose (50 mg/ml) containing petri plates and their statistical evaluation.

	Time (h)							
Glucose (50 mg/ml), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value (μ)	853,27	1169,83	1594,82	2449,7	5437,5	7812,5	10687,5	17875
Absolute Error (%)	32,19	30,01	20,62	10,80	2,30	12,08	7,96	8,35
T-test	0,362	0,256	0,462	0,272	0,072	0,039	0,033	0,003
F-test	0,768	0,632	0,463	0,285	0,003	0,671	0,263	0,544

Table B2: Average linear extension of colonies in mycelial solution inoculated, maximum PEG 4000 (0.025 M) plates and their statistical evaluation.

	Time (h)							
PEG 4000 (0,025M), MyS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value (μ)	663,52	823,72	1229,76	1797,3	2992,19	4812,5	6250	10625
Absolute Error (%)	12,90	23,29	9,44	5,51	18,38	5,44	20,20	18,58
T-test	0,075	0,027	0,026	0,348	0,111	0,252	0,127	0,465
F-test	0,567	0,791	0,728	0,018	0,199	0,920	0,599	0,867

Table B3: Average linear extension of colonies in spore solution inoculated, minimum glucose (10 mg/ml) containing petri plates and their statistical evaluation.

	time (h)							
Glucose (10 mg/ml), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	610,33	878,99	996,77	1315,43	3410,5	6493,84	9000	15625
Absolute Error (%)	27,68	22,90	2,37	4,50	15,71	29,99	18,65	21,92
T-test	0,129	0,180	0,089	0,121	0,274	0,312	0,333	0,210
F-test	0,298	0,358	0,352	0,747	0,591	0,613	0,462	0,913

Table B4: Average linear extension of colonies in spore solution inoculated, minimum polyethylene glycol 4000 (0.005 M) containing petri plates and their statistical evaluation.

	time (h)							
PEG 4000 (0,005 M), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	689,89	959,35	1304,19	1333,14	4416,67	6375	7416,67	13541,67
Absolute Error (%)	26,27	19,95	14,45	15,70	23,57	20,20	12,86	20,82
T-test	0,104	0,105	0,053	0,100	0,097	0,109	0,024	0,255
F-test	*0,295*	*0,076*	*0,726*	*0,834*	*0,816*	*0,660*	*0,717*	*0,934*

Table B5: Average linear extension of colonies in spore solution inoculated, maximum polyethylene 4000 (0.025 M) containing petri plates and their statistical evaluation.

	time (h)							
PEG 4000 (0,025 M), SpS	*0*	*5*	*10*	*15*	*25*	*30*	*35*	*48*
Average Value	667,28	765,11	1163,49	1319,25	3489,7	4830,92	6604,17	12708,33
Absolute Error (%)	35,92	24,00	27,03	22,68	10,96	12,86	20,40	14,19
T-test	0,207	0,185	0,232	0,237	0,275	0,263	0,029	0,439
F-test	0,150	0,384	0,701	0,443	0,285	0,929	0,782	0,841

AUTOBIOGRAPHY

İbrahim GÜLSEREN was born in 1980. He has graduated from Kadıköy Anadolu Lisesi in 1997 and from Food Engineering Dept. of İTÜ in Spring 2001. He has started his MS studies as a student and research assistant in Fall 2001 at Molecular Biology and Genetics Dept. of İTÜ.