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The Regulation of Secondary Metabolism from *Tolypocladium inflatum*: A Study on Strain Improvement in Cyclosporin A Productivity and its Relation to Growth and Glucose Metabolism

By

Ken Swidinsky

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

Master of Science

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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The Regulation of Secondary Metabolism from *Tolypocladium inflatum*: A Study on Strain Improvement in Cyclosporin A Productivity and its Relation to Growth and Glucose Metabolism

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KEN SWIDINSKY

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

The fungal product cyclosporin A is a powerful immunosuppressive with antifungal activity produced as a secondary metabolite from *Tolypocladium inflatum*. As its role in transplantation surgery increases, the demands on industry to improve yields intensifies. Mutants showing increased production are compared with strain, MS-1, the original strain for this work for glucose (dextrose) consumption and growth and a relationship with enzymes of the Embden-Meyerhof Pathway is investigated. Statistical analysis is performed to ensure significance.

Classical methods of mutation and selection for strain improvement are utilized. Increased cyclosporin A-producing mutants display decreased glucose consumption and slower biomass build-up when compared to strain MS-1 ($P<0.001$) suggesting a slower rate of growth supports higher production.

Coarse control of the enzymes hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) in cell-free extracts of *T. inflatum* was investigated. Decreased activities of HK, PFK and PK activity was observed in higher cyclosporin A-producing strains with probabilities of significance of <0.01 for HK and PFK and <0.05 for PK. A peak of activity early in the fermentation was also observed with probabilities of <0.01 that change by day in enzyme activity is significant for HK, PFK and PK. This suggests a decreased role by the EM pathway and the early establishment of the biochemical route for glucose catabolism.

First and foremost, I would like to express my gratitude to my advisor Dr. Xinfa Xiao who supervised this project and sacrificed many hours of his time. His guidance and advice was always appreciated and won't be forgotten.

Also my appreciation to Dr. David J. Cox and Apotex Fermentation Inc. who supported Dr. Xiao's proposal.

I would like to thank the members of my committee, Dr. Michael Butler and Dr. David Punter for their useful suggestions and comments.

Also, Dr. Jerry Su for making statistics a little easier.

And I especially want to thank my wife Shelly and son Brett - what a wonderful team. Their patience, understanding and support were my inspiration.

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ADP	-	adenosine-5'-diphosphate
AMP	-	adenosine-5'-monophosphate
ATCC	-	American Type Culture Collection
ATP	-	adenosine-5'-triphosphate
CFU/mL	-	colony forming units per millilitre
conidia/mL	-	conidia per millilitre
CsA	-	cyclosporin A
DF	-	degrees of freedom
DNA	-	deoxyribonucleic acid
ED	-	Entner-Doudoroff Pathway
EM	-	Embden-Meyerhof-Parnas Pathway
EMS	-	ethyl methane sulfonate
g	-	gram
g/L	-	grams per litre
GTP	-	guanosine-5'-triphosphate
HK	-	hexokinase
HMP	-	hexose monophosphate pathway
HPLC	-	high performance liquid chromatography
hrs	-	hours
KCl	-	potassium chloride
M.I.C.	-	minimum inhibitory concentration
$MgCl_2$	-	magnesium chloride, anhydrous
$MgSO_4 \cdot 7H_2O$	-	magnesium sulfate, heptahydrate
min	-	minute
mL	-	millilitre
mM	-	millimolar
mU/mg	-	milliunit per milligram
mV	-	millivolt
n	-	number of values
NAD/NADH	-	nicotinamide adenine dinucleotide (oxidized/reduced form)
NADP/NADPH	-	nicotinamide adenine dinucleotide phosphate (oxidized/reduced form)
nm	-	nanometre
NTG	-	1-methyl-3-nitro-1-nitrosoguanidine
°C	-	degrees celsius
P. chrysogenum	-	*Penicillium chrysogenum*
P-value	-	probability value
PEP	-	phospho(enol)pyruvate
PFK	-	phosphofructokinase
PK	-	pyruvate kinase
PP	-	pentose phosphate pathway
Prob	-	probability
Prob>F value	-	probability of a larger F-value
psi	-	pounds per square inch

r-value	-	correlation coefficient
RNA	-	ribonucleic acid
RPM	-	rotations per minute
SEM	-	standard error of the mean
T. inflatum	-	*Tolypocladium inflatum*
Tris	-	Tris(hydroxymethyl)-aminomethane
U	-	international unit of enzyme activity
UV	-	ultraviolet
ΔA/min	-	change in absorbance per minute
μg/mL	-	microgram per millilitre
μL	-	microlitre
μmole	-	micromole
$\mu W/cm^2$	-	microwatts per square centimetre

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1.0. INTRODUCTION

There are many fungal products of economic importance. Some are listed in Table 1 and have diverse natures and functions. The cyclic undecapeptide, cyclosporin A, an immunosuppressive with a weak, narrow spectrum of antifungal activity, is produced non-ribosomally from a multi-functional enzyme template, cyclosporin synthetase, by the filamentous fungus, *Tolypocladium inflatum* Gams (Kobel and Traber, 1982). The history of cyclosporin A has been reviewed (Bernard et al, 1986, Rehacek and De-Xiu, 1991) and the enzyme has been well-researched, purified and cloned (Zocher et al, 1986, Billich and Zocher, 1987, Lawen and Zocher, 1990, Dittmann et al, 1994, Weber and Leitner, 1994, Weber et al, 1994).

The fungal product cyclosporin A is recognized as a secondary metabolite (Dreyfuss et al, 1976, Rehacek and De-Xiu, 1991). As defined by Demain (1996) "a secondary metabolite is not 'secondary' because it is produced after growth, but because it is not involved in the growth of the producing culture. Thus, elimination of production of a secondary metabolite by mutation will not stop or slow down growth; indeed it may increase the growth rate".

1.1. Strain Improvement

The purpose of an industrial fungal strain improvement programme is to alter a specific characteristic or trait as desired through repeated cycles of genetic alteration and screening for selection. The selected strain is then scaled-

Table 1

Fungal products and their producing organism (Elander and Lowe, 1992)

Organism	Product
	Antibiotic
Cephalosporium acremonium	Cephalosporin C
Emericellopsis sp.	Penicillin N
Fusidium coccineum	Fusidic Acid
Paecilomyces varioti	Variotin
Penicillium chrysogenum	Penicillins G and V
Penicillium patulum	Griseofulvin
Tolypocladium inflatum	Cyclosporin
	Metabolites Other Than Antibiotics
Ashbya gossypii	Riboflavin
Aspergillus niger	Citric Acid
Aspergillus oryzae	Kojic Acid
Aspergillus terreus	Itaconic Acid
Ceratocystis virescens	Flavors and fragrances
Claviceps purpurea	Alkaloids
Fusarium moniliforme	Gibberellins
Penicillium stoloniferum	Mycophenolic Acid
Phycomyces blakesleanus	β-Carotene
Rhizopus sp.	Fumaric Acid
	Enzymes
Aspergillus sp.	Glucoamylase
Aspergillus sp.	Lipase
Aspergillus sp.	Pectinase
Aspergillus sp.	Pentosanase
Aspergillus niger	β-Glucanase
Aspergillus niger	Glucose oxidase
Aspergillus niger	Lactase
Aspergillus oryzae	α-Amylase
Aspergillus oryzae	Protease
Fusarium sp.	Penicillin acylase
Mucor sp.	Rennet
Penicillium sp.	Dextranase
Trichoderma reesei	Cellulase

	Miscellaneous Compounds
Aspergillus sp.	Polyols
Aureobasidium pullulans	Pullulan
Fusarium sp.	Microbial protein
Metarrhizium anisopliae	Microbial insecticides
Neurospora crassa	Insulin
Penicillium chrysogenum	Double-stranded RNA
Penicillium roquefortii	Cheese manufacturer
Rhizopus nigricans	Steroid conversion
Saccharomyces sp.	Ethanol
Saccharomyces cerevisiae	Interferons
Saccharomyces cerevisiae	Vaccines
Schizophyllum commune	Schizophyllan
Trigonopsis sp.	Glycerol

up commercially. Some of these traits to be considered are productivity, genetic stability, morphology, substrate efficiency, oxygen requirements, pigment production and shear sensitivity (Rowlands, 1992).

A strain's fermentation conditions can be optimized to enhance or reduce a specific trait by modifying the physical and/or chemical growth parameters (Calam, 1964, Lein, 1986). The most efficient method is to use fermentation modification in conjunction with a strain improvement programme (Calam, 1970, Lein 1986).

Besides the use of recombinant DNA technology, there are basically three ways by which a strain's genotype can be genetically altered resulting in the generation of a unique genotype: (1) by selection alone, (2) by the application of the techniques of hybridization followed by selection and (3) by mutation with selection (Johnston, 1975).

Significant improvements can be realized by the selection alone of natural spontaneous variation or mutation (Bos and Stadler, 1996, Elander, 1966) and has been successfully used in a penicillin strain improvement programme (Alikhanian, 1962).

Techniques involving breeding by hybridization and selection act via the parasexual cycle (Ball, 1984, Calam, 1970) but have made relatively little impact in relation to its potential (Rowlands, 1983).

The classical method of fungal strain improvement involves repeated cycles of mutation and selection. This undoubtedly is the most important method for obtaining improved strains and has been extensively reviewed by Alikhanian

(1962), Calam (1964), Davies (1964), Elander (1966), Rowlands (1983), Lein (1986), Rowlands (1992).

1.1.1. Induced and Spontaneous Mutation

Mutation is a change in a heritable trait (Bos and Stadler, 1996) spontaneously introduced or induced (Rowlands, 1983) either by physical means, such as near UV light (UV_{254}) or high-energy radiation, or chemical methods, such as 1-methyl-3-nitro-1-nitrosoguanidine (NTG) or ethyl methanesulfonate (EMS) (Calam, 1970, Baltz, 1986). Mutation is necessary in order to provide an effective input into a strain improvement programme (Rowlands, 1983). Once variation has been introduced, strains with the desired characteristic are detected by screening and subsequently selected for commercial use in large-scale fermentation or chosen as the start strains for another round of mutation and selection. All mutation and selection is a form of recycling (Simpson and Caten, 1979b, Rowlands and Normansell, 1983).

The source of all genetic variation is mutation, whether spontaneous or induced. Spontaneous variants are screened as single-colony isolates within a population and result in cultures that are pure and have the highest productivity or other commercially useful characteristic. To facilitate the detection of improved strains it is necessary to begin a mutation and selection cycle with a pure culture (Hopwood, 1970, Rowlands, 1992).

As previously stated, genetic variation or mutagenesis of a genotype can be induced by both physical and chemical treatments. In the example of

germicidal ultraviolet light (UV_{254}), chromosomal aberrations are introduced to the DNA as thymidine dimers and are "fixed" into place by an error-prone excision repair mechanism (Rowlands, 1983, Bridges, 1976, Rowlands, 1992, Bos and Stadler, 1996). The procedure for mutagenesis by germicidal light is quite straightforward. A conidial suspension is exposed with stirring for a length of time in order to reach a certain amount of killing. The process is carried out in the dark to prevent photoreactivation or the reversal of pyrimidine dimers caused by exposure to visible light (Baltz, 1986). For convenience, a consistent initial conidial concentration of 10^7/mL is recommended (Calam, 1970). A kill in the order of 90-99% or more is suggested because it is believed to result in mutants with increased productivity (Calam, 1970, Bos and Stadler, 1996) although this claim is still subject to differences in opinion by authors that agree low kill rates are better then high (Rowlands, 1983). The survivors are plated out and incubated.

1.1.2. Screening for Improved Mutants

Screening is required to detect, from hundreds and thousands of individuals, those mutants with the trait of interest, for example, productivity. Those mutants exhibiting the trait of interest are then selected. A screen can be divided into two types: (1) a direct screen where product is directly analysed and (2) an indirect, rational or pre-screen. In the second case, a known biochemical or genetic property is assayed that is associated with the product of interest rather then the actual product (Rowlands, 1983, Rowlands and Normansell,

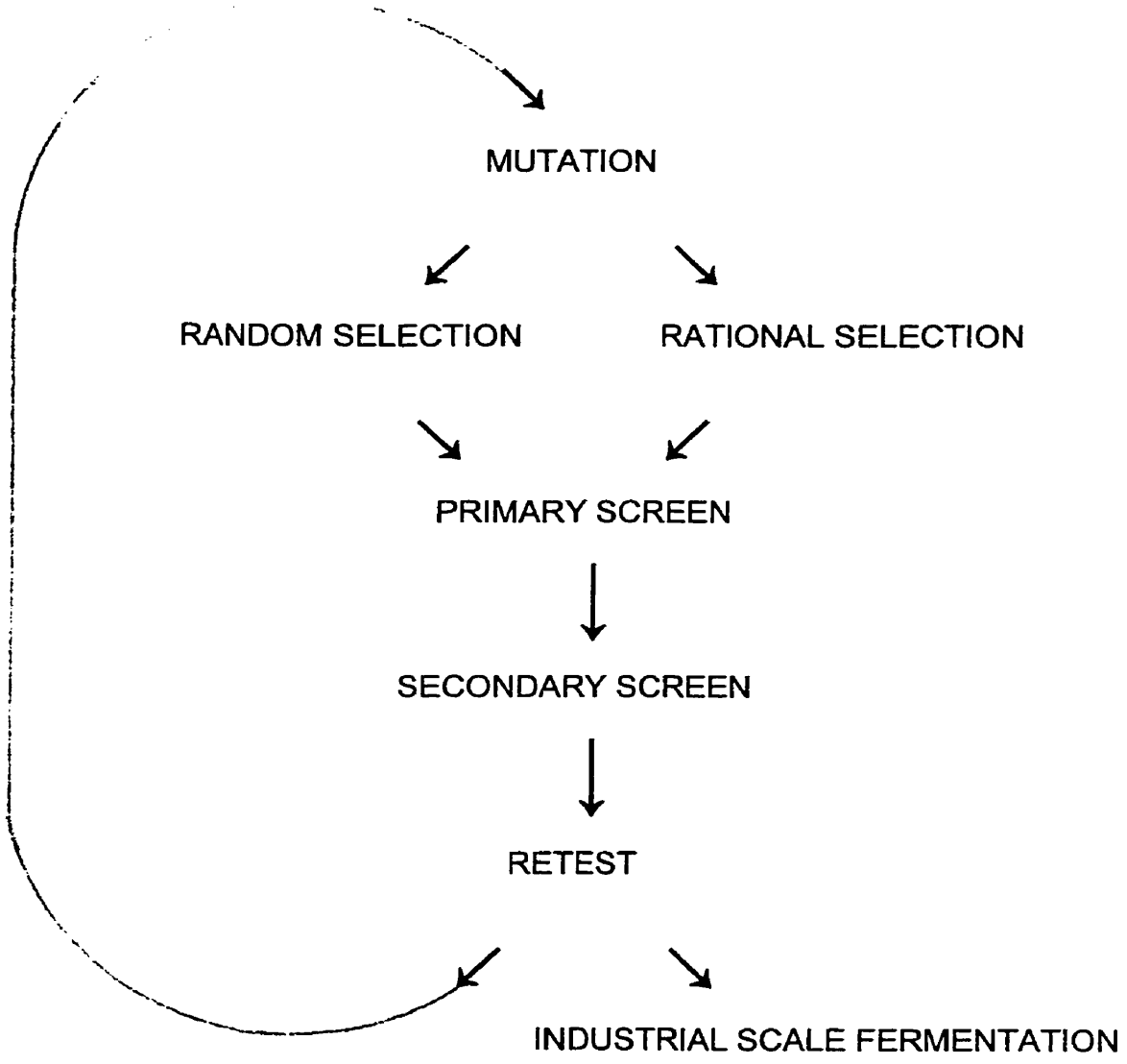
1983, Rowlands, 1992).

A direct screen is commonly referred to as a random screen or random selection and can be organized into a multi-level screen (Figure 1). A multi-level screen is a useful screening strategy when improved mutants are rare and the error of testing of, or production from, the screen is high (Calam, 1964, Davies, 1964, Rowlands, 1992). Following incubation of the mutagenized conidia, isolated colonies are selected and streaked onto solid medium. After incubation, each colony is inoculated as a single replicate and incubated. Primary screening is designed with a lower resolution to allow the maximum throughput of isolates to be assayed. The low-resolution screen can be performed with agar plugs or plates, miniature shake flasks or in tubes (Rowlands, 1992) and differentiates the high producers from the low producers with the high producers being selected for further analysis in a mid-resolution screen (Calam, 1964). At this secondary level, the number of replicates is increased and approximately 10% of the most active producers are selected for assay with the maximum allowable number of replicates in a high-resolution screen at the retest level. The number of replicates is increased to improve accuracy and screening (Calam, 1970). From this high-resolution screen, the top producing strains are then selected as start strains in the next cycle (Davies, 1964, Calam, 1964, Queener and Lively, 1986).

A rational screen is a pre-screen and is highly effective because it kills those mutants with low productivity (Rowlands, 1992). In this way, the application of a pre-screen will significantly concentrate the improved mutants of interest within the mutated population thus making the entire screening process more

Figure 1

Flow diagram for fungal strain improvement using a multi-level screen



efficient (Chang and Elander, 1979). An effective pre-screen removes the empiricism and randomness of a direct screen (Rowlands, 1992). A number of methods have been recognized as effective for screening of commercial strains of *Acremonium chrysogenum* for industrial production of cephalosporin C: (1) direct selection of colonies based on antibacterial activities, (2) selection of mutants resistant to amino acid analogs or metabolic inhibitors, (3) selection of mutants sensitive to antimetabolites or growth inhibitors, (4) isolation of mutants resistant to metallic ions, (5) isolation of specific morphological mutants, (6) selection of auxotrophs followed by reversion to prototrophy, (7) isolation of spontaneous or induced sector variants and (8) isolation of presumptive homozygous diploids (Chang and Elander, 1979). Strains selected based on one of the above criteria are still assayed directly for production in shake flasks in a multi-level screen (Figure 1) to protect against "false positives" (Rowlands, 1992). Agathos et al (1986) successfully employed the selection of auxotrophs followed by reversion to prototrophy with *T. inflatum* ATCC 34921 as a method of increasing the yields of cyclosporin A. Auxotrophy was lost in the second generation but increased productivity was retained.

The rational screening method used effectively for this thesis was resistance to fungal growth inhibitors, AF1 and AF2. These antagonists are classified as polyene antifungal agents. Polyenes have been used to increase titres of penicillin G in *Penicillium chrysogenum* and cephalosporin C in *Cephalosporium acremonium* (Rowlands and Normansell, 1983). The effect of polyene antibiotics on cells of fungal origin is well-established (Grindle, 1974,

Aperecida de Resende and Alterthum, 1990). The active sites are sterols in the cell membrane and binding them alters the permeability of membranes causing the cell to leak low molecular weight compounds and ions such as small proteins, phosphates and potassium. The result is cell death. Polyene antibiotic-resistant mutants achieve resistance by a reduction in or loss of a binding site for the antagonizing agent and are therefore identified by their germination and growth in its presence. Intermediates and the primary metabolic machinery normally required for ergosterol biosynthesis would be considered as redirected to secondary metabolism resulting in overproduction and increased yield (Queener et al, 1982, Ozaki et al, 1987, Grunwald-Raij and Margalith, 1990).

