THE EXTRACELLULAR LIPASES OF THERMOPHILIC *STREPTOMYCES*

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The extracellular lipases of thermophilic *Streptomyces*

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor in Philosophy

by

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Declaration

I declare that this thesis is my own account of my research and contains as its contents work which has not previously been submitted for a degree at any university.

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I would like to dedicate this thesis to my parents Georgios and Niki Papas and to my sisters Heleni and Maria.

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Abstract

The genus *Streptomyces* is defined as Gram-positive bacteria that produce extensively branching hyphae and reproduce primarily by means of asexual spores. Thermophilic *Streptomyces* are widely distributed in nature, particularly in compost heaps and decaying vegetable matter, and thrive best at temperatures between 45 and 60°C. They often rely on degradation of polymeric materials for their nutrition by secretion of a wide variety of extracellular hydrolytic enzymes. An important function of these bacteria is the degradation of a wide range of organic compounds that are resistant to breakdown by other microbes.

Lipases are enzymes that hydrolyse oils and fats to glycerol and free fatty acids, carbon and energy sources that can easily be utilised by many organisms. The synthesis and secretion of lipases by bacteria are influenced by a variety of environmental factors such as ions, carbon sources or presence of non-metabolizable polysaccharides. Bacterial lipases, with few exceptions, are able to completely hydrolyse a tiacylglycerol substrate although a certain preference for primary ester bond has been observed. They are mostly extracellular in nature and in some cases are inducible by the inclusion of lipid substrates in the growth media.

This study describes the preliminary characterisation of extracellular lipases of *Streptomyces thermoviolaceus, S. thermodiastaticus, S. thermoflavus, S. thermovulgaris* and *S. thermonitrificans* on defined media. Growth on a range of lipid sources at 50°C revealed that some gave preferentially higher growth and lipase secretion and the bulk of the work described have employed cultures growing with olive oil or Tween 80 as sole lipid source. The organisms secreted lipase activity during exponential and stationary phases and reached maximum levels when biomass production was at its highest level. For all species used the optimum pH for lipase activity was 8.0 and profound thermostability was found at 70°C. Inhibitors were used in an attempt to resolve the type of lipase produced and suggested the presence of a metallo-lipase and a serine-lipase. The effects of divalent metals suggested that magnesium and/or calcium is important for enzyme activity.

Laboratory fermentation studies, based on *Streptomyces thermoviolaceus and S. thermodiastaticus*, revealed that growth on defined media was biphasic and the faster the growth rate of the second phase of growth the greater the production of volumetric lipase activity. Also, it was found that the growth temperature of 50°C was the ideal temperature for secretion of volumetric lipase activity.

Preliminary experiments attempting to isolate and characterise lipases from *Streptomyces thermoviolaceus* suggested that two lipases were present with molecular weights of 36 and 49 kDa respectively.

Plasmid pIJ702 was used for cloning of chromosomal DNA from *Streptomyces thermoviolaceus* in *S. lividans,* and the cloned DNA was found to be 6 kbs but attempts to grow the recombinant clone in salts media used for *Streptomyces* growth were unsuccessful.

Abbreviations

BDH	British Drug Houses
BSA	bovine serum albumin
°C	degree centigrade
CIAP	calf intestine alkaline phosphatase
cm	centimetre(s)
CTP	chymotrypsin protease
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
g	gram(s)
g	gravitational force
G+C	guanine+cytosine
GES	guanidium thiocyanate reagent
GSI	glutamine synthetase I
h	hour(s)
kbs	kilobase(s)
kDa	kilodalton
l	litre(s)

μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mmol	millimolecule(s)
MOPS	3-(N-morpholino) propanesulphonic acid
MTP	metallo-protease
n	sample size
NADH	NADH-dehydrogenase
nm	nanometre(s)
nmol	nanomolecule(s)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pCMB	p-chloromercuribenzoate
PEG	polyethylene glycol
pH	-log $[H^+]$
PMSF	phenylmethylsulphonylfluoride

RNA	ribonucleic acid
RNAse	ribonuclease
rRNA	ribosomal RNA
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-SB	SDS sample buffer
$T_{(50)}$	time taken for the loss of 50% of the total lipase activity
TEMED	NNN'N'-tetramethyl-ethylenediamine
TLCK	tosyl-lysine chloromethyl ketone
TLP	trypsin-protease
Tris	Tris (hydroxymethyl) aminomethane
Tween	polyoxyethylenesorbitan
U	units
UV	ultraviolet
v/v	volume/volume
W	watts
w/v	weight/volume
YEME	yeast extract-malt extract medium
%	percentage
z1	growth rate of the first phase of growth
z2	growth rate of the second phase of growth

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Chapter 1

Introduction

1.1 *Actinomycetales*

The *Actimomycetales* differ from most other bacteria in that true branching is normally found in all of its species. None of the *Actinomycetales* is stalked or photosynthetic, none appears to accumulate sulphur, iron or other free elements in or on the cells. All of the organisms in the order are chemoorganotrophic, although many will grow in simple mineral media with an organic carbon source. *Actinomycetales* include aerobic, facultatively anaerobic and strictly anaerobic species.

1.2 Actinomycetes

Actinomycetes are a heterogeneous group of Gram-positive, predominantly mycelial bacteria. Although it is of bacterial dimensions, the mycelium is in some ways analogous to the mycelium formed by the filamentous fungi. The following properties establish the actinomycetes as bacteria: 1) they are prokaryotic 2) their cell walls contain muramic acid and diaminopimelic acid, which are characteristic of bacterial cell walls and they lack the chitin and glucans which are characteristic of the cell walls of fungi 3) their growth is inhibited by penicillins, tetracyclines, sulphonamides and antibacterial drugs that have no inhibitory effects on fungi and 5) the filaments of actinomycetes readily segment into bacillary and twig-like forms, with dimensions typical of bacteria.

Although most are strict saprophytes, some form parasitic or symbiotic associations with plants or animals (Williams, *et al.*, 1984). Due to their metabolic diversity and evolution of specific mechanisms for dispersal, actinomycetes occur in a wide range of natural environments (McCarthy and Williams, 1990). Actinomycetes isolated from soil and related substrates show primary biodegradative activity, secreting a range of extracellular enzymes and exhibiting the capacity to metabolise recalcitrant molecules

(McCarthy and Williams, 1992). Studies by Goodfellow and Williams, (1983), based on phylogenetic trees produced from 16S rRNA sequences, lead to the definition of actinomycetes as Gram-positive bacteria with a G+C ratio of >55 Mol%. Differentiation of the actinomycetes from one another is determined by morphological and physiological traits.

The actinomycetes are physiologically active, utilising a variety of carbon and nitrogen compounds and many are actively proteolytic. The optimum temperature for growth is usually 20 to 30°C, although some of the pathogenic species grow at 37°C. Actinomycetes have received considerable interest as potential producers of antibiotics. They secrete extracellular enzymes, proteins, nucleic acids, polysaccharides and plant polymers in natural environments (McCarthy, *et al.*, 1985), which are important in the breakdown of complex organic materials.