1.2. Metabolic Pathways in Fungal Glucose Catabolism

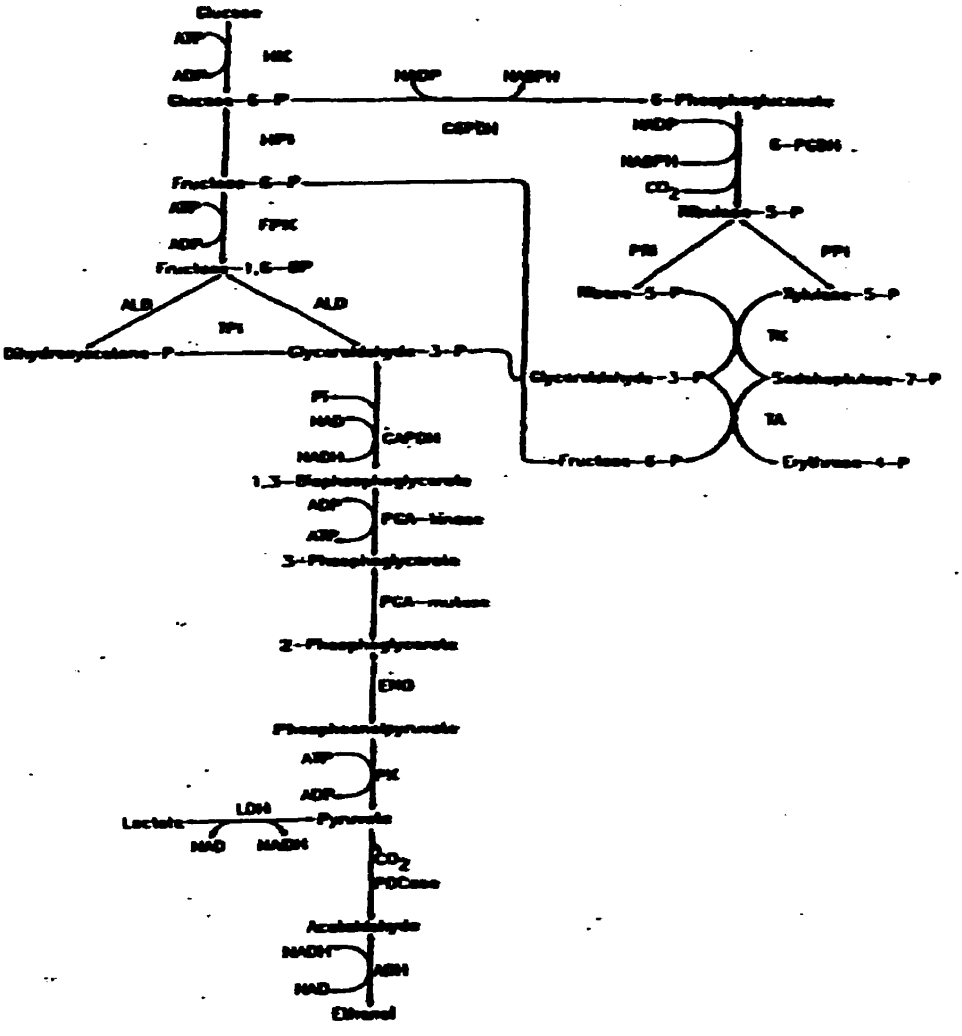
In order to understand the concepts of the regulation of secondary metabolism a brief description of the intracellular catabolism of glucose and the role it plays in primary metabolism would be useful.

The main metabolic pathways universally recognized in glycolysis or for the breakdown of glucose by the cell are the Embden-Meyerhof-Parnas (EM) pathway, the Pentose Phosphate (PP) pathway (Cochrane, 1976) (Figure 2) and the Entner-Doudoroff (ED) pathway.

The ED pathway has been established as a pathway for the anaerobic breakdown of glucose by bacteria (Cochrane, 1976, Fraenkel, 1986) and its presence is only suggested in the fungi, *Tilletia caries* and *Caldariomyces fumago* (Cochrane, 1976).

Figure 2

Metabolic reactions of the Embden-Meyerhof-Parnas Pathway and Pentose Phosphate Pathway for the catabolism of glucose (Miernyk, 1990)



Embden-Meyerhof pathway enzymes HK = hexokinase, HPI = hexose phosphate isomerase, PFK = phosphofructokinase, ALD = fructose 1,6-bisphosphate aldolase, GAPDH = NAD glyceraldehyde 3-phosphate dehydrogenase, PGA = phosphoglycerate, PK = pyruvate kinase, PDCase = pyruvate decarboxylase, ADH = alcohol dehydrogenase, LDH = lactate dehydrogenase, Pentose Phosphate pathway enzymes G6PDH = glucose 6-phosphate dehydrogenase, 6PGDH = 6-phosphogluconate dehydrogenase, PRI = phosphoriboisomerase, PPE = phosphopentose epimerase, TK = transketolase, TA = transaldolase

Many of the pioneering studies establishing the existence of the Embden-Meyerhof-Parnas pathway were performed on yeast. It has been recognized from the 1960's that the EM pathway exists (Blumenthal, 1965) and is very common in filamentous fungi (Cochrane, 1976). The EM pathway involves the sequence of catabolic reactions between glucose and the resultant two moles of pyruvate. Its role is the production of ATP, from substrate-level phosphorylation, pyruvate, used in the formation of acetyl CoA and NADH, which can be converted to NAD^+ by oxidative phosphorylation resulting in ATP or reoxidized anaerobically (Griffin, 1993, Voet and Voet, 1995).

The first EM pathway step involves a family of hexose-phosphorylating enzymes commonly known as hexokinase (ATP:D-Hexose-6-phosphotransferase; EC 2.7.1.1), which exists in two forms, P-I and P-II (Colowick, 1973) or A and B (Ramel et al, 1971) with molecular weights of 108,000 each (Easterby and Rosemeyer, 1972), as well as glucokinase (ATP:D-Glucose-6-phosphotransferase; EC 2.7.1.2) (Turner and Turner, 1980). The hexokinase irreversibly phosphorylates several hexoses including fructose (Fraenkel, 1986, Turner and Turner, 1980):

$$ATP + Glucose \rightarrow ADP + Glucose\text{-}6\text{-}phosphate$$

Glucokinase phosphorylates only glucose. Hexokinase from yeasts has been extensively studied by Colowick (1973). A double mutant lacking any two of the three enzymes grew on glucose but a triple mutant did not. Therefore, individual

enzymes can be used for growth (Fraenkel, 1986). The P-I form phosphorylates fructose 2.6 times faster than glucose and the P-II form 1.3 (Turner and Turner, 1980). According to Fraenkel (1986) in some wild-type yeast strains, P-II is mainly required for growth and the P-I form is carbon catabolite repressed and is only expressed upon glucose depletion or growth in ethanol.

Low concentrations of a number of metabolites including inorganic phosphate, citrate, malate, glycerate-3-phosphate and ATP activate P-II in a sulphonate buffer with a pH below 7.0 (Turner and Turner, 1980). P-II is inhibited by ADP and GTP in concentrations of 0.2-0.5 mM. ADP and GTP activate the enzyme without sulphonated buffers (Colowick, 1973). Other roles for hexokinase, in addition to the phosphorylation of hexoses, have been proposed including catabolite repression and transport (Fraenkel, 1986).

The second EM enzyme is phosphofructokinase (ATP:D-Fructose-6-phosphate 1-phosphotransferase; EC 2.7.1.11). Phosphofructokinase (PFK) catalyses an irreversible glycolytic step:

ATP + D-Fructose-6-phosphate → ADP + D-Fructose-1,6-diphosphate

Its regulation could determine the rate of glycolysis (Turner and Turner, 1980, Lobo and Maitra, 1983).

It is the second largest glycolytic enzyme in yeast with a molecular weight of approximately 800,000 and an $\alpha_4\beta_4$ structure. The subunits have similar sizes

with slightly different composition. It has been suggested that α and β subunits have different functions, regulatory and catalytic, respectively (Fraenkel, 1986). Since PFK is under metabolic control there is a variety of effectors. Inhibition of PFK is by ATP and activation is by AMP in *Candida albicans* and an important influence in controlling the use of hexose phosphates is the activity of PFK (Chattaway et al, 1973). In *Neurospora crassa*, it is inhibited by ATP, ADP, AMP, citrate and P_i (Tsao and Madley, 1972, Tsao et al, 1969).

The third of three glycolytic enzymes to be assayed is pyruvate kinase (ATP:Pyruvate 2-O-phosphotransferase; EC 2.7.1.40). Pyruvate kinase (PK) catalyses an energy-yielding reaction in a slightly reversible step:

ADP + Phosphoenolpyruvate → ATP + Pyruvate

and is responsible for the balance between glycolysis and gluconeogenesis (Kayne, 1973, Barwell et al, 1971). Pyruvate kinase of *Neurospora crassa* has been purified (Kapoor and Tronsgaard, 1972) and the enzyme has been reviewed (Kayne, 1973). Yeast PK has a molecular weight of 162,000 - 168,000 (Kuczenski and Suelter, 1970) and exists in multiple conformations probably involved in regulation (Kayne, 1973).

Pyruvate kinase in yeast has a number of effectors and is positively activated by phosphoenolpyruvate with the cations K^+ (or NH_4^+) and Mg^{++} (Turner and Turner, 1980) and inhibited by ATP, AMP, 3',5'-AMP, NADP, citrate and

Ca^{++} (Kuczenski and Suelter, 1970).

An alternative to glycolysis, the pentose phosphate (PP) pathway, also referred to as the phosphogluconate oxidation pathway and the hexose monophosphate shunt, exists in the higher fungi and there is evidence to support its existence in imperfect fungi (Malca et al, 1968, Greene, 1969). A paper by Casselton (1966) reports on the evidence for EM and PP pathway enzymes in cell-free extracts of *Polyporus brumalis* when grown in a carbohydrate-based medium. According to Griffin (1993) the PP pathway is widespread if not universal in the fungi. The PP pathway is so-called because of the existence of pentose-phosphate intermediates, ribose-5-phosphate and xylulose-5-phosphate. There exists two varieties of the PP pathway - a reductive and an oxidative pathway. The reductive sequence is found in plants and some bacteria and is involved in the photosynthetic fixation of CO_2 (Miernyk, 1990). The major role of the oxidative PP pathway is to supply reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the cell for biosynthetic reactions (Cochrane, 1976, Miernyk, 1990, Turner and Turner, 1980, Griffin, 1993). It is also acknowledged that the PP pathway supplies pentose phosphates for the production of aromatic amino acids and nucleic acid synthesis (Turner and Turner, 1980, Wood, 1986) and its control is not fully understood (Barnett, 1976). A final intermediate for the PP pathway does not exist as in the EM pathway where the end-product is never in doubt. Intermediates in the PP pathway are utilized based on the biochemical requirements of the cell (Cochrane, 1976).

Overall, information surrounding enzymatic regulation in fungi is not

extensive but is partially in agreement with that for bacterial and animal cells. The control mechanism balancing the direction or pathway by which glucose is catabolized is uncertain. A detailed review of the literature is not possible and generalizations are difficult (Cochrane, 1976).

In aerobically growing *Saccharomyces cerevisiae* cells, 12% of D-glucose is catabolized by the PP pathway and the remainder by the EM pathway. In resting cells of *Saccharomyces cerevisiae,* all oxidation of D-glucose took place in the EM pathway although an earlier study indicated that 17% its breakdown occurred in the PP pathway and a later estimate 9.7% with subsequent studies suggesting even lower percentages. For *Candida (Torulopsis) utilis* and *Candida albicans* the figure is 35% and 50%, respectively (Barnett, 1976).

Brady and Chambliss (1967) and Osmond and Ap Rees (1969) both agree that as the enzyme activity of the EM pathway decreases there is an increase in activity in PP enzymatic activity. This would result in an increase in intracellular NADPH. Evidence of the inverse relationship for specific activity in EM and PP pathway enzymes was found in differentiating cultures of *Aspergillus niger* (Smith et al, 1971). They also state that the enzymatic differences are mainly limited to young mycelium in a period of growth of 24-48 hours. According to Fuscaldo et al (1971) a "eucaryotic cell is estimated to produce about 50% of the reduced cofactor via the HMP (PP) shunt pathway".

1.3. Regulation of Secondary Metabolism

The regulation of secondary metabolism involves many aspects

(Rowlands, 1992). I will be considering carbon regulation, ATP regulation and regulation by growth as they relate to the context of this thesis.

1.3.1. Carbon Regulation

Glucose is an excellent and rapidly used source of carbon for microbial growth while its utilization interferes with secondary metabolism (Demain, 1991). The interference was also observed with other quickly catabolized carbohydrate sources in the biosynthesis of antibiotics (Table 2). In fermentation medium containing two carbon sources, one rapidly consumed and another consumed more slowly, the rapidly catabolized source is preferentially utilized for growth without any secondary metabolite production. Following depletion, the second source is used for antibiotic biosynthesis in the built-up mycelial mass. Well-known cases are the regulation of penicillin and cephalosporin production by glucose. Glucose is preferentially consumed by *P. chrysogenum* and is most suitable for growth but not penicillin production . Lactose exhibits the opposite behaviour and is consumed after the depletion of glucose (Soltero and Johnson, 1953). This suggests that glucose represses the enzymes required for penicillin biosynthesis (Demain, 1991).

The repression of enzymes responsible for the synthesis of secondary metabolites subject to glucose control are well-known and the direct inhibition of some enzymes is also thought to be controlled by carbon (Demain, 1996). Glucose represses single enzymes of biosynthetic pathways. Phenoxazinone synthase, involved in the phenoxazinone ring of actinomycin, is repressed by

Table 2

The regulation of antibiotic biosynthesis by sources of carbon (Martin and Demain, 1980)

Antibiotic	Interfering Carbon Source	Noninterfering Carbon Source
Penicillin	Glucose	Lactose
Actinomycin	Glucose	Galactose
Streptomycin	Glucose	Mannan Slowly fed glucose
Siomycin	Glucose	Maltose
Indolmycin	Glucose	Fructose
Bacitracin	Glucose	Citrate
Cephalosporin C	Glucose	Sucrose
Chloramphenicol	Glucose	Glycerol
Violacein	Glucose	Maltose
Prodigiosin	Glucose	Galactose
Mitomycin	Glucose	Low glucose
Neomycin	Glucose	Maltose
Kanamycin	Glucose	Galactose
Enniatin	Glucose	Lactose
Puromycin	Glucose	Glycerol
Novobiocin	Citrate	Glucose
Candidin	Glucose	Slowly fed glucose
Candihexin	Glucose	Slowly fed glucose
Butirosin	Glucose	Glycerol
Cephamycin	Glycerol	Asparagine, starch

glucose resulting in reduced actinomycin biosynthesis. Also, phosphatase(s) required for neomycin production are repressed by glucose (Martin and Demain, 1980).

The direct carbon catabolite inhibition and a number of phosphorylated carbon intermediates inhibit δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine (acyl) synthetase, the first enzyme of cephalosporin biosynthesis in crude extracts of *C. acremonium* (although no inhibition was observed with the purified enzyme) (Demain, 1996). The action of penicillin-forming enzymes in *P. chrysogenum* was not inhibited by glucose (Demain, 1991).

1.3.2. ATP Regulation

Similar to carbon regulation, there is much written concerning ATP regulation. Evidence exists that too much ATP produced and accumulated affects secondary metabolite production (Butler et al, 1997). For example, (1) intracellular concentrations of ATP increase rapidly in *Streptomyces griseus*, a producer of the polyene antibiotic candicidin, after addition of phosphate to the medium and the increase occurs just before the inhibition of antibiotic synthesis (Martin and Demain, 1976), (2) time course studies on ATP concentration reveal a decrease in intracellular ATP prior to the onset of antibiotic synthesis (Liras et al, 1977) and (3) intracellular concentrations of ATP are lower in improved-producing strains than in their low-producing ancestors (Saliva et al, 1965, Janglova et al, 1969).

1.3.3. Growth Rate Regulation

Carbon and ATP regulation of antibiotic synthesis via repression or inhibition may also involve growth rate although mechanisms are not well understood (Demain, 1991, Demain, 1996). It is stated by Martin and Demain (1980) that an important characteristic of secondary metabolism is that these metabolites are produced at low specific growth rates suggesting that antibiotic synthetases are not formed or repressed or, if formed, the activity of the synthetase is inhibited when the growth rate is high. A slow growth rate during penicillin biosynthesis can be obtained from a fermentation and interference by glucose can be prevented with the slow feeding of glucose (Soltero and Johnson, 1954). This fermentation procedure can also be used to increase the production of the polyene antifungals, candidin and candihexin (Martin and McDaniel, 1974). It is concluded by maintaining a constant lower glucose concentration in the medium from slow-feeding, product formation is favoured at the expense of cell growth (Table 2).

In general, production of a secondary metabolite is delayed either by repression or inhibition during the early stages of growth or during logarithmic growth (Demain, 1996). Producing organisms are sensitive to their metabolic product especially in the early exponential stages of growth and therefore a delay in production offers the organism a survival advantage by preventing its suicide (Demain, 1982).

Much research has been performed on the mode of action of cyclosporin A (Foxwell and Ryffel, 1989) and some aspects of its regulation have been

studied (Kobel and Traber, 1982, Agathos et al, 1986, Chun and Agathos, 1991, Lee and Agathos, 1991) but there appears to be a lack of evidence specifically for *Tolypocladium inflatum* on the regulation of this metabolite's production by carbon utilization, adenosine-5'-triphosphate (ATP) and amount of growth in comparison with the quantity of information available for these topics in relation to other secondary metabolite producers. The regulation of cyclosporin A productivity-improved mutants derived from the classical techniques of strain improvement is involved in this research. Its focuses are:

1) The classical methods of mutation and selection to improve yields of cyclosporin A from strains of *T. inflatum;*

2) Yields of cyclosporin A associated with carbon consumption and the growth of the producing organism, and

3) The activity of enzymes involved in glucose catabolism and their relationship with increased yields of cyclosporin A.

A combination of the classical strain improvement techniques of mutation and selection resulting in increased yields of secondary metabolite and the concurrent elucidation of the intracellular routes for catabolites has been used with success for the overproduction of β-lactam antibiotics such as penicillin from the fungi, *Aspergillus nidulans* and *Penicillium chrysogenum* (Ball, 1984). That philosophy is applied to this study using improved production strains of *T. inflatum.*

2.0. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microorganism

The filamentous fungus *Tolypocladium inflatum* ATCC 34921, listed as *Beauveria nivea* in the ATCC Catalog of Fungi/Yeasts (17th Edition, 1987, Rockville, Maryland, USA) was the organism used for this research. The fungus *Trichoderma polysporum* (Link ex Pers.) Rifai was discovered as the original producer of the metabolite cyclosporin A (Dreyfuss et al, 1976). It was later reclassified as *Tolypocladium inflatum* Gams (Kobel and Traber, 1982) and is now more correctly referred to as *Tolypocladium niveum* Rostrup (Isaac et al, 1990).

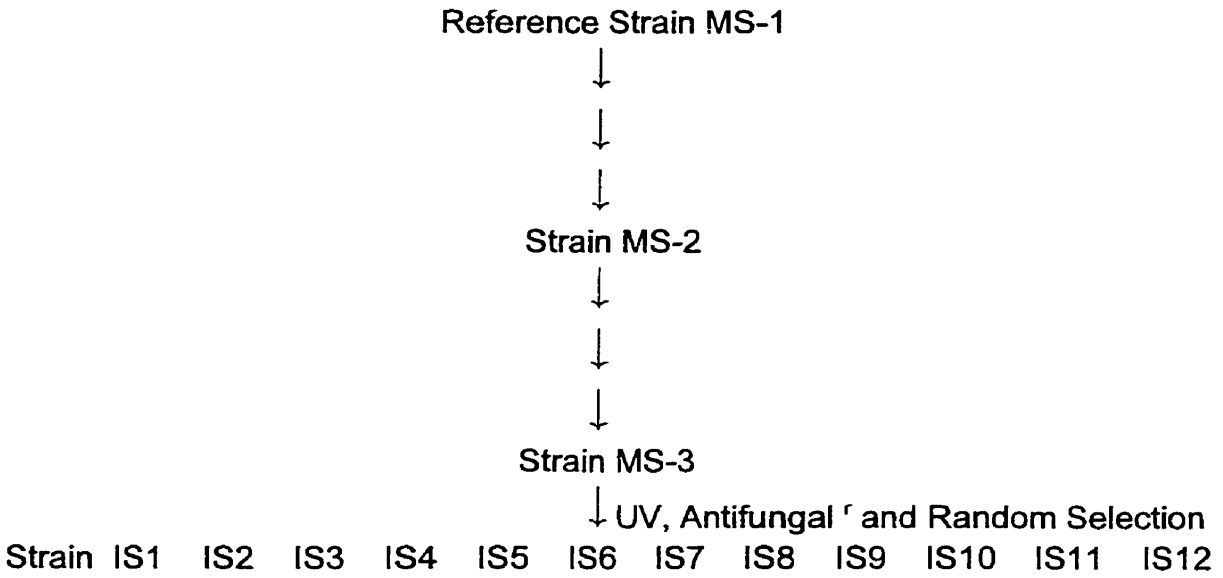
From the reference cyclosporin-producing strain, MS-1, involved in this work active cyclosporin A-producing mutants MS-2 and MS-3 and subsequently improved strains were obtained through a series of mutations and selections (Figure 3). The parent MS-3 was the start strain for the last cycle of strain improvement and yielded mutants IS1 to IS12.

2.1.2. Chemicals

All chemicals in this research were purchased commercially. Adenosine 5'-triphosphate (ATP), β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phospho(enol)pyruvate,

Figure 3

Lineage for the improvement of cyclosporin A production from strains of *Tolypocladium inflatum*



↓ = mutation and selection cycle, UV = ultraviolet (254 nm) light irradiation, Antifungal^r = AF1 and AF2 resistant

fructose-6-phosphate, β-nicotinamide adenine dinucleotide, reduced form (β-NADH), pyruvate kinase (EC 2.7.1.40), L-lactic dehydrogenase (EC 1.1.1.27), adenosine 5'-diphosphate (ADP), antifungals AF1 and AF2 were purchased from Sigma Chemical Company. Magnesium chloride was purchased from Aldrich Chemical Company, $MgSO_4 \cdot 7H_2O$ and KCl from Caledon Laboratories Limited from Biorad Laboratories.

2.2. Assays

2.2.1. Dry Cell Weight Determination

Dry cell weight for the determination of biomass or amount of growth was obtained from culture filtrates using a GF/A glass micro fibre filter (Whatman International Limited). The biomass reported in this thesis has been converted to relative biomass units with the 10 day harvest of the MS-1 culture being one unit. This harvest biomass was the reference for the calculation of relative values of all other dry cell weights.

2.2.2. Dextrose (Glucose) Concentration

The dextrose concentration in submerged culture broth was measured using a YSI Select Biochemistry Analyser Model 2700 equipped with a YSI Immobilized Enzyme Membrane specific for dextrose (D-glucose) (YSI Incorporated).

2.2.3. pH

The pH was measured using an EC10 Portable pH/mV/Temperature Metre Model 50050 equipped with a Combination pH Electrode with Temperature Model 50200 (Hach Company).

2.2.4. Protein Concentration

Protein concentration in cell-free extracts was measured with the Biorad Protein Assay (Biorad Laboratories) procedure using bovine serum albumin (standard II). The procedure was based on the method by Bradford (1976).

2.2.5. Cyclosporin A HPLC Assay and Conversion of Titre to Relative Cyclosporin A Units

A methanol extract from a sample of whole broth was measured for cyclosporin A content. Cyclosporin A was measured using the HPLC method according to Kreuzig (1984) with modifications. The titres reported in this thesis have been converted to relative titre units with the 10 day harvest of the MS-1 culture being one unit. This harvest titre was the reference for the calculation of relative values of all other titres.

2.2.6. Glycolytic Enzymes Activity Assays

The assay time for each enzyme was 10 seconds with a wavelength of 339 nm using a Shimadzu UV-160 UV-Vis Recording Spectrophotometer (Shimadzu Corporation).

One international enzyme unit (U) of enzyme activity was defined as 1μmole of product formed in one minute at 27.5°C in the conditions described (Perkampus, 1992). Specific activity is recorded as milliunit/mg of protein (mU/mg).

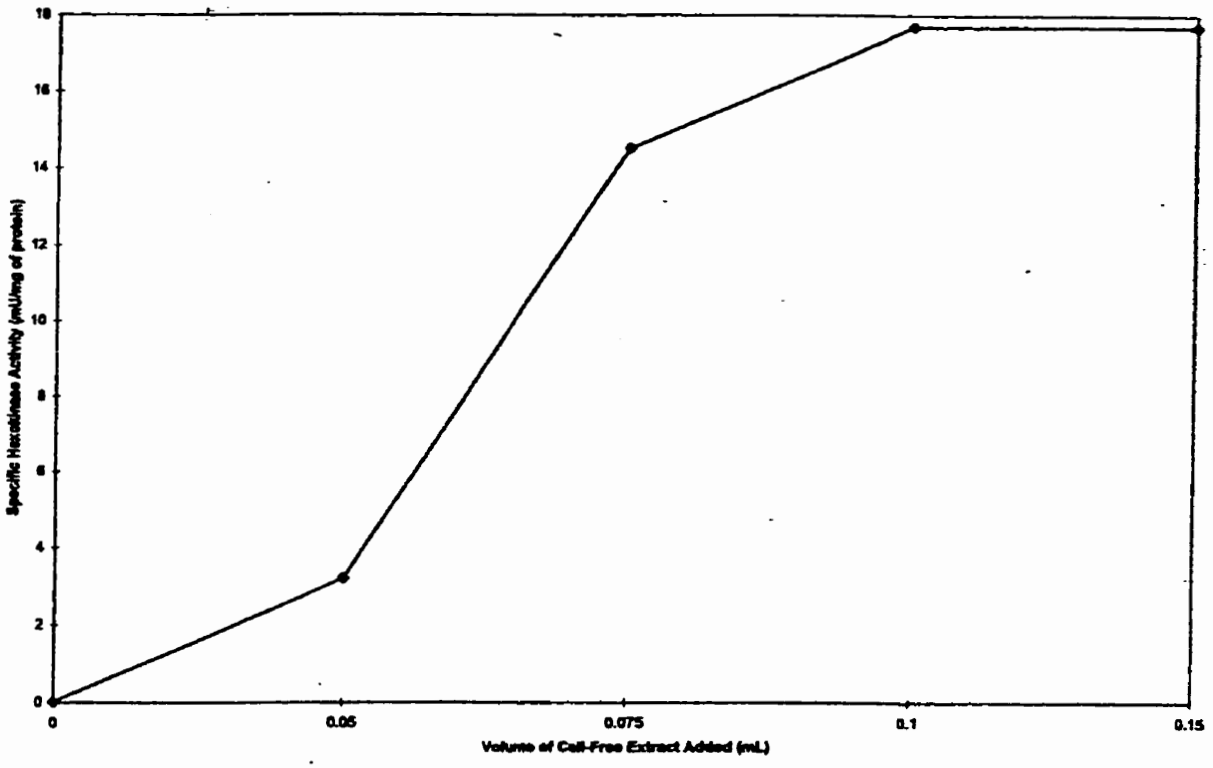
2.2.6.1. Hexokinase

Hexokinase assay was carried out as described by Fraenkel and Horecker (1964), Bergmeyer et al (1974a) and Martin and Russel (1986). Hexokinase activity was determined from a spectrophotometric assay using a coupled reaction of glucose-6-phosphate dehydrogenase. The amount of product (glucose-6-phosphate) formed was directly proportional to the amount of reduced β-NADP formed (Porter et al, 1980). Each parameter was optimized by varying only the studied factor while all other conditions were kept constant. The enzyme assay for determination of hexokinase activity was 0.10 mL 5 mM dextrose, 0.10 mL 250 mM ATP, 0.10 mL 10 mM β-NADP, 0.10 mL 500 mM $MgCl_2$, 4.6 units of glucose-6-phosphate dehydrogenase and 0.10 mL cell-free extract in Tris buffer, pH 7.5 to a final volume of 1.00 mL in an acryl-cuvette (Sarstedt No. 67.740) . Assay temperature was 27.5°C.

The assay was tested for HK activity prior to optimization (Figure 4). The specific enzyme activity was measured each time an increased volume of cell-free extract from a mutant strain was added to the assay. Activity increased with increasing volumes of cell-free extract up to 0.10 mL suggesting a validated assay.

Figure 4

Validation of the hexokinase assay by increasing the volume of cell-free extract added to the system



Hexokinase assay was validated prior to optimization by measuring the specific activity of the enzyme when adding increasing volumes of cell-free extract prepared from a mutant strain to an acryl-cuvette containing 0.10 mL each of 0.1 mM dextrose, 0.1 mM ATP, 0.01 mM β-NADP, 1 M $MgSO_4 \cdot 7H_2O$, 7.6 units of glucose-6-phosphate dehydrogenase in buffer, pH 7.5 to a final volume of 1.00 mL, 30°C at 339 nm.

2.2.6.2. Phosphofructokinase

Phosphofructokinase assay was carried out as described by Bergmeyer et al (1974b). The activity of PFK was determined by a spectrophotometric assay using the coupled reaction of pyruvate kinase and L-lactic dehydrogenase. The amount of product (fructose-1, 6-diphosphate) formed was directly proportional to the amount of β-NADPH oxidized. Assay modifications were determined from a factorial design performed on Design-Ease Software, Version 5.0 (Stat-Ease Inc). Factorial designs are an efficient type of experiment allowing all possible combinations of the levels of the factors to be investigated (Montgomery, 1991). The PFK assay was not optimized. Modifications were determined by varying the concentrations of 6 assay factors for two levels in a 2^{6-1}, standard order fractional factorial design (Table 12). A 2^{6-1} design allows 32 of the possible 64 combinations to be tested. Each combination or PFK assay in the design was tested for a response. Response was measured as PFK activity (ΔA/min). The values of the assay factors $MgSO_4 \cdot 7H_2O$, KCl, phospho(enol)pyruvate, fructose-6-phosphate, ATP and β-NADH were arbitrarily selected to be 5 times less then the control at low levels and 5 times more then the control at high levels. The trial with the greatest response was selected as the enzyme assay. Analysis of variance indicates the model's Prob>F value was <0.0001 suggesting a 99.99% confidence level that the model's results are significant (Table 13). The enzyme assay selected for determination of phosphofructokinase activity was 0.10 mL 70 mM $MgSO_4 \cdot 7H_2O$, 0.10 mL 225 mM KCl, 0.10 mL 35 mM phospho(enol)pyruvate, 0.10 mL 90 mM fructose-6-phosphate, 0.10 mL 2.2 mM

ATP, 0.10 mL 0.8 mM β-NADH, 19.68 units of pyruvate kinase, 8.8 units of L-lactic dehydrogenase and 0.10 mL cell-free extract in buffer, pH 7.5 to a final volume of 1.00 mL in an acryl-cuvette (Sarstedt No. 67.740). Assay temperature was 27.5°C.

The assay was tested for PFK activity after it was developed (Figure 5). The specific enzyme activity was measured each time an increased volume of cell-free extract from a mutant strain was added to the assay. Activity increased with increasing volumes of cell-free extract up to 0.15 mL suggesting a validated assay.

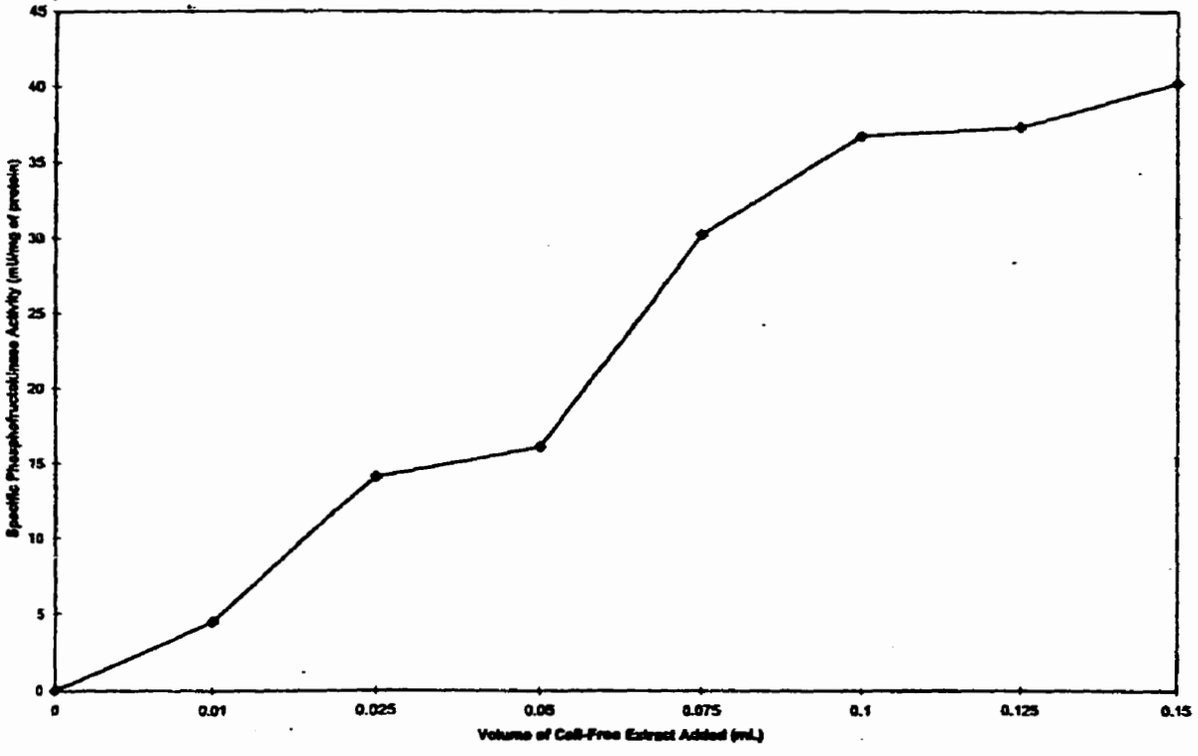
2.2.6.3. Pyruvate Kinase

Pyruvate Kinase assay was carried out as described by Fujii and Miwa (1983). The PK activity was determined by a spectrophotometric assay using the coupled reaction of L-lactic dehydrogenase. The amount of product (pyruvate) is directly proportional to the amount of β-NADPH oxidized. Modifications to the PK assay were determined from sequential factorial designs performed on Stat-Ease Software, Version 5.0 (Stat-Ease Inc). The PK assay was not optimized.

Modifications were determined by varying the concentrations of 5 assay factors for two levels in a 2^{5-1}, standard order fractional factorial design (Table 14). A 2^{5-1} design allows 16 of the possible 32 combinations to be tested. Each combination or PK assay in the design was tested for a response. Response was measured as PK activity (ΔA/min). The values of the assay factors $MgCl_2$, KCl, phospho(enol)pyruvate, β-NADH and ADP were arbitrarily selected to be 10

Figure 5

Validation of the phosphofructokinase assay by increasing the volume of cell-free extract added to the system



Phosphofructokinase assay was validated by measuring the specific activity of the enzyme when adding increasing volumes of cell-free extract prepared from a mutant strain to an acryl-cuvette containing 0.10 mL each of 70 mM $MgSO_4 \cdot 7H_2O$, 225 mM KCl, 35 mM phospho(enol)pyruvate, 90 mM fructose-6-phosphate, 2.2 mM ATP, 0.8 mM β-NADH, 19.68 units pyruvate kinase and 8.8 units L-lactic dehydrogenase in buffer, pH 7.5 to a final volume of 1.00 mL, 27.5°C at 339 nm.

times less then the control at low levels and 10 times higher then the control at high levels. The significant interactions were identified (Table 15) with an analysis of variance confidence level of 92.76% where the model's Prob>F value was 0.0733. Low levels of $MgCl_2$ and KCl (Figure 20), a low level of KCl and high level of β-NADH (Figure 21), low levels of KCl and ADP (Figure 22), high levels of phospho(enol)pyruvate and β-NADH (Figure 23) and a high level of phospho(enol)pyruvate and low level of ADP (Figure 24) increased PK activity.

The factor levels from the interactions that favour PK activity were selected as control factors in a second two level 2^{5-1}, standard order fractional factorial design (Table 16). The levels of the control factors were 0.02 mM $MgCl_2$, 0.1 mM KCl, 1 mM phospho(enol)pyruvate, 0.05 mM β-NADH and 0.005 mM ADP. The values of the factors in this assay were arbitrarily selected to be 5 times less then the control at low levels and 5 times higher then the control at high levels. Each combination or PK assay in the design was tested for a response. Response was measured as PK activity (ΔA/min). Analysis of variance indicates the model's Prob>F value was <0.0001 suggesting a 99.99% confidence level that the model's results are significant (Table 17). The trial with the greatest decrease in absorbance was selected as the enzyme assay. The enzyme assay for determination of pyruvate kinase activity was 0.10 mL 0.1 mM $MgCl_2$, 0.10 mL 0.5 mM KCl, 0.10 mL 0.2 mM phospho(enol)pyruvate, 0.10 mL 0.25 mM NADH, 0.10mL 0.001 mM ADP, 8.8 units of L-lactic dehydrogenase and 0.10 mL cell-free extract in buffer, pH 7.5 to a final volume of 1.00 mL in an acryl-cuvette (Sarstedt No. 67.740). Assay temperature was 27.5°C.

The assay was tested for PK activity after it was developed (Figure 6). The specific enzyme activity was measured each time an increased volume of cell-free extract from a mutant strain was added to the assay. Activity increased with increasing volumes of cell-free extract up to 0.075 mL suggesting a validated assay.

2.3. General Methods

2.3.1. Seed and Fermentation Medium

The strains of *Tolypocladium inflatum* were grown and maintained on surface culture on plates and slants composed of solid phase medium (XYZ) supplemented with agar. Agar was purchased commercially from Difco Laboratories.

Seed medium for submerged culture contained undefined organic carbon and nitrogen sources and was named Medium S.

Fermentation or production medium for submerged culture was named Medium F.

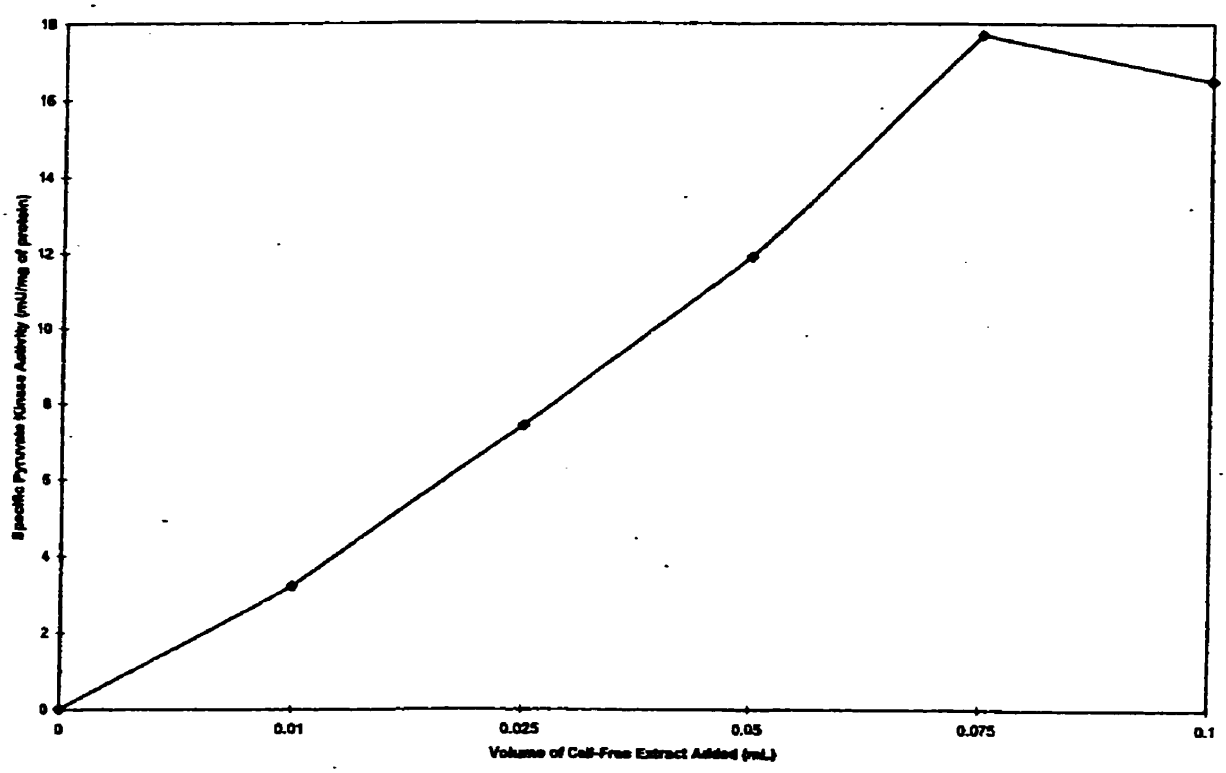
Due to their proprietary nature, detailed compositions of media XYZ, S and F are not listed.

2.3.2. One-Step Fermentation for Primary Screening

Aseptically, half-colonies of selected isolated mutants grown on the 9-location XYZ template are directly inoculated by crushing into a volume of

Figure 6

Validation of the pyruvate kinase assay by increasing the volume of cell-free extract added to the system



Pyruvate kinase assay was validated by measuring the specific activity of the enzyme when adding increasing volumes of cell-free extract prepared from a mutant strain to an acryl-cuvette containing 0.10 mL each of 0.1 mM $MgCl_2$, 0.5 mM KCl, 0.2 mM phospho(enol)pyruvate, 0.25 mM β-NADH, 0.001 mM ADP and 8.8 units L-lactic dehydrogenase in 100 mM Tris buffer, pH 7.5 to a final volume of 1.00 mL, 27.5°C at 339 nm.

fermentation medium F contained in a sterile centrifuge tube for the one-step fermentation. The culture was incubated with agitation. The best producers are picked off the template with the remaining half-colony and streaked completely over the surface of a XYZ slant and incubated.

2.3.3. Two-Step Fermentation for Secondary Screening and Experimentation

Conidia were aseptically harvested from XYZ slants. Conidia concentration in the inoculum was not adjusted for the secondary screen or retest. For other experimentation, the conidia concentration was determined by counting on a Double Neubauer Counting Chamber (VWR Scientific) and a consistent concentration of conidia was used to inoculate Medium S.

Submerged seed culture was grown in seed medium S in Erlenmeyer flasks for a period of time with controlled temperature and agitation then transferred to fermentation medium. The production shake flask fermentations were incubated for the indicated days with controlled temperature and agitation. Flask agitation was carried out on a Model G-53 orbital shaker with 2 inch throw (New Brunswick Scientific). All procedures are performed aseptically except the counting of conidia.

2.3.4. Mutagenesis and Post-mutagenesis Treatment

Conidia were harvested from a slant. The conidial suspension was filtered to remove mycelia and the conidia were adjusted to a final concentration of 1 X 10^6 conidia/ml. An aliquot of 10 ml was aseptically transferred to a glass petri

dish and with the lid removed was exposed with stirring to a far UV germicidal lamp (General Electric Co.) with an ultraviolet light intensity of 18 $\mu W/cm^2$ for 240 seconds. The intensity of ultraviolet energy was measured on a J-225 Shortwave Metre (Blak-Ray Ultraviolet Intensity Metre). In the dark, the treated suspension was serially diluted 1:10 and XYZ agar plates were spread with aliquots from the diluted suspensions. Untreated conidial suspensions were handled similarly. After incubation in the dark, colony forming units were determined and the percent kill was calculated. The surviving isolated colonies were selected and plated onto 9-location XYZ templates and incubated.

2.3.5. Minimum Inhibitory Concentration (M.I.C.) of Metabolic Inhibitors

The minimum inhibitory concentration of metabolic inhibitors was determined from surface culture using the double-dilution technique (The American Phytopathological Society. Committee on the Standardization of Fungicidal Tests, 1947) modified for surface culture.

2.3.6. Random and Rational Screen

Aliquots from UV-treated conidial suspensions were spread on XYZ plates without antifungal agent and with AF1 at a M.I.C. of 2.5 μg/ml and AF2 at a M.I.C. of 1.56 μg/ml for random selection and rational selection, respectively. Surviving colonies were selected and plated onto 9-location XYZ templates. Due to their proprietary nature, the names of AF1 and AF2 are not listed.

2.3.7. Multi-Level Screen

Large numbers of mutants were analysed for high or increased cyclosporin productivity in the primary or low-resolution screen. Approximately 10% were selected for secondary screening. This was followed by the retest to confirm the most active producers from the secondary screen. The multi-level screen is as described by Calam (1964), Davies (1964), Calam (1970), Simpson and Caten (1979b), Rowlands and Normansell (1983) and Rowlands (1992).

2.3.8. Preparation of Cell-Free Extracts

The mycelia of *T. inflatum* were harvested at different ages by centrifugation at 3000 RPM for 10 minutes. The weight of wet biomass cake was standardized to 10 g. The cake was washed three times by resuspension in 15 ml 100 mM Tris buffer, pH 7.5 and centrifugation at 3000 RPM for 10 min. The washed cake was resuspended in 15 ml 100 mM Tris buffer, pH 7.5 and homogenized on ice for 30 seconds with a T25 Ultra-Turrax Homogenizer equipped with a SDT182EN Shaft (VWR Canlab) to facilitate the transfer of suspension into the french press. The fungal cells were disrupted by two passages through a French Press Cell Press (American Instruments Co. Inc.) at 16,000 psi and the cell-free extracts were obtained by centrifugation at 14,000 RPM for 30 min at 4°C. The cell-free extracts were stored at 4°C.

3.0. RESULTS

3.1. Mutation of *T. inflatum* Strain MS-3 and the Selection of Improved Mutants from a Multi-Level Screen

A conidial suspension of *T. inflatum* strain MS-3 was UV-irradiated with a wavelength of 254 nm at 18 $\mu W/cm^2$ for 240 seconds. This irradiation resulted in 99.84% kill (Table 3).

The M.I.C. of antifungal agents AF1 and AF2 for the *T. inflatum* strain MS-3 was found to be 2.5 μg/ml and 1.56 μg/ml, respectively (Table 4).

Isolated mutant colonies that germinated and grew in the presence of antifungal agent at its M.I.C. were selected as rationally screened mutants. Isolated mutant colonies that germinated and grew on XYZ in the absence of the antagonist were selected as randomly-selected mutants. A total of 570 colonies were screened in the primary screen. High versus low production of cyclosporin A within the population was the selection criterion. The total number of strains selected for the secondary screen was 48 (Table 5). Specifically, 15 randomly-selected mutants, 15 AF2-resistant mutants and 18 AF1-resistant mutants were selected.

After secondary screening, 12 strains were selected based on high cyclosporin A productivity for retest. The retest represents the highest resolution using an assay with 10 replicates per strain and fermentation conditions similar to the secondary screen for confirmation of the yields of cyclosporin A. In the secondary screen (Table 6), all 12 mutants had higher titres then the parent

Table 3

Percent killing from ultraviolet light (254 nm) induced mutagenesis for *T. inflatum* strain MS-3

Pre-Mutagenesis Results	
Haemacytometer Count	Colony Count
1.325×10^6	4.4×10^5

Post-Mutagenesis Results				
UV_{254} Light Intensity ($\mu W/cm^2$)	Exposure Time (sec.)	Colony Count (CFU/ml)	% Survival	% Kill
18	240	700	0.159	99.84

Table 4

Minimum inhibitory concentration results for growth inhibitors of *T. inflatum* strain MS-3

Growth Inhibitor	Minimum Inhibitory Concentration (μg/mL)
AF1	2.5
AF2	1.56

Table 5

Ranges of relative volumetric cyclosporin A production from the primary screen and number of strains selected for secondary screening for mutants of *T. inflatum* strain MS-3

Mutants Randomly Selected		
Number of Mutants	Titre Range (Relative CsA Units)	Number of Strains
99	0.6-20.3	15

Selected Mutants Resistant to (μg/mL)					
AF1 (2.5)			AF2 (1.56)		
Number of Mutants	Titre Range (Relative CsA Units)	Number of Strains Selected	Number of Mutants	Titre Range (Relative CsA Units)	Number of Strains Selected
192	0.6-18.6	18	279	0-24.2	15

Table 6

Relative volumetric cyclosporin A production of *T. inflatum* strain MS-3 mutants and their screening conditions selected from the secondary screen for retest

Strain	Screening Condition	Relative Cyclosporin A Units	Change in Yield of Cyclosporin A From MS-3 (Control)
IS1	Random Selection	51.4	+5%
IS2	Random Selection	51.0	+4%
IS3	Random Selection	58.3	+9%
IS4	Random selection	52.1	+6%
IS5	Random Selection	51.5	+5%
IS6	AF1 (2.5 μg/mL)	51.0	+4%
IS7	AF1 (2.5 μg/mL)	51.4	+5%
IS8	AF1 (2.5 μg/mL)	53.4	+9%
IS9	AF2 (1.56 μg/mL)	62.5	+27%
IS10	AF2 (1.56 μg/mL)	59.0	+20%
IS11	AF2 (1.56 μg/mL)	60.1	+22%
IS12	AF1 (2.5 μg/mL)	54.4	+11%
MS-3	Control	49.1	

strain MS-3.

The retest confirmed that 9 of the 12 mutants had higher titres then the parent with titre increases of 15-80% (Table 7). Four of the improved strains were from random screening and five were from rational screening. The strains developed from AF2-resistant screening expressed the largest titre increment. Strains IS11 and IS12 have yields increased by 63% and 80%, respectively, relative to the parental control strain MS-3.

3.2. Comparison Between Strain MS-1 and all Mutants for Cyclosporin A Productivity, Growth and Dextrose Consumption

Figure 7 compares the cyclosporin A productivity for all of the improved strains from Figure 3 to strain MS-1 in a physiological profile. Harvesting of shake flasks was performed every second day for 10 days and culture was analysed for cyclosporin A productivity, growth and dextrose consumption. We can see all the mutants have a 27.9 (strain MS-2) to 55.4 times (strain IS10) increased productivity from strain MS-1. Simultaneously, there is a significant decrease in the rate of dextrose consumption (Figure 8) and biomass build-up (Figure 9) for the 14 mutants in comparison with strain MS-1. Strain MS-1 had the lowest titre and most rapid biomass build-up and dextrose consumption.

The difference in cyclosporin A productivity between all the mutants was small, approximately 2 times (27.9-55.4 relative cyclosporin A units). The comparison among mutants for growth and dextrose consumption showed no clear coordination with the differences in productivity.

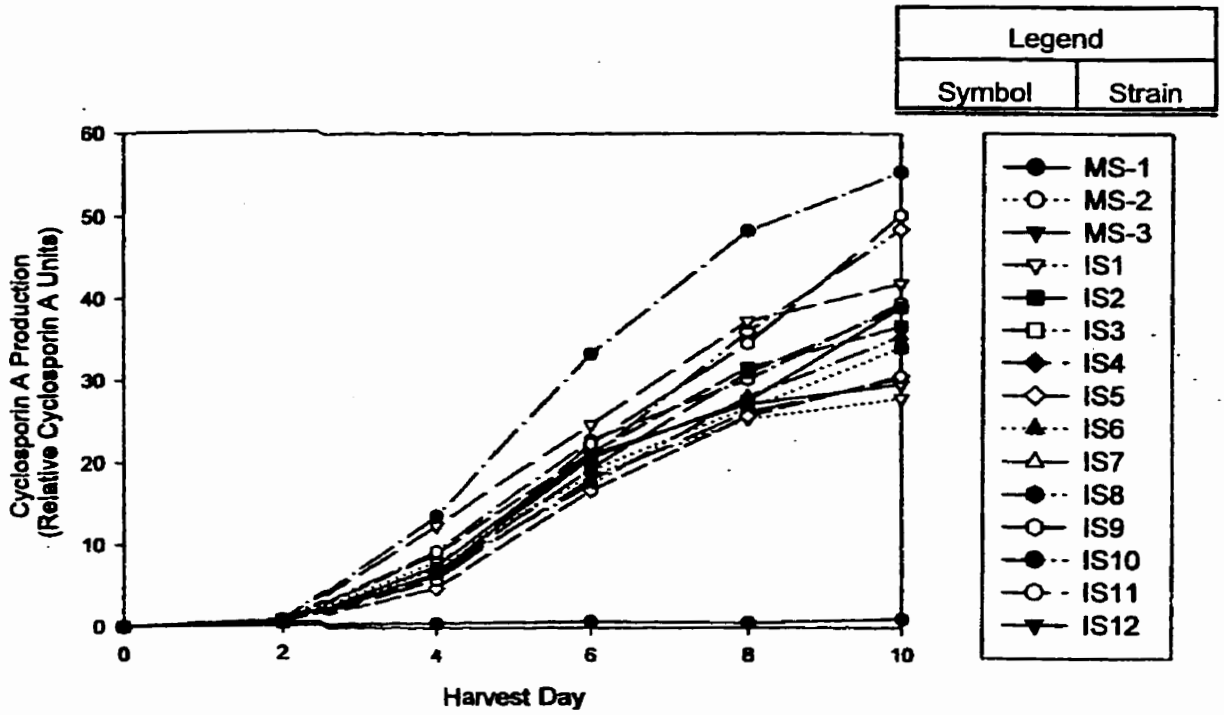
Table 7

Relative volumetric cyclosporin A production from the retest and screening conditions from mutants of *T. inflatum* strain MS-3

Strain	Screening Condition	Relative Cyclosporin A Units	Change in Yield of Cyclosporin A From MS-3 (Control)
IS1	Random Selection	42.6	+23%
IS2	Random Selection	40.8	+18%
IS3	Random Selection	54.7	+58%
IS4	Random selection	32.6	-6%
IS5	Random Selection	39.8	+15%
IS6	AF1 (2.5 μg/mL)	17.7	-49%
IS7	AF1 (2.5 μg/mL)	29.1	-16%
IS8	AF1 (2.5 μg/mL)	43.0	+24%
IS9	AF2 (1.56 μg/mL)	52.1	+51%
IS10	AF2 (1.56 μg/mL)	56.4	+63%
IS11	AF2 (1.56 μg/mL)	62.1	+80%
IS12	AF1 (2.5 μg/mL)	44.2	+28%
MS-3	Control	34.6	

Figure 7

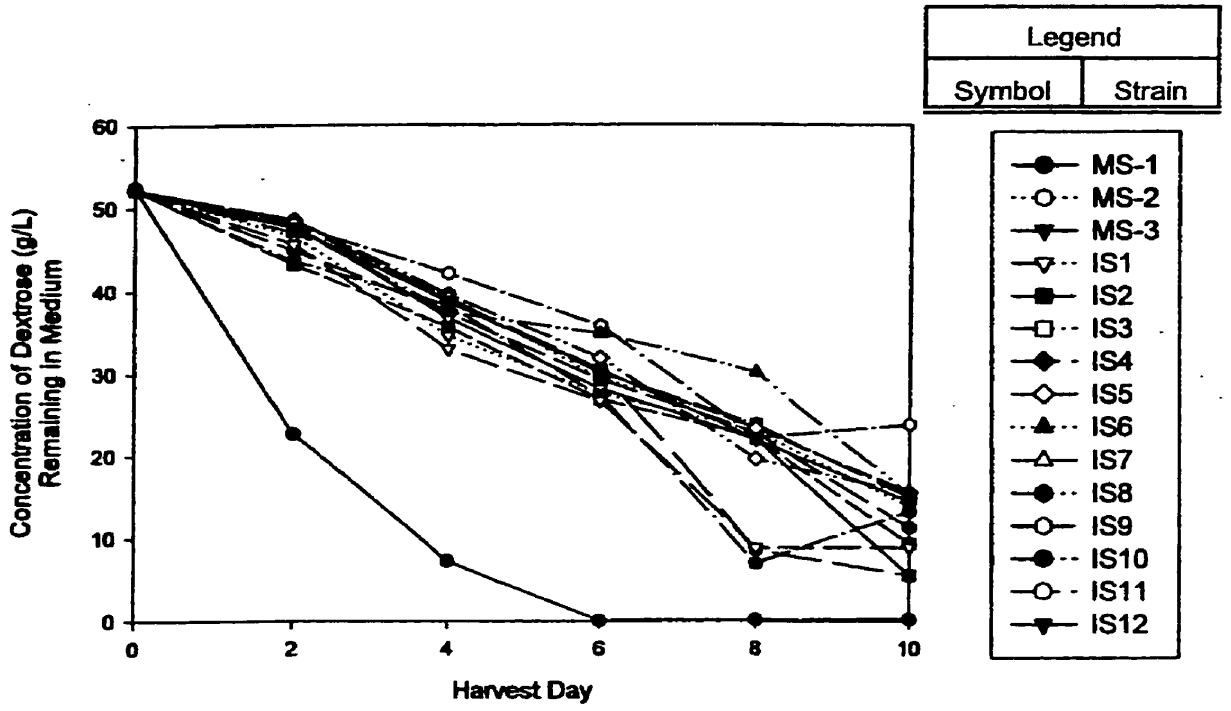
Physiological profile comparing the relative cyclosporin A production between MS-1 and mutant strains of *T. inflatum*



Triplicate shake flasks with strain MS-1 and 14 mutant strains grown in fermentation medium F were harvested every second day for 10 days. The broth from each shake flask had cyclosporin A extracted and the productivity was analysed by HPLC.

Figure 8

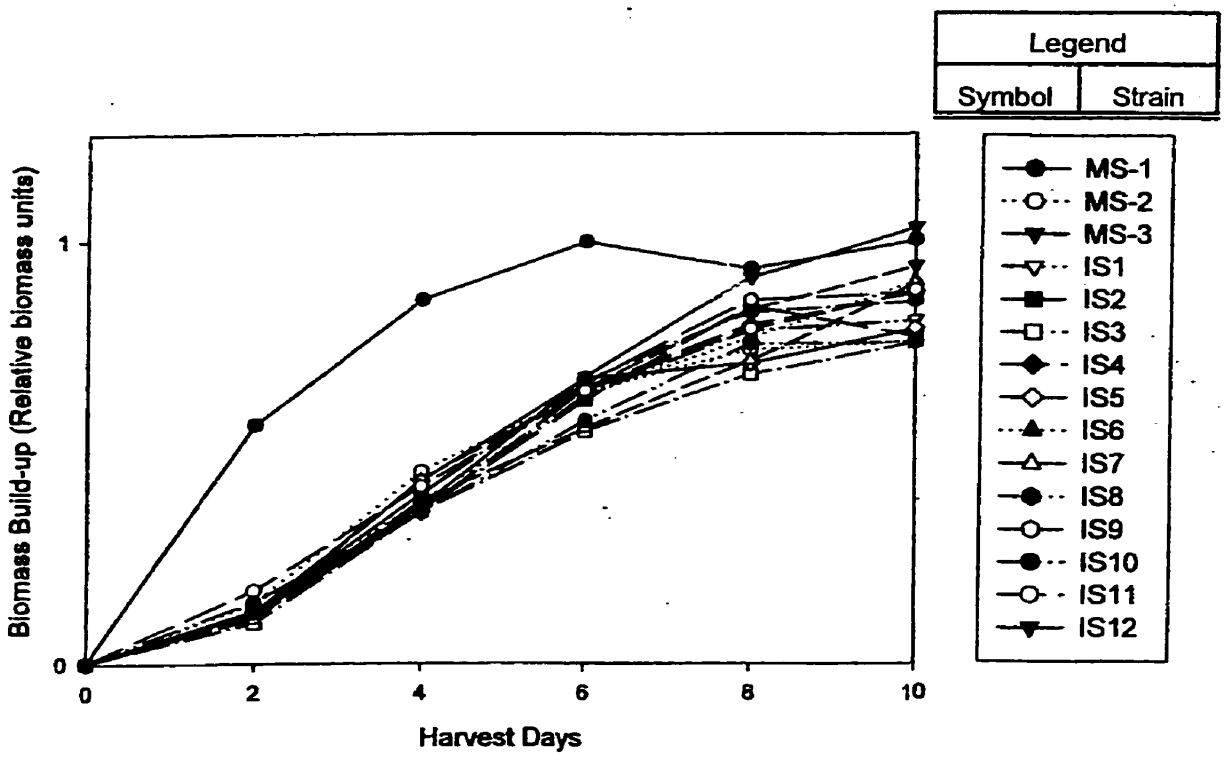
Physiological profile comparing the rate of dextrose consumption between MS-1 and mutant strains of *T. inflatum*



Triplicate shake flasks with strain MS-1and 14 mutant strains grown in fermentation medium F were harvested every second day for 10 days. The broth from each strain was pooled and a sample was analysed in triplicate for dextrose consumption.

Figure 9

Physiological profile comparing biomass build-up between MS-1 and mutant strains of *T. inflatum*



Triplicate shake flasks with strain MS-1and 14 mutant strains grown in fermentation medium F were harvested every second day for 10 days. The broth from each strain was pooled and the dry cell weight of duplicate samples was determined.

The statistical relationship of specific productivity and specific glucose consumption rate between strain MS-1 and 14 mutant strains was investigated. Statistical analysis was performed according to Zar (1984) and Khazanie (1986). Integrals were determined using SigmaPlot^{R} Scientific Graphing Software, Version 2.0 (Jandel Scientific Software) with user-defined modifications kindly donated by Dr. M. Butler.

The integral of biomass and time was determined from Figure 9 and plotted against cyclosporin A production. Specific productivity was calculated as the slope of the linear regression for each strain (Table 8). An example of the data and plot for MS-1 is given in Figure 25. The specific productivity for the high-producing mutant strains increased approximately 100-fold compared with the MS-1 strain (0.000125 ± 0.0000325 relative cyclosporin A units/relative units biomass-day). The specific productivity for strain MS-1 was statistically significant compared with the other strains and distinctly low. The strain with the highest relative volumetric productivity, IS10 with 55.4 relative cyclosporin A units, had the highest overall specific productivity (0.0171 ± 0.0031 relative cyclosporin A units/relative biomass units-day). The specific productivity of strain IS10 was statistically significant against strains MS-1, MS-2, MS-3, IS2, IS5, IS6, IS7, IS8 and IS12.

The integral of biomass and time from Figure 9 was also plotted against glucose consumption. Specific glucose consumption rate was calculated as the slope of the linear regression for each strain (Table 8). An example of the data and plot for strain MS-1 is given in Figure 25. For MS-1 only, the specific

Table 8

Specific cyclosporin A productivity and dextrose consumption rate for strain MS-1 and 14 mutant strains and the calculated P-value

Strain	Specific Cyclosporin A Productivity (Relative cyclosporin A units/Relative biomass units-day)	Specific Dextrose Consumption Rate (g/L dextrose/Relative biomass units-day)
MS-1	0.000125 ± 0.0000325	-0.0919
MS-2	0.009 ± 0.0016	-0.0097 ± 0.0016
MS-3	0.0111 ± 0.0019	-0.0097 ± 0.0012
IS1	0.016 ± 0.0012	-0.0109 ± 0.0016
IS2	0.010 ± 0.0019	-0.0100 ± 0.0012
IS3	0.014 ± 0.0019	-0.0096 ± 0.0023
IS4	0.013 ± 0.0019	-0.0103 ± 0.0012
IS5	0.0098 ± 0.0019	-0.0103 ± 0.0016
IS6	0.0109 ± 0.0016	-0.0101 ± 0.0016
IS7	0.0109 ± 0.0019	-0.0109 ± 0.002
IS8	0.0125 ± 0.0012	-0.0099 ± 0.0016
IS9	0.0156 ± 0.0012	-0.0139 ± 0.0023
IS10	0.0171 ± 0.0031	-0.0109 ± 0.0031
IS11	0.0129 ± 0.0023	-0.0118 ± 0.0023
IS12	0.0111 ± 0.0016	-0.0117 ± 0.0008
r value	0.782	
P-value	<0.001	
Degrees of Freedom	14	

dextrose consumption rate was calculated from 0 to 0.565 relative biomass units-day because at this stage of the fermentation the rate of consumption is most rapid. Between 0.565 and 3.850 relative biomass units-day the rate of glucose consumption is slowing down and for 3.850 to 7.713 relative biomass units-day no relationship exists. The specific glucose consumption rate for the high-producing mutant strains was statistically significant when compared with strain MS-1 (0.0919 g/L dextrose/relative biomass units-day). There was no correlation between specific dextrose consumption rate and volumetric cyclosporin A production for the 14 mutant strains although there does appear to be an inverse relationship between strain MS-1 and the 14 mutants. This is observed in Figure 10. The relationship between specific dextrose consumption rate and cyclosporin A productivity for strain MS-1 is distinct from the 14 mutant strains. There is a probability of a relationship of <0.001 (Table 8) for a decreased rate of specific dextrose consumption and increased specific cyclosporin A production as calculated in Figure 26.

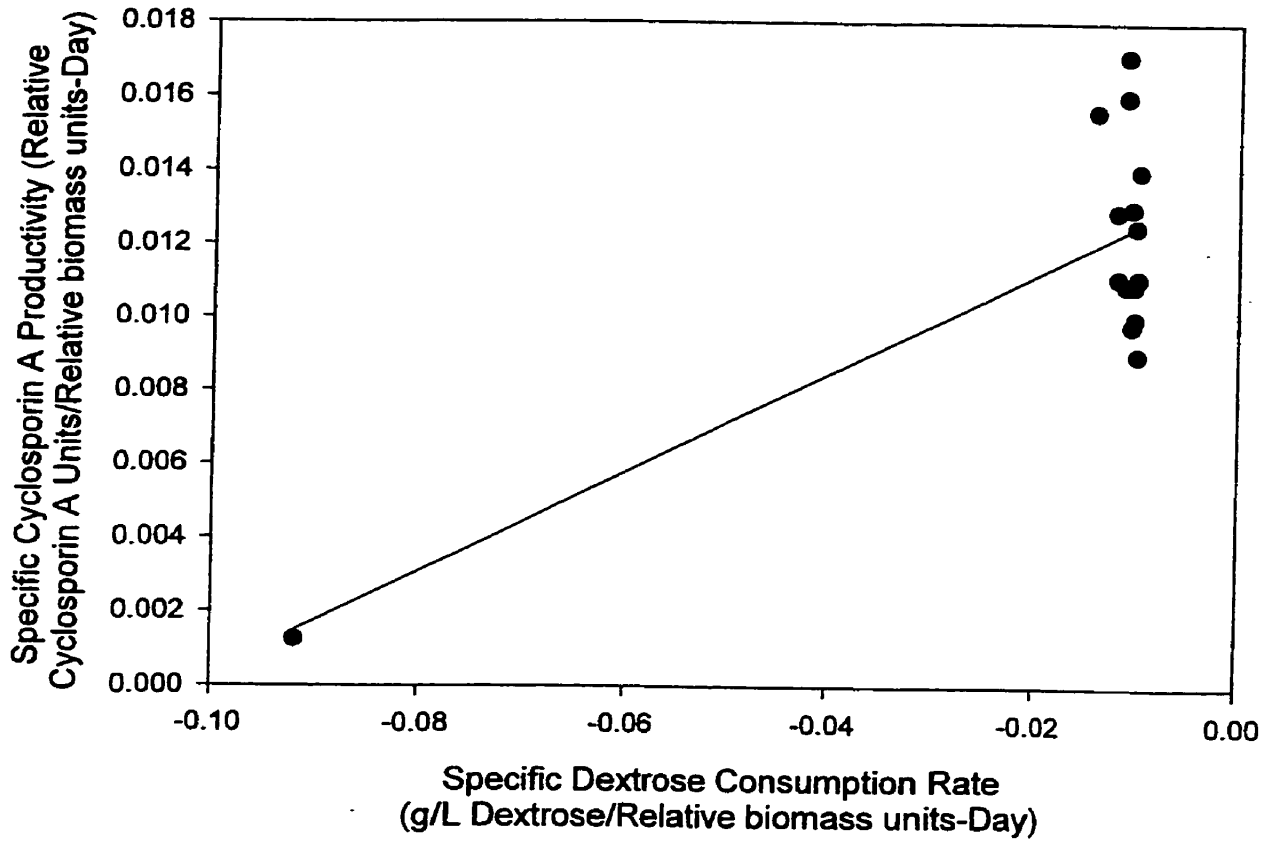
The enzymes involved in glucose catabolism were analysed in order to correlate their activity with the inverse relationship that was established.

3.3. Coarse Control of Glucose Catabolism in the Embden-Meyerhof Pathway by Activity Levels of Hexokinase, Phosphofructokinase and Pyruvate Kinase and Their Relationship with Increased Yields of Cyclosporin A

Figures 11, 12 and 13 feature the enzymatic activities of hexokinase, phosphofructokinase and pyruvate kinase, respectively, in a physiological growth

Figure 10

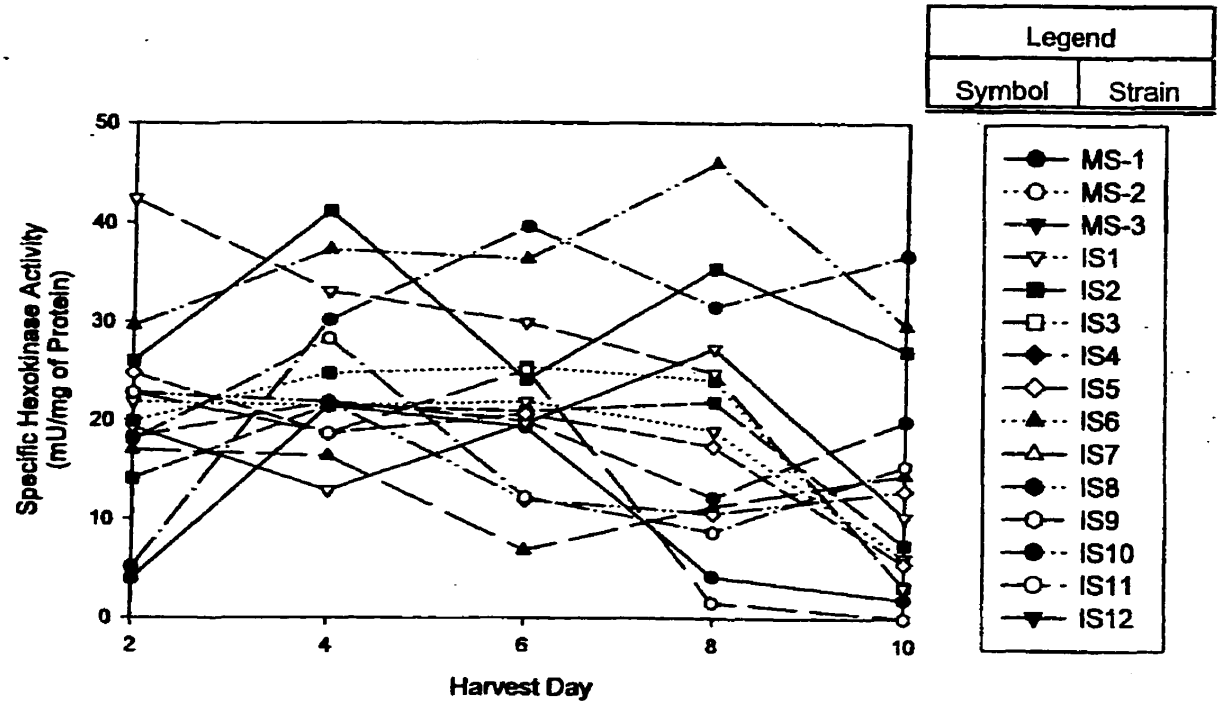
Statistical correlation between specific cyclosporin A productivity and specific dextrose consumption rate for strain MS-1 and 14 mutant strains



The statistical correlation was determined by plotting the specific cyclosporin A productivity against the specific dextrose consumption rate for each strain. Specific productivity was determined from a linear regression of cyclosporin A productivity and the integral of biomass and harvest days. Specific dextrose consumption rate was determined from a linear regression of the amount of dextrose consumed and the integral of biomass and harvest days.

Figure 11

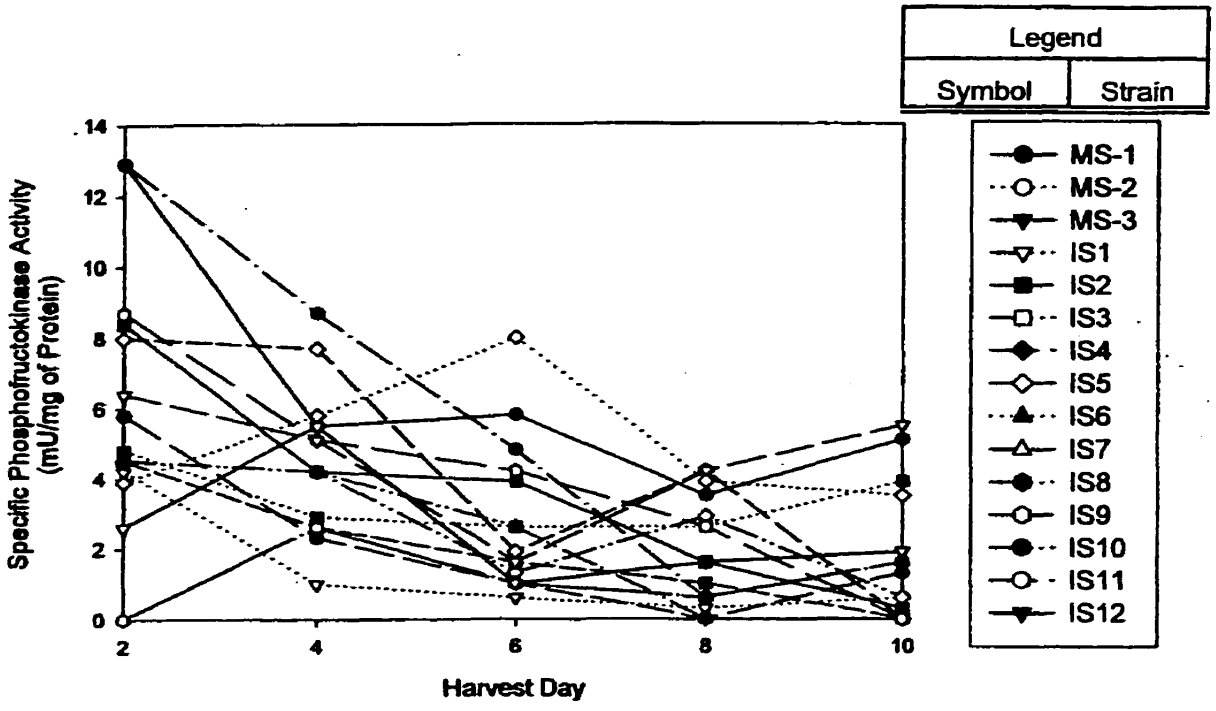
Physiological profile of strain MS-1 and
14 mutant strains and their specific hexokinase activity



Triplicate shake flasks with strain MS-1 and 14 mutant strains from fermentation medium F were harvested every second day for 10 days. The broth from each strain was pooled and cell-free extract was prepared. The activity of hexokinase was determined in 0.10 mL cell-free extract in an assay with 0.10 mL each of 5 mM dextrose, 250 mM ATP, 10 mM β-NADP, 500 mM $MgCl_2$, 4.6 units of glucose-6-phosphate dehydrogenase in buffer, pH 7.5 to a final volume of 1.00 mL, 27.5°C at 339 nm.

Figure 12

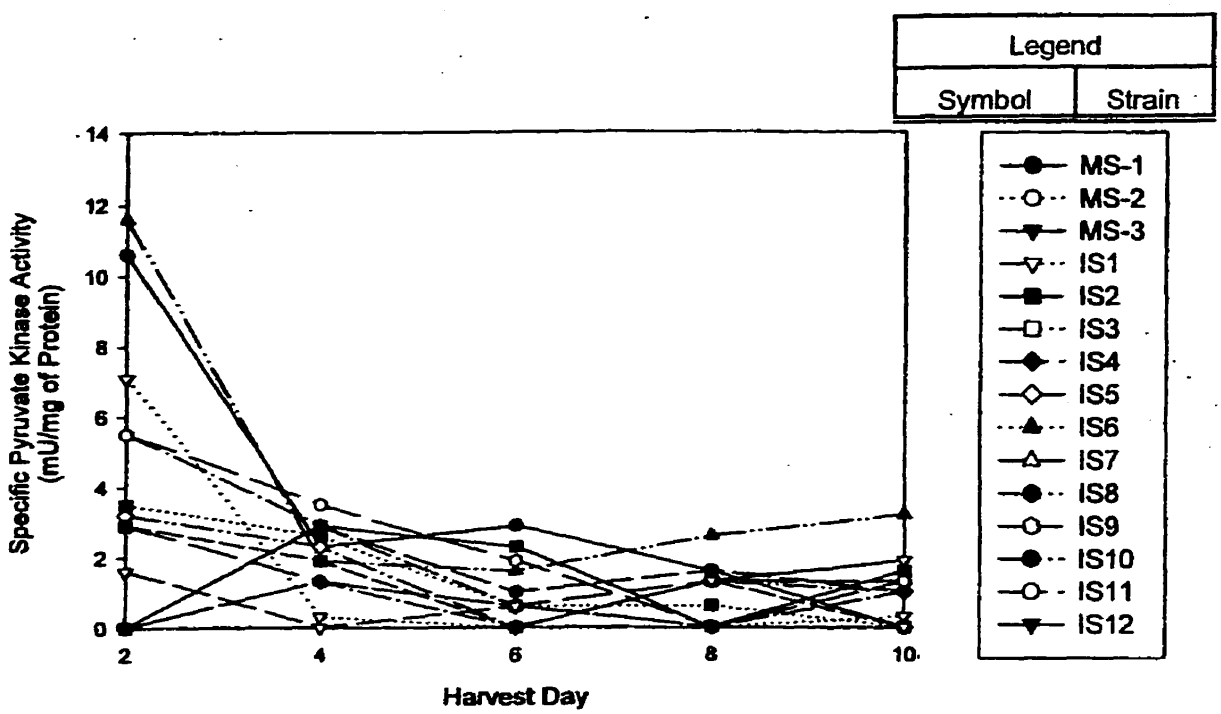
Physiological profile of strain MS-1 and
14 mutant strains and their specific phosphofructokinase activity



Triplicate shake flasks with strain MS-1 and 14 mutant strains from fermentation medium F were harvested every second day for 10 days. The broth from each strain was pooled and cell-free extract was prepared. The activity of phosphofructokinase was determined in 0.10 mL cell-free extract in an assay with 0.10 mL each of 70 mM $MgSO_4 \cdot 7H_2O$, 225 mM KCl, 35 mM phospho(enol)pyruvate, 90 mM fructose-6-phosphate, 2.2 mM ATP, 0.8 mM β-NADH, 19.68 units pyruvate kinase and 8.8 units L-lactic dehydrogenase in buffer, pH 7.5 to a final volume of 1.00 mL, 27.5°C at 339 nm.

Figure 13

Physiological profile of strain MS-1 and
14 mutant strains and their specific pyruvate kinase activity



Triplicate shake flasks with strain MS-1 and 14 mutant strains from fermentation medium F were harvested every second day for 10 days. The broth from each strain was pooled and cell-free extract was prepared. The activity of pyruvate kinase was determined in 0.10 mL cell-free extract in an assay with 0.10 mL each of 0.01 mM $MgCl_2$, 0.05 mM KCl; 0.02 mM phospho(enol)pyruvate, 0.025 mM β-NADH, 0.001 mM ADP and 8.8 units of L-lactic dehydrogenase in buffer, pH 7.5 to a final volume of 1.00 mL, 27.5°C at 339 nm.

profile. Harvests of shake flasks were performed as in Section 4.2 and cell-free extracts were prepared for strain MS-1 and 14 mutants. The extracts were tested for enzyme activity.

The specific hexokinase activity for all 14 mutant strains (Figure 11) demonstrates activity higher than strain MS-1 (3.9 mU/mg) early in the fermentation period suggesting that hexokinase is not the enzyme limiting the consumption of dextrose. The data from Figure 11 from the harvest days and strains assayed were analysed in an analysis of variance to determine the significance of differences in activity (Table 9). The difference in activities were significant for each with a P-value of <0.01 at 99% confidence levels. The means and standard error of the means for each harvest day (Figure 14) and strain (Figure 15) were plotted. The specific hexokinase activity is statistically significant on days 2 and 4 against day 10 and strain MS-1 has activity that is not statistically significant only with strains IS2 and IS9.

The tendency of the specific activities for phosphofructokinase (Figure 12) and pyruvate kinase (Figure 13) is to peak early (day 2) in the fermentation and a general trend of these enzymes is for less activity from the high cyclosporin A-producing strains than strain MS-1.

The data from Figure 12 from the harvest days and strains assayed were analysed in an analysis of variance to determine the significance of differences in specific phosphofructokinase activity (Table 10). The difference in activities were significant for each with a P-value of <0.01 at 99% confidence levels. The means and standard error of the means for each harvest day (Figure 16) and strain

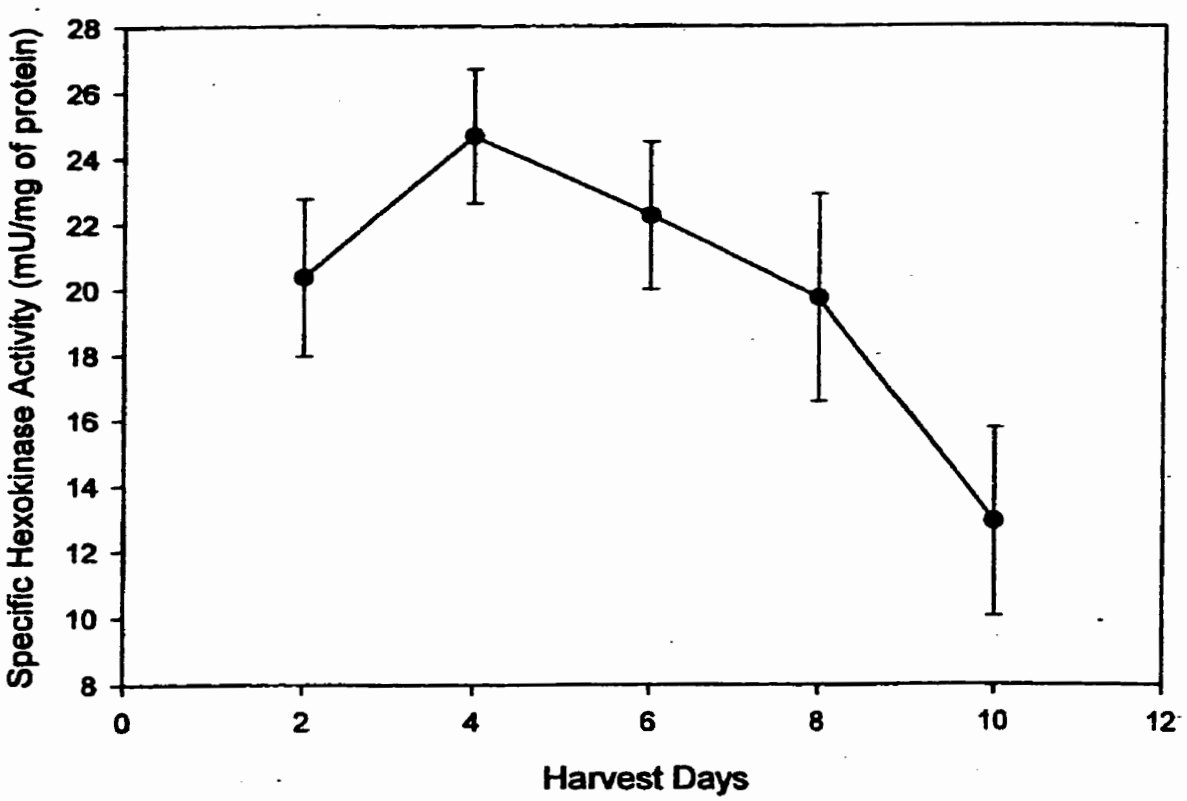
Table 9

Harvest day and strain analysis of variance of the specific activity from hexokinase for MS-1 and 14 mutant strains

Hexokinase					
Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F Value	Prob
Total	74	8086			
Harvest Day	4	1159	289.8	4.9	<0.01
Strain	14	3602	257.3	4.3	<0.01
Residual	56	3325	59.38		

Figure 14

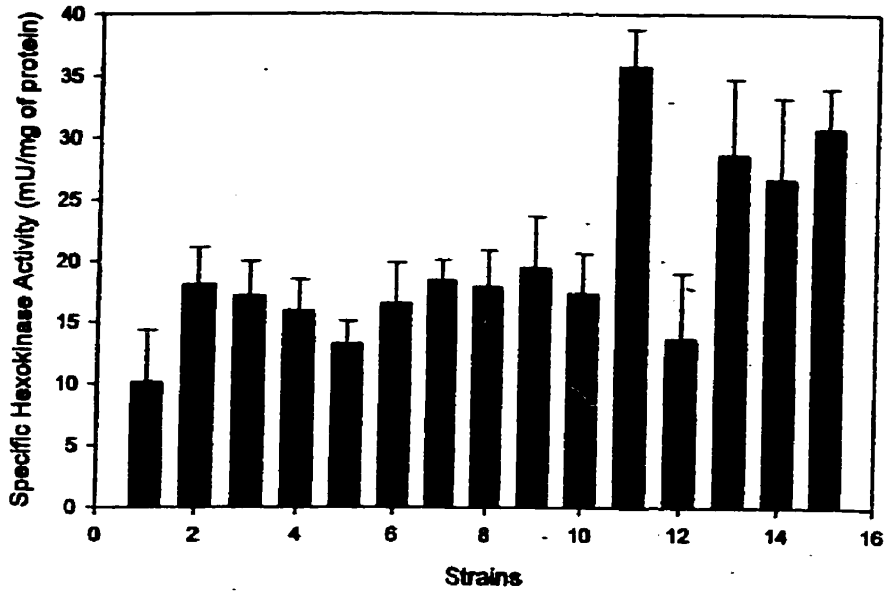
Specific hexokinase activity means and standard error
of the means from MS-1 and 14 mutant strains for each harvest day



Values are means (+SEM) of activities for 15 strains.

Figure 15

Specific hexokinase activity means and standard error of the means from MS-1 and 14 mutant strains for each strain



Legend	
Symbol	Strain
1	MS-1
2	MS-2
3	MS-3
4	IS1
5	IS2
6	IS3
7	IS4
8	IS5
9	IS6
10	IS7
11	IS8
12	IS9
13	IS10
14	IS11
15	IS12

Values are means (+SEM) over 10 days of growth (n=5).

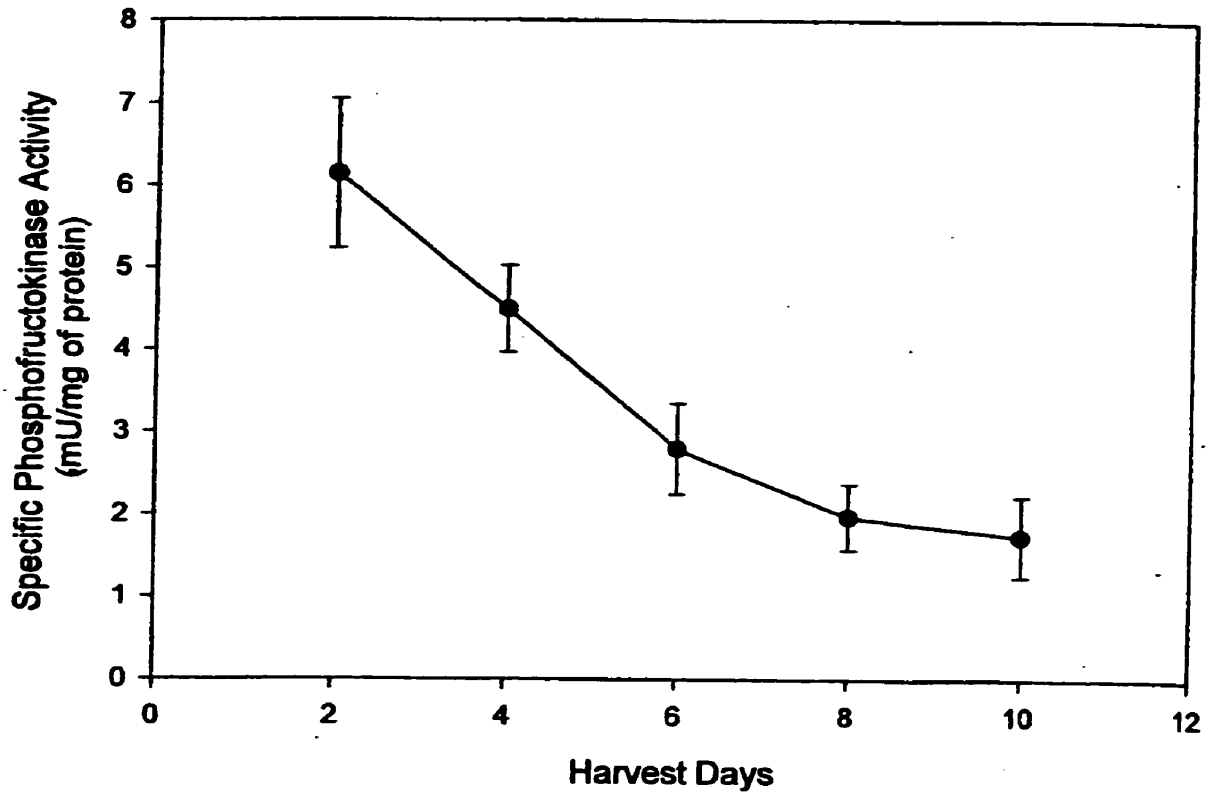
Table 10

Harvest day and strain analysis of variance of the specific activity from phosphofructokinase for MS-1 and 14 mutant strains

Phosphofructokinase					
Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F Value	Prob
Total	74	590.76			
Harvest Day	4	208.56	52.14	14.6	<0.01
Strain	14	182.74	13.05	3.67	<0.01
Residual	56	199.46	3.56		

Figure 16

Specific phosphofructokinase activity means and standard error of the means from MS-1 and 14 mutant strains for each harvest day



Values are means (+SEM) of activities for 15 strains.

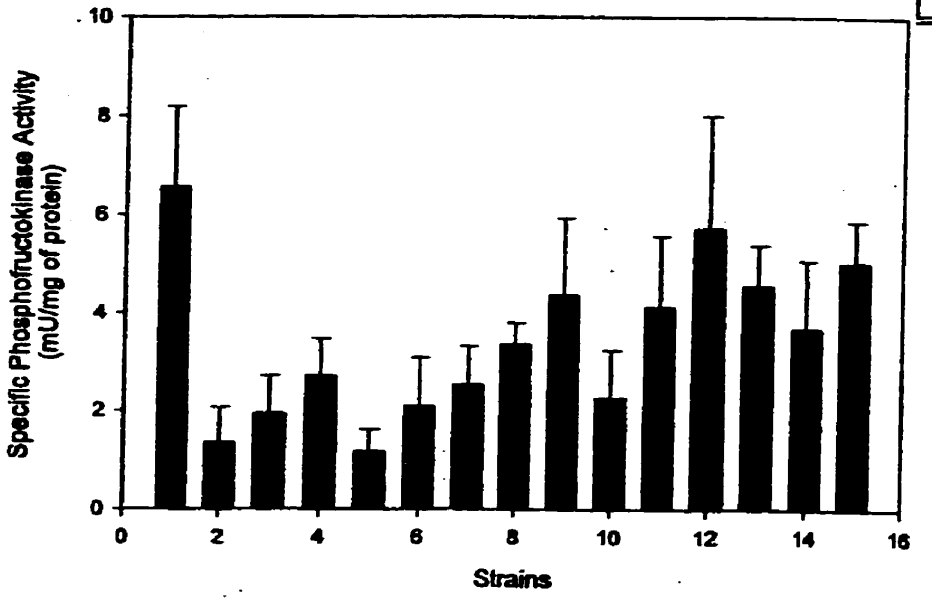
(Figure 17) were plotted. The specific phosphofructokinase activity is statistically significant on day 2 against days 4, 6, 8 and 10 with a highly active enzyme early in the fermentation and strain MS-1 has activity that is statistically significant with all mutant strains with the exception of strain IS9.

The data from Figure 13 from the harvest days and strains assayed were analysed in an analysis of variance to determine the significance of differences in specific pyruvate kinase activity (Table 11). The difference in activities were significant for the harvest days with a P-value of <0.01 at 99% confidence level. The difference in activities were significant for the strains with a P-value of <0.05 at a 95% confidence level. The means and standard error of the means for each harvest day (Figure 18) and strain (Figure 19) were plotted. The specific pyruvate kinase activity is statistically significant on day 2 against days 4, 6, 8 and 10 with a highly active enzyme early in the fermentation and strain MS-1 has activity that is statistically significant with all mutant strains with the exception of strains MS-1 and IS8.

In general, PFK and PK play important roles in controlling dextrose consumption and appear to be the rate-limiting enzymes.

Figure 17

Specific phosphofructokinase activity means and standard error of the means from MS-1 and 14 mutant strains for each strain



Legend	
Symbol	Strain
1	MS-1
2	MS-2
3	MS-3
4	IS1
5	IS2
6	IS3
7	IS4
8	IS5
9	IS6
10	IS7
11	IS8
12	IS9
13	IS10
14	IS11
15	IS12

Values are means (+SEM) over 10 days of culture (n=5).

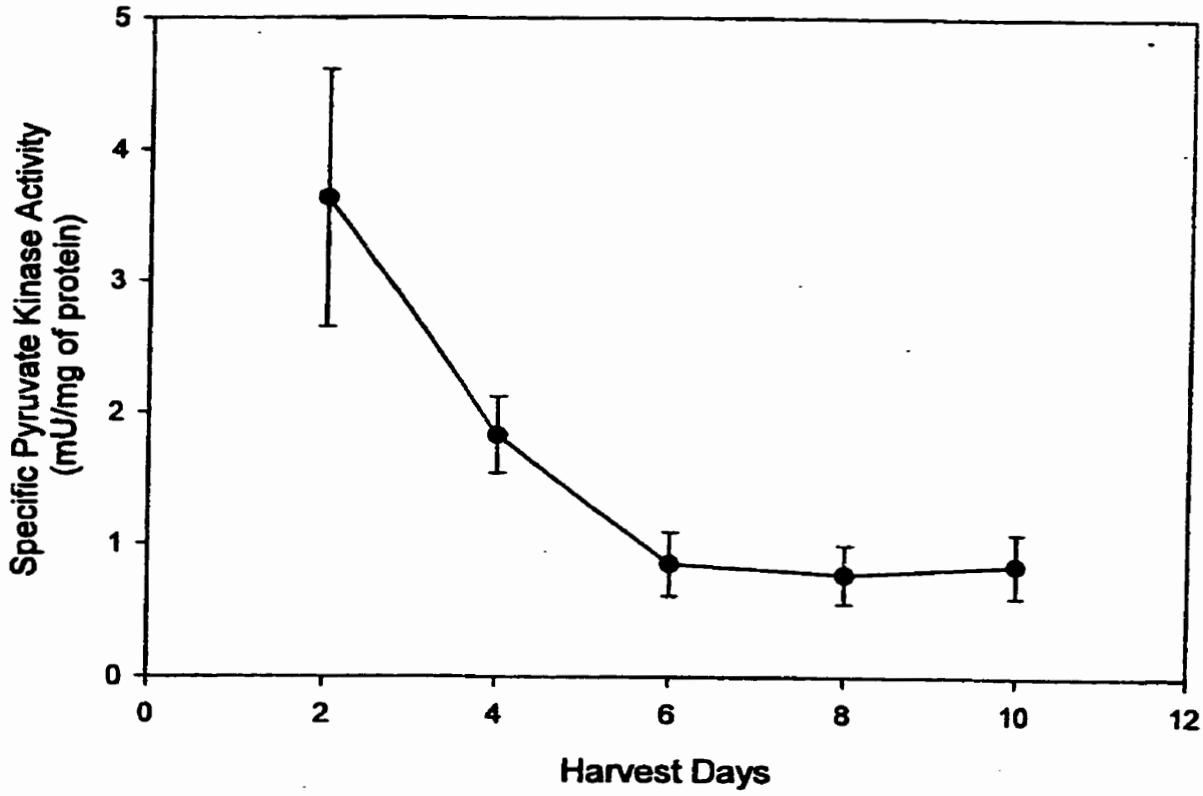
Table 11

Harvest day and strain analysis of variance of the specific activity from pyruvate kinase for MS-1 and 14 mutant strains

Pyruvate Kinase					
Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F Value	Prob
Total	74	34.37			
Harvest Day	4	89.83	22.46	7.44	<0.01
Strain	14	84.71	6.05	2.0	<0.05
Residual	56	169.16	3.02		

Figure 18

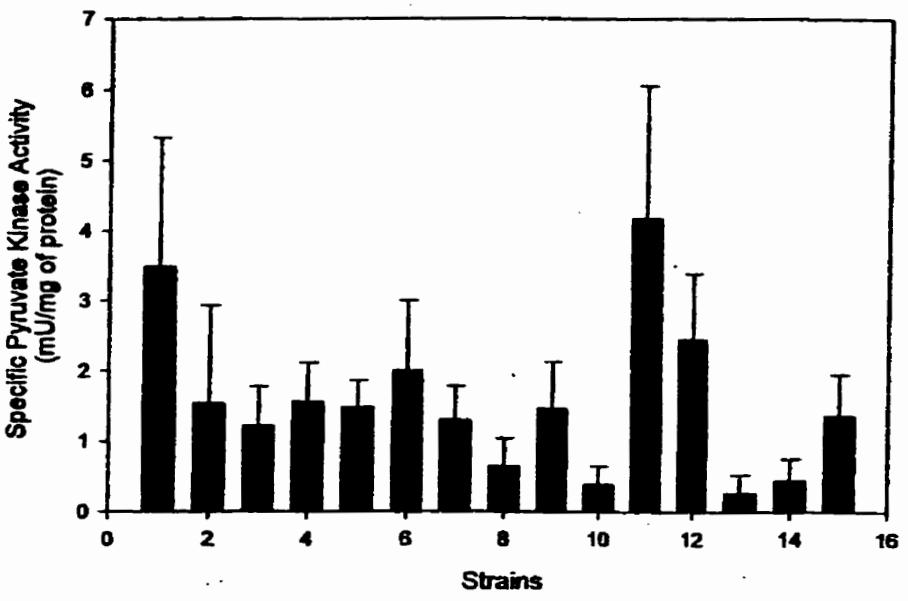
Specific pyruvate kinase activity means and standard error of the means from MS-1 and 14 mutant strains for each harvest day



Values are means (+SEM) of activities for 15 strains.

Figure 19

Specific pyruvate kinase activity means and standard error of the means from MS-1 and 14 mutant strains for each strain



Legend	
Symbol	Strain
1	MS-1
2	MS-2
3	MS-3
4	IS1
5	IS2
6	IS3
7	IS4
8	IS5
9	IS6
10	IS7
11	IS8
12	IS9
13	IS10
14	IS11
15	IS12

Values are means (+SEM) over 10 days of culture (n=5).

4.0. DISCUSSION

4.1. The Mutation and Selection of *T. Inflatum* Strain MS-3

Ultraviolet light (254 nm) is a convenient mutagen (Hopwood, 1970). With an ultraviolet light intensity of 18 $\mu W/cm^2$ and exposure time of 240 seconds, 99.84% kill is achieved (Table 3). The suspension is stirred during exposure to ensure all conidia are exposed to the action of the rays since they only penetrate a short distance into the suspension (Calam, 1970). A conidial suspension of 1.325×10^6 conidia/mL with 4.4×10^5 viable conidia/mL was less then the 10^7 conidial concentration recommended by Calam (1970) but the purpose was similar - facilitate plating of treated suspension on to culture medium. A conidial suspension containing 10^6 conidia/mL and 8-methoxypsoralen was irradiated with far UV light (UV_{365}) and stirring by Simpson and Caten (1979a).

I have demonstrated that better-yielding mutants can be obtained by treating a suspension of conidia with a high dosage of near UV radiation. The common industrial practice is to select increased production strains from a sample subjected to the action of a mutagen resulting in a high degree of kill (often >>99%). In general, the yield of mutants per surviving cells increases with increasing dose of mutagen (Bos and Stadler, 1996). The proper amount is still subject to debate. According to Calam (1970) there are differences in opinion as to the amount of kill. A greater number of mutants is obtained with a relatively mild dosage with a kill range of 30-70%, although by the experience of strain improvement workers a greater kill in the order of 90-99% is suggested to obtain

cultures with a higher productivity. Rowlands (1983) suggested there was a general agreement that low doses are better then high in order to obtain an improvement in productivity. This is especially true for high-producing strains with uninucleate material that have a large number of mutations contributing to the increase in titre and any induced change to the current mutations will be deleterious.

Both random and rational selection methods following UV mutagenesis have obtained improved strains. Two hundred and seventy nine AF2-resistant mutants, 192 AF1-resistant mutants and 99 randomly-selected colonies were tested in the primary screen (Table 5) and the cyclosporin A range was 0.6-20.3 relative units, 0-24.2 relative units and 0.6-18.6 relative units, respectively. The largest range of relative titre from the primary screen was for AF2-resistant screening. I could not conclude that AF2-resistant screening created a larger titre range among the mutants because of a larger population of AF2-resistant mutants screened in the primary screen than for the others. But according to the retest data (Table 7) the superior most-improved mutants were derived from AF2 screening. This suggests the screening method is highly efficient. In general, rational screening of mutants for resistance to antifungal inhibitors results in higher yielding strains. The evidence surrounding this claim remains circumstantial. A study as to why certain screens work and others don't would be useful.

Other important applications of rational screening techniques for industrial purposes would be to locate *T. inflatum* mutants that are derepressed for

cyclosporin production in the presence of high concentrations of glucose. A derepressed mutant would be useful at the production scale employing a process of glucose feeding.

Among the total of 12 selected mutants from secondary screening, 9 were confirmed in the retest and the 6 most active-producing mutants were the same from both secondary screening and retest. This result indicates the multi-level screening strategy can be successfully applied for strain improvement (Calam, 1964, Davies, 1964, Rowlands, 1992).

4.2. Aspects of Regulation on Cyclosporin A Production by *T. inflatum*

4.2.1. The Inverse Relationship of Growth and Glucose Consumption With Cyclosporin A Titre

Demain (1996) defines a secondary metabolite by suggesting a secondary metabolite is not 'secondary' because it is produced after growth, but because it is not involved in the growth of the producing culture. Thus, elimination of production of a secondary metabolite by mutation will not stop or slow down growth; indeed, it may increase the growth rate or as he more succinctly states (1991), a secondary metabolite is secondary only because it is not essential for vegetative growth of the producing culture. This research found that a significant cyclosporin A productivity increase for 14 mutants (Figure 7) is associated with reduced glucose consumption (Figure 8) and amount of growth (Figure 9) in comparison with strain MS-1 and the relationship is a statistically

significant with a P-value of <0.001 (Table 8). The coordination of a physiological state with increased secondary metabolism agrees with the statement of Martin and Demain (1980) that an important characteristic of secondary metabolism is that these metabolites are produced at low specific growth rates suggesting that antibiotic synthetases are not formed or repressed or, if formed, the activity of the synthetase is inhibited when the growth rate is high. Slow feeding of glucose to lower the growth rate can increase the biosynthesis of penicillin (Soltero and Johnson, 1954) and the polyene antifungals, candidin and candihexin (Martin and McDaniel, 1974). This discovery suggests in order to achieve higher cyclosporin A production, the growth rate should be continually lowered either through continued strain improvement or from fermentation process control and is reflected in slower dextrose consumption.

The determination of the specific productivity per strain for 14 high-producing mutant strains compared with strain MS-1 revealed a difference in the production efficiency. The specific productivity for strain MS-1 was considerably lower (0.000125 ± 0.0000325 relative cyclosporin A units/relative biomass units-day) than that for the other strains suggesting productivity that was less efficient (Table 8).

The determination of the specific glucose consumption rate per strain for the same strains revealed an increase in the rate of glucose consumption in strain MS-1 of approximately 10 times (Table 8).

The P-value (P<0.001) from Table 8 suggested with a high probability an inverse relationship existed for specific cyclosporin A production and dextrose

consumption rate for the wild-type strain in comparison to the 14 mutants (Figure 10). The relationship does not appear to exist among the population of 14 mutant strains.

The establishment of this inverse relationship validates Demain's definition of a secondary metabolite.

4.2.2. Levels of Activity of Catabolic Enzymes in the Embden-Meyerhof Pathway and Their Association With Levels of Relative Volumetric Cyclosporin A Production

The specific activity levels of phosphofructokinase were analysed from strain MS-1 and 14 mutants (Figure 12). The specific PFK activity of 12 strains, including strain MS-1, was maximum on day 2. The statistical analysis of phosphofructokinase revealed the overall trend of a peak of activity early in the incubation for all the strains (Figure 16) and generally lower activity levels for the 14 mutants compared with strain MS-1 (Figure 17). This suggests when cyclosporin A is being actively produced, PFK activity is decreased. Also, PFK activity is low for high-producing mutant strains of *T. inflatum*.

It has been shown that PFK plays a key regulatory role in fungi in general, and in *Neurospora crassa* in particular (Tsao and Madley, 1972). Changes in PFK activity are observed with changes in levels of substrate. The enzyme is activated when concentrations of substrate are high and allows the conversion of fructose 6-phosphate to fructose 1,6-diphosphate. Also, continued development of mycelium was favoured by low phosphofructokinase activity (Chattaway et al,

1973). This suggests that low PFK activity (Figure 12) is due to decreased substrate levels since metabolites are being rerouted.

The specific activity levels of pyruvate kinase were also analysed from strain MS-1 and 14 mutants (Figure 13). The specific PK activity of 10 strains, including MS-1, was maximum on day 2. Five strains had maximum PK activity on day 4. The statistical analysis of pyruvate kinase revealed the overall trend of a peak of activity early in the incubation for all the strains (Figure 18) and generally lower activity levels for the 14 mutants compared with strain MS-1 (Figure 19). This suggests when cyclosporin A is being actively produced, PK activity is also decreased and PK activity is low for high-producing mutant strains of *T. inflatum*.

Overall, the trend for PK activity is similar to that observed for PFK. Peak PK activity is reached early in the fermentation suggesting that when cyclosporin A is being actively produced PK levels are low and that PK activity is generally lower in the higher producing mutant strains than the low producing strain, MS-1.

Although regulatory patterns for PK differ in various organisms it is acknowledged that PK is a key regulatory enzyme (Kapoor and Tronsgaard, 1972) responsible for balancing glycolysis and gluconeogenesis in yeast.

The analysis of variance performed for each enzyme's specific activity (Table 9, 10 and 11) from each harvest day and on all 15 strains of *T. inflatum* suggests that the data from Figures 10, 11 and 12 have statistically significant differences for time and strain.

Maximum PFK and PK activity at approximately day 2 of the fermentation

would suggest the metabolic route for glucose catabolism is determined early. This agrees with Smith et al (1971) who found enzymic differences mainly limited to young mycelium.

In strain MS-1, high PFK and PK activities are required to convert all phosphorylated dextrose to pyruvate. In the high cyclosporin A-producing mutants, PFK or PK activity is not as high as HK activity suggesting that phosphorylated intermediates of the EM pathway are limiting the uptake of dextrose even though the phosphorylating activity of HK is high. Another possibility is dextrose catabolites are being rerouted to a pathway other then the EM pathway. In either case, reduced pyruvate formation could result in less TCA activity and decreased formation of ATP. Decreased production of intracellular ATP is acknowledged an important effector of secondary metabolism (Butler et al, 1997, Liras et al 1977) and that ATP concentrations are lower in improved-producing strains (Saliva et al, 1965, Janglova et al, 1969).

Coordination of activity with enzymatic activity from other pathways, for example, the pentose phosphate pathway could provide insight into the fate of the small molecule intermediates of glycolysis in an actively differentiating and producing strain of *T. inflatum*. An inverse relationship between enzymes of the EM and PP pathways in sporulating and non-sporulating mycelium of *Aspergillus niger* has been observed at 24 hours. At later stages of growth, 48-96 hours, enzyme levels are similar suggesting a balance between the pathways (Smith et al, 1971). Studies on glucose catabolism in *Aspergillus nidulans* reveal increased PP activity during exponential growth of batch cultures (Carter and Bull, 1969).

Low specific phosphofructokinase and pyruvate kinase activity to high cyclosporin A production suggests:

1. Less glucose is being catabolized by the EM pathway.
2. Less activity by the EM pathway suggests a reduced source of ATP and NADH, which is metabolically oxidized to NAD^+ in the formation of ATP (Voet and Voet, 1995).
3. The PP pathway, as an alternative route for glycolysis, is assuming a larger role in the biosynthesis of cyclosporin A.
4. Increased activity of the PP pathway suggests a greater generation of NADPH necessary for cyclosporin A production.

Measurement of the activities of the enzymes of the pentose phosphate pathway, specifically, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, would be required and a correlation of their activity with enzymes of the EM pathway determined.

Since it is acknowledged that the PP pathway is an alternate route for glycolysis the evidence for its role within this thesis in the catabolism of glucose by strains of *T. inflatum* is circumstantial. When performing measurements on the relative flux of radioactively-labelled glucose through glycolysis, Senn et al (1991) calculate that 40% of all glucose molecules are cycled through the pentose phosphate pathway by a high producing mutant of *T. inflatum* NRRL 8044. Increased PP pathway activity would yield increased concentrations of NADPH for cellular biosynthesis (Cochrane, 1976, Miernyk, 1990, Turner and Turner, 1980), the formation of amino acids (Smith et al, 1971) which could be

used in the synthesis of cyclosporin A, a cyclic undecapeptide, and nucleic acid synthesis (Turner and Turner, 1980, Wood, 1986). Rationally screening with inhibitors of the Pentose Phosphate Pathway in order to find overproducing mutants of NADPH + H^+.

5.0. SUMMARY

Tolypocladium inflatum is a producer of the cyclic undecapeptide cyclosporin A, a powerful immunosuppressive with antifungal properties. With the interest in cyclosporin A as a therapeutic agent and its commercial production comes the demands for increased productivity from the producing organism. Traditional methods of mutation and selection are useful elements in the field of strain improvement and was applied to strains of *T. inflatum* in order to increase the titres of cyclosporin A. Ultraviolet light mutagenesis with random and rational selection developed cyclosporin A titre-increased mutants. Screening with the antifungal agent, AF2 resulted in mutants with the most improved yields. This indicates the potential of using rational screening in cyclosporin A strain improvement.

The regulatory aspects of cyclosporin A production indicate with increased yields of cyclosporin A from mutant strains of *T. inflatum* there is a reduction in the amount of cellular growth and this is reflected in slowed biomass build-up and decreased glucose consumption ($P<0.001$).

The lower activity from the Embden-Meyerhof pathway enzymes hexokinase ($P<0.10$), phosphofructokinase ($P<0.01$) and pyruvate kinase ($P<0.05$) in mutants with high specific cyclosporin A productivity compared with strain MS-1 suggests that they, specifically PFK, are involved in the regulating dextrose catabolism.

Also, the trends of maximum activity of hexokinase ($P<0.01$),

phosphofructokinase ($P<0.01$) and pyruvate kinase ($P<0.01$) is achieved during the early stages of fermentation suggesting that the biochemical routes of dextrose catabolism are decided early.

The evidence for a larger role for the Pentose Phosphate pathway is suggestive. A reduction in the activity of EM pathway enzymes often results in increased PP pathway activity. The higher NADPH production associated with increased PP pathway activity would be necessary to supply the reducing power for the many metabolic reactions, specifically the production of cyclosporin A, in an actively growing and differentiating cell.

6.0. REFERENCES

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APPENDIX

Table 12

Levels for factors in a 2^{6-1}, standard order fractional factorial design for phosphofructokinase

Factor	Initial Level of Factor in the Assay (mM)		
	-	Control	+
$MgSO_4 \cdot 7H_2O$	2.8	14	70
KCl	9	45	225
Phospho(enol)pyruvate	1.42	7.1	35.5
Fructose-6-phosphate	3.6	18	90
ATP	2.2	11	55
NADH	0.8	4	20

- = low factor level
+ = high factor level

Table 13

Analysis of variance of a 2^{6-1}, standard order fractional factorial design for phosphofructokinase

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	0.052065	2	0.0260325	28.66	<0.0001
Residual	0.026338	29	0.0009082		
Cor Total	0.078404	31			

Table 14

Levels for factors in a 2^{5-1}, standard order fractional factorial design for pyruvate kinase

Factor	Initial Level of Factor in the Assay (mM)		
	-	Control	+
A-$MgCl_2$	0.02	0.2	2
B-KCl	0.1	1	10
C-Phospho(enol)pyruvate	0.01	0.1	1
D-NADH	0.0005	0.005	0.05
E-ADP	0.005	0.05	0.5

- = low factor level
+ = high factor level

Table 15

Analysis of variance and statistical analysis of main effects and interactions from a 2^{5-1}, standard order fractional factorial design for pyruvate kinase

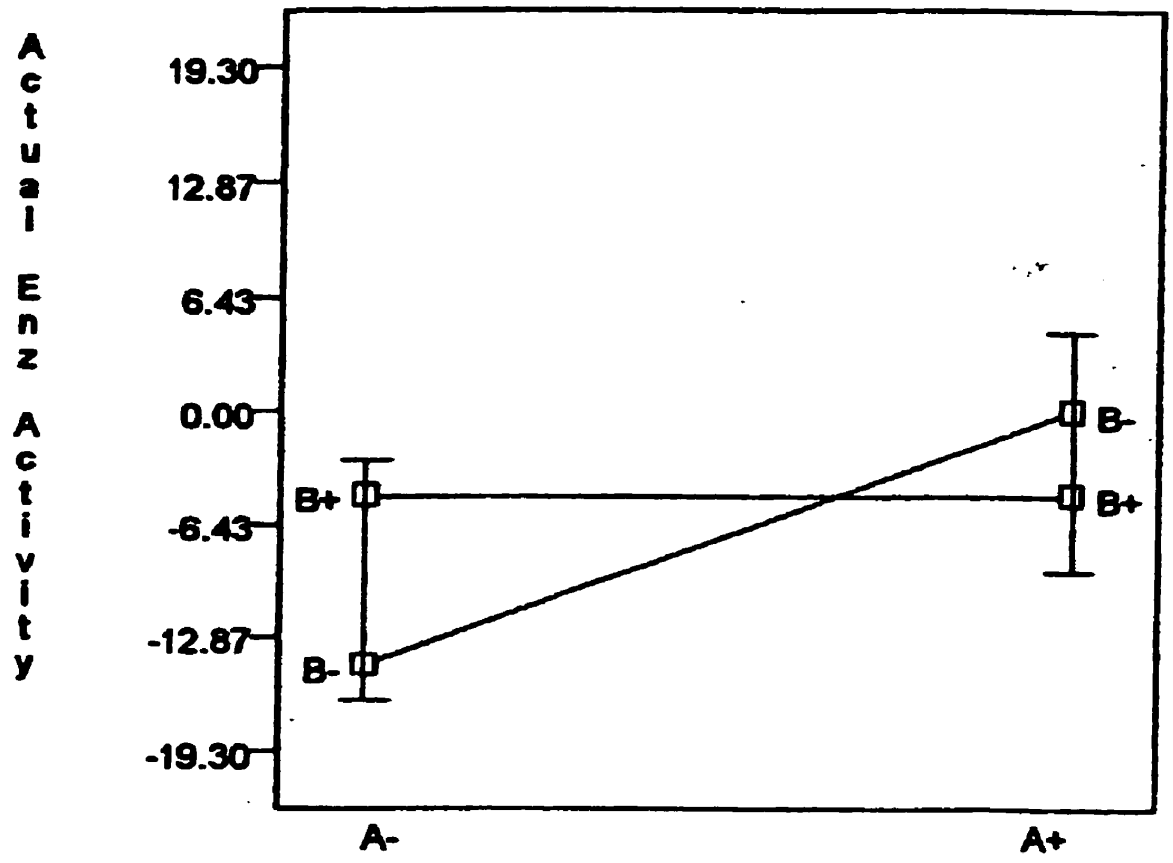
Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	1466.6794	7	209.53	3.00	0.0733
Residual	558.7350	8	69.84		
Cor Total	2025.4144	15			

Factor	Coefficient Estimate	DF	Standard Error	t for H_0 Coefficient=0	Prob>| t |
Intercept	-6.031250	1	2.089286	1.73	0.1215
A	3.618750	1	2.089286	1.73	0.1215
E	3.618750	1	2.089286	1.73	0.1215
AB	-3.618750	1	2.089286	1.73	0.1215
BD	3.618750	1	2.089286	1.73	0.1215
BE	-3.618750	1	2.089286	1.73	0.1215
CD	-3.618750	1	2.089286	1.73	0.1215
CE	3.618750	1	2.089286	1.73	0.1215

where A = $MgCl_2$
B = KCl
C = phospho(enol)pyruvate
D = β-NADH
E = ADP

Figure 20

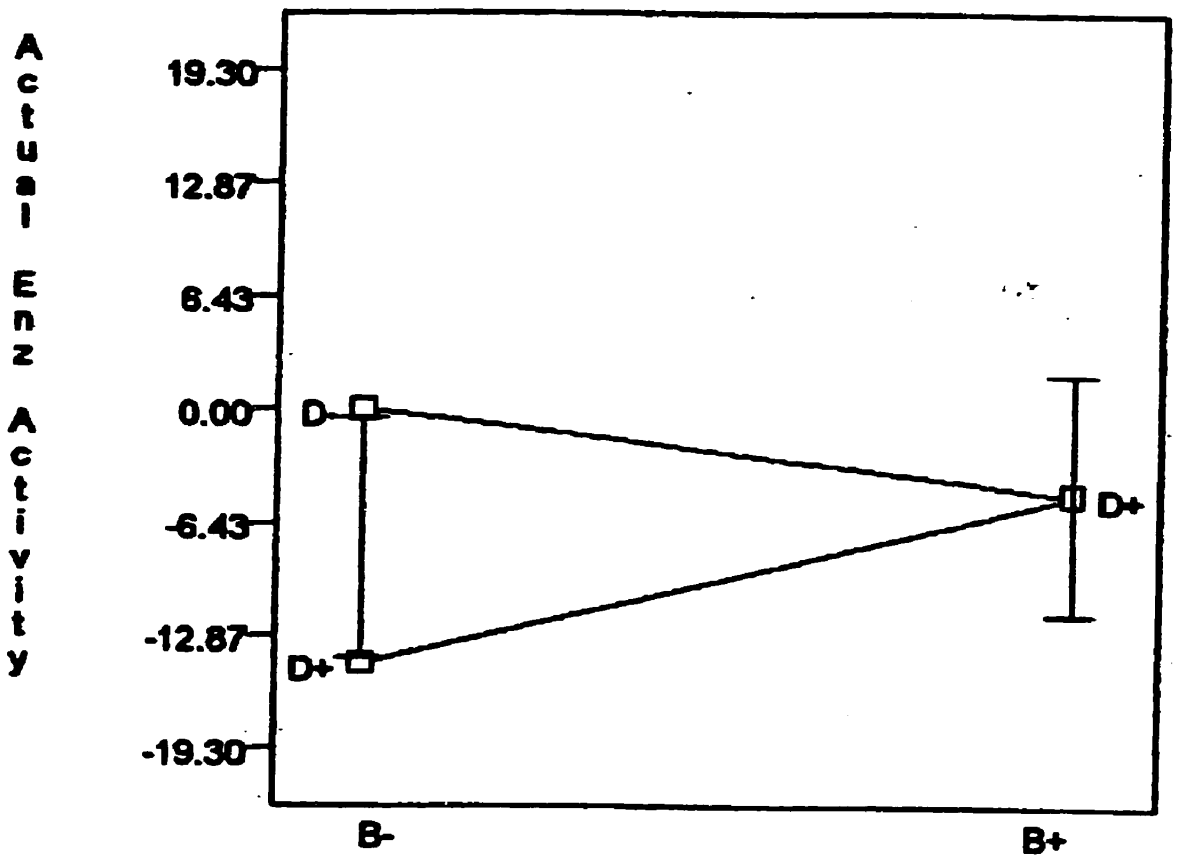
Interaction plot for $MgCl_2$ and KCl from the statistical analysis of a 2^{5-1}, standard order fractional factorial design for the modification of a pyruvate kinase activity assay



-A = 0.02 mM $MgCl_2$ and +A = 2 mM $MgCl_2$
-B = 0.1 mM KCl and +B = 10 mM KCl

Figure 21

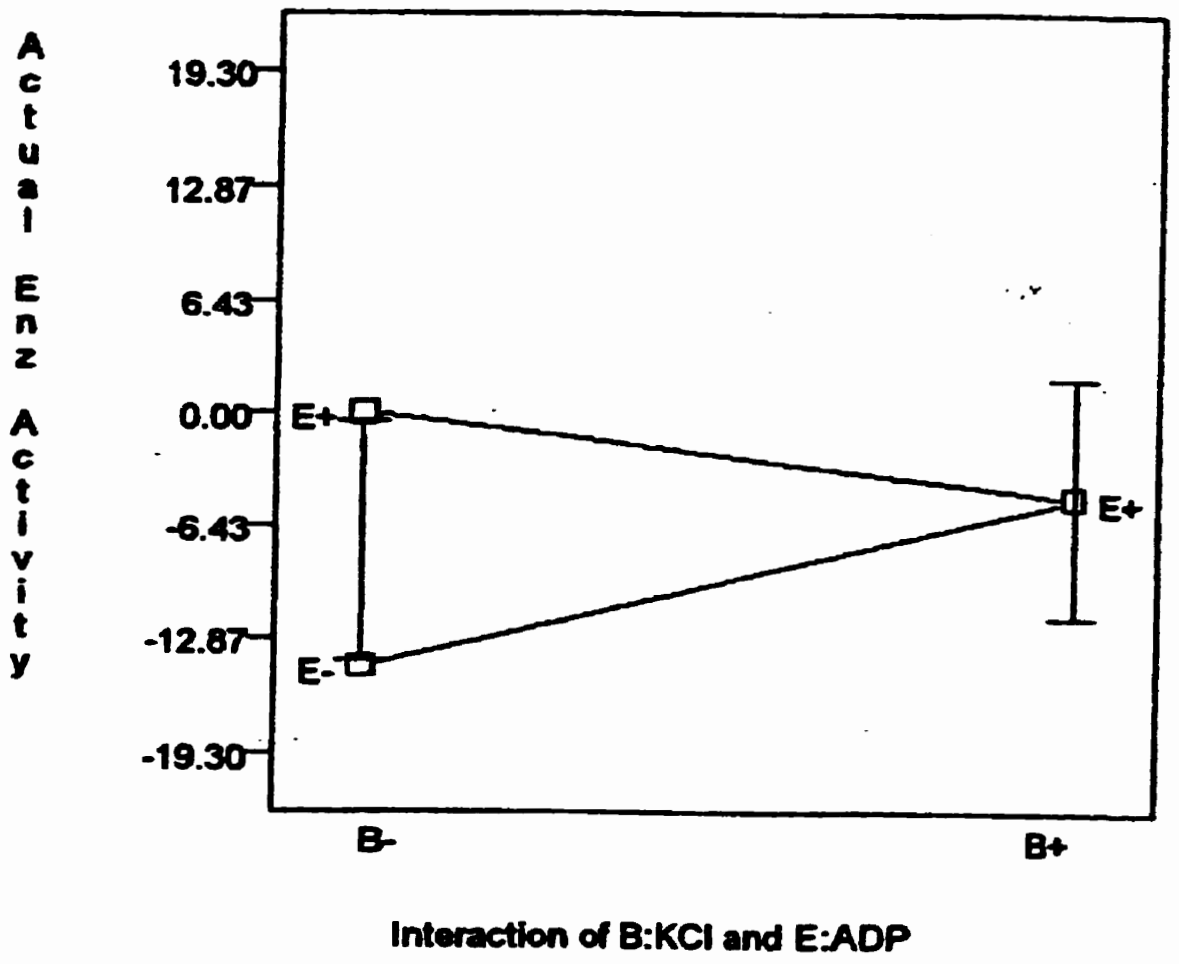
Interaction plot for KCl and β-NADH from the statistical analysis of a 2^{5-1}, standard order fractional factorial design for the modification of a pyruvate kinase activity assay



-B = 0.1 mM KCl and +B = 10 mM KCl
-D = 0.0005 mM β-NADH and +B = 0.05 mM β- NADH

Figure 22

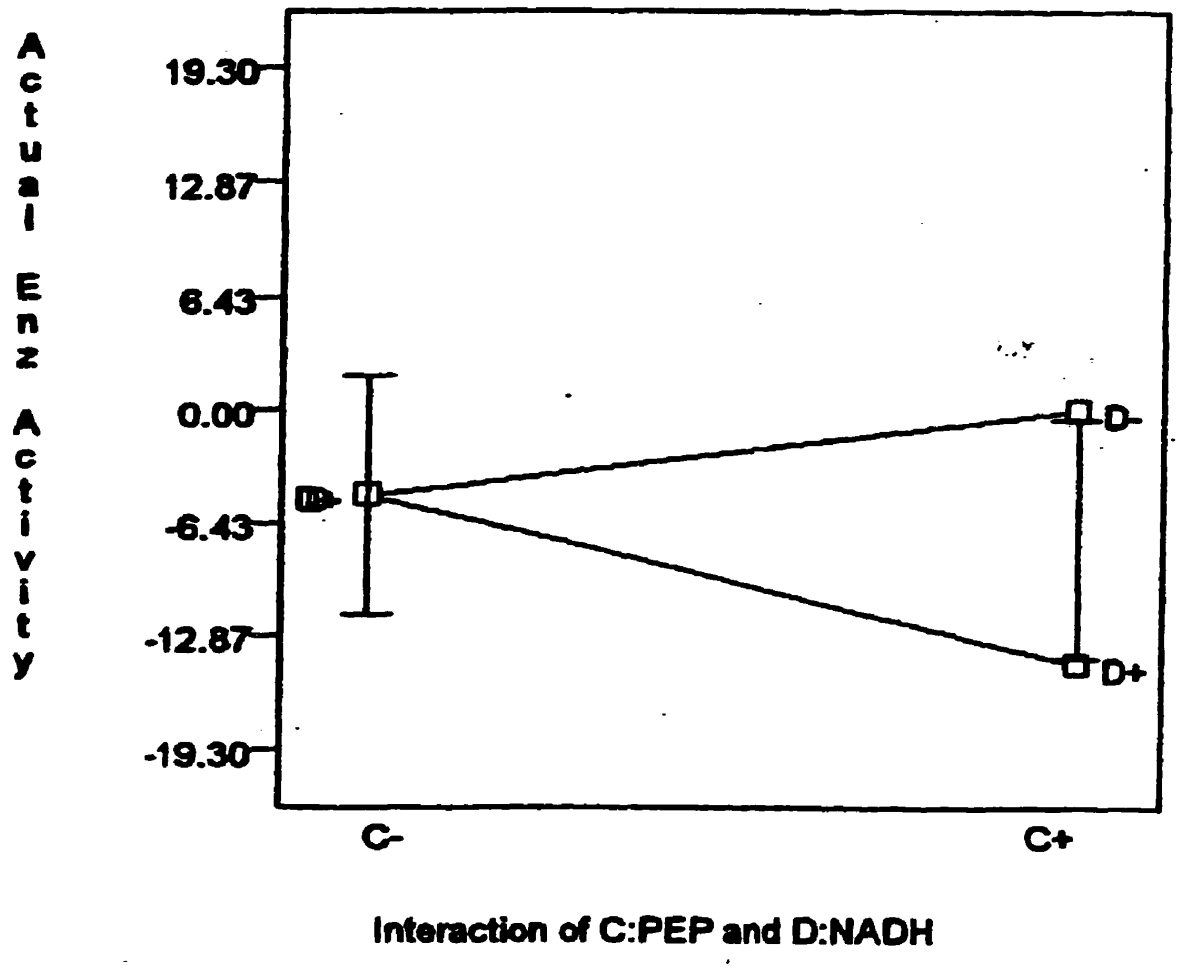
Interaction plot for KCl and ADP from the statistical analysis of a 2^{5-1}, standard order fractional factorial design for the modification of a pyruvate kinase activity assay



-B = 0.1 mM KCl and +B = 10 mM KCl
-E = 0.005 mM ADP and +E = 0.5 mM ADP

Figure 23

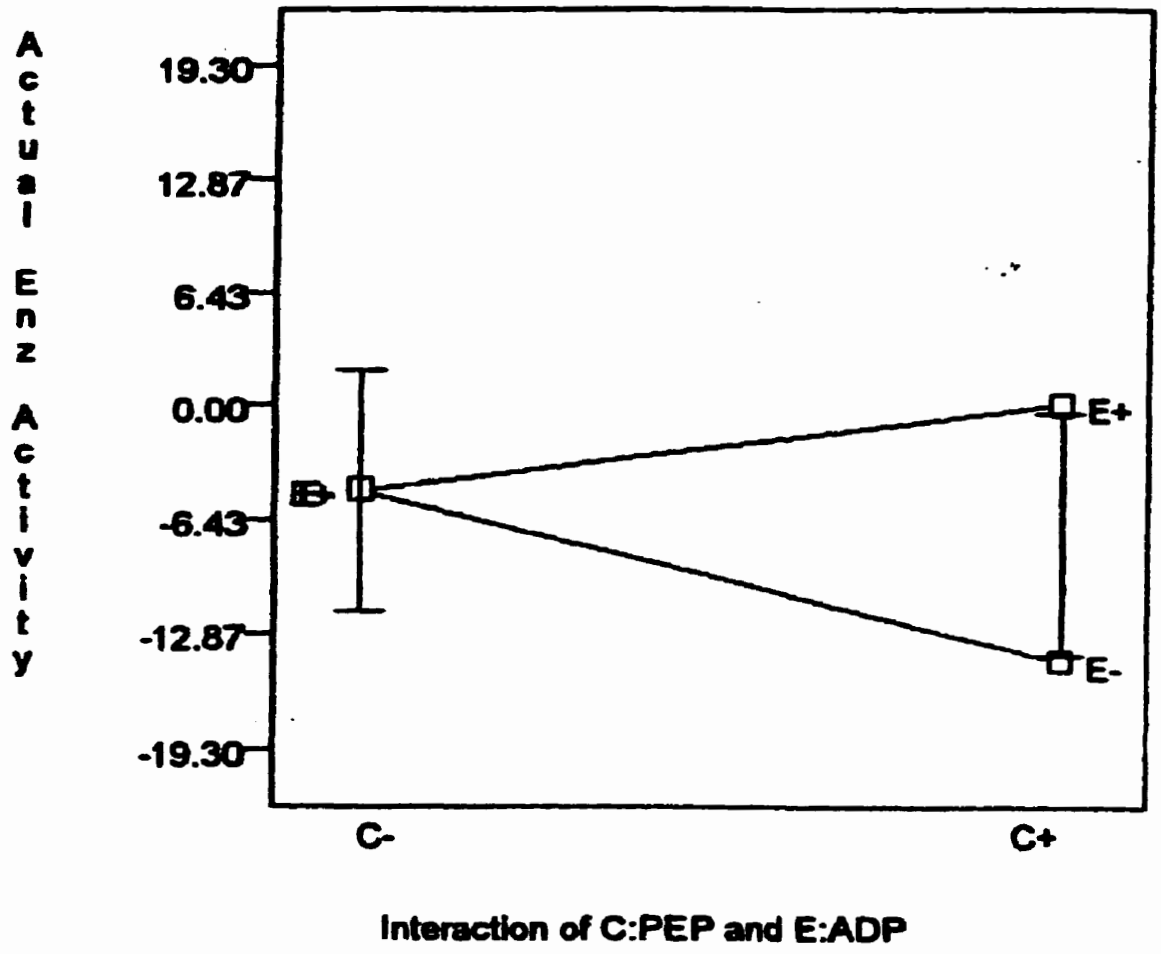
Interaction plot for phospho(enol)pyruvate and β-NADP from the statistical analysis of a 2^{5-1}, standard order fractional factorial design for the modification of a pyruvate kinase activity assay



-C = 0.01 mM phospho(enol)pyruvate and +C = 1 mM phospho(enol)pyruvate
-D = 0.0005 mM β-NADH and +D = 0.05 mM β- NADH

Figure 24

Interaction plot for phospho(enol)pyruvate and ADP from the statistical analysis of a 2^{5-1}, standard order fractional factorial design for the modification of a pyruvate kinase activity assay



-C = 0.01 mM phospho(enol)pyruvate and +C = 1 mM phospho(enol)pyruvate
-E = 0.005 mM ADP and +E = 0.5 mM ADP

Table 16

Levels for factors in a 2^{5-1}, standard order fractional factorial design for pyruvate kinase

Factor	Initial Level of Factor in the Assay (mM)		
	-	Control	+
$MgCl_2$	0.004	0.02	0.1
KCl	0.02	0.1	0.5
Phospho(enol)pyruvate	0.2	1	5
NADH	0.01	0.05	0.25
ADP	0.001	0.005	0.025

- = low factor level
+ = high factor level

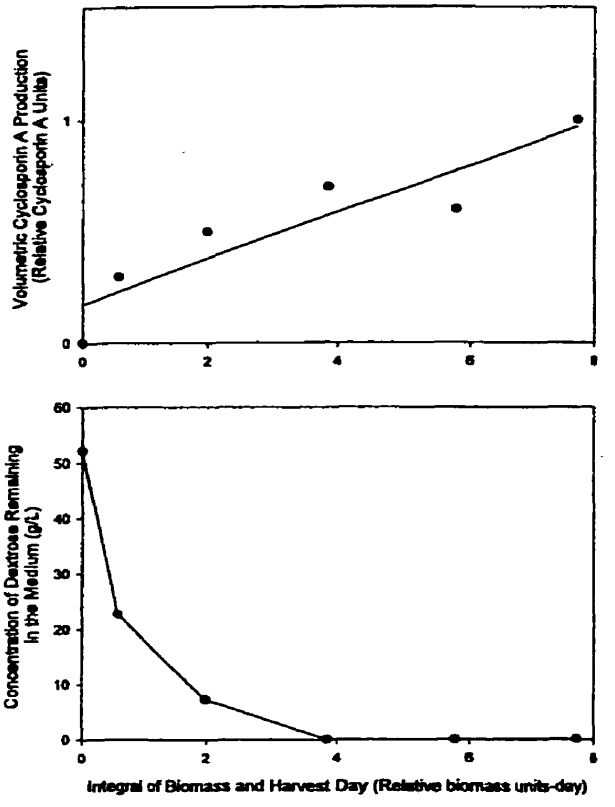
Table 17

Analysis of variance from a 2^{5-1}, standard order fractional factorial design for pyruvate kinase

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F
Model	32010.212	3	10670.1	34.30	<0.0001
Residual	3721.813	12	310.2		
Cor Total	35732.025	15			

Figure 25

An example of the data and plot for strain MS-1
for the determination of specific cyclosporin A productivity
and dextrose consumption rate



T. inflatum strain MS-1				
Harvest Day	Biomass (relative biomass units)	Integral of time and biomass (relative biomass units-day)	Volumetric Cyclosporin A Production (Relative Cyclosporin A Units)	Concentration of Dextrose (g/L) Remaining in the Production Medium
0	0	0	0	52.20
2	0.565	0.565	0.3	22.76
4	0.861	1.992	0.5	7.25
6	0.997	3.850	0.7	0.01
8	0.933	5.780	0.6	0.07
10	1.000	7.713	1	0.156

Figure 26

Example of the determination of the P-value for the statistical correlation between specific cyclosporin A productivity and specific dextrose consumption rate for MS-1 and 14 mutant strains

Where H_o = increasing specific cyclosporin A productivity is associated with decreasing specific dextrose consumption rate
and H_A = increasing specific cyclosporin A productivity is not associated with specific dextrose consumption rate.

Calculation of the coefficient of linear correlation (r) between specific cyclosporin A productivity and specific dextrose consumption rate:

$$r = \frac{n\sum xy - \sum x \sum y}{([n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]}$$

where the sum of specific dextrose consumption rate ($\sum x$) = -6.203
the sum of specific cyclosporin A productivity ($\sum y$) = 4.473
the sum of the square of specific dextrose consumption rate ($\sum x^2$) = 6.589
the sum of the square of specific cyclosporin A productivity ($\sum y^2$) = 1.482
and the sum of the product of specific dextrose consumption rate and specific cyclosporin A productivity ($\sum xy$) = -1.25

therefore r = 0.782
using standard error of $r = s_r = (1 - r/n - 2)^{½}$ where n = the size of the population with 2 degrees of freedom (n - 2),
and s_r = 0.13

And $t = \frac{r}{s_r} = \frac{0.782}{0.13} = 6.015$

$t_{0.05\,(2),\,13} = 4.221$, therefore accept H_o with $P < 0.001$.