1.3 *Streptomyces*

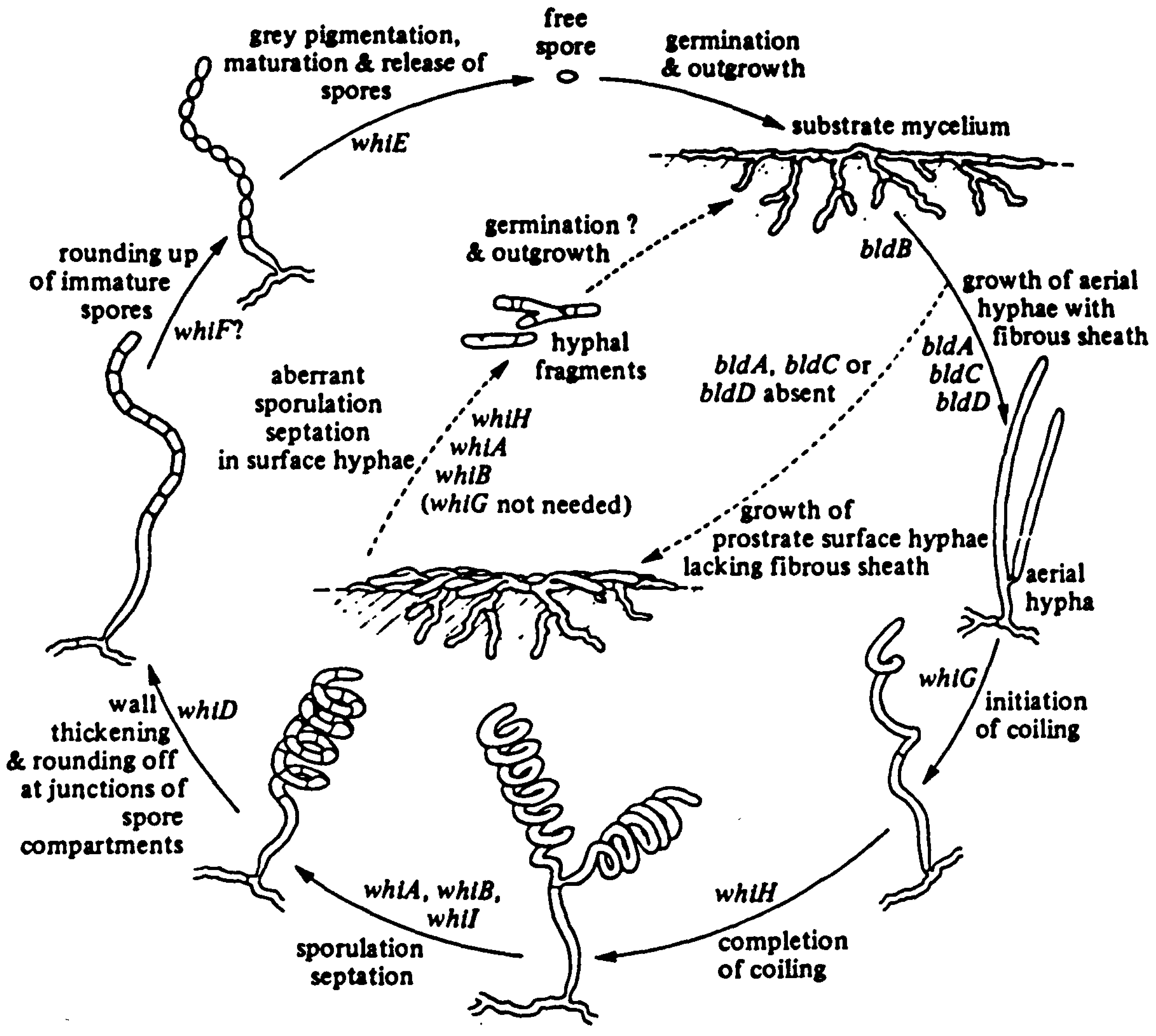
The genus *Streptomyces* is defined by Gram-positive bacteria that produce extensively branching hyphae and reproduce primarily by means of aerial asexual spores. Members of this genus form long, much branched, aerial mycelia. Differences in morphology, reproduction and chemical composition distinguishes this genus from other actinomycete genera; the peptidoglycan of their cell wall is characterised by an interpeptide bridge of glycine molecules which is lacking in other actinomycetes. Also their mycelial growth is more complex and differentiated.

Williams, *et al.*, (1983) performed a large scale numerical phenetic survey to clarify the infrastructure of the *Streptomyces* genus. The results indicated that the type strains of *Streptomyces* species could be distributed into 23 major clusters (containing 4 or more strains), 20 minor clusters (containing 2 or 3 strains) and 25 clusters containing a single member. Lambert and Loria, (1989) demonstrated that the majority of

pathogenic *Streptomyces* form a distinct genus consistent with the original genus description and provided phenotypic criteria which lead to the differentiation of this genus. Kämpfer, *et al.*, (1991) performed a numerical classification of the genus *Streptomyces* by using miniaturised physiological tests, and the resulting phenetic data, in most cases, confirmed the major phena of the study of Williams, *et al.*, (1983). Ochi, (1995) attempted a different taxonomic study of the genus *Streptomyces* by analysis of ribosomal protein AT-L30 and classified the strains into 4 groups and a non-group category. He found that there was considerable agreement between these results and those of numerical phenetic classification although there were some disagreements. Genetic manipulations can provide useful information on the phenetic identification of *Streptomyces* species (Clarke, *et al.*, 1995). Phenetic characters most stable to genetic manipulation are morphological and biochemical, while those most susceptible are the utilisation of carbon and nitrogen sources and melanin pigment production. More recently, Oh, *et al.*, (1996) used the relative electrophoretic mobilities of various enzymes from different *Streptomyces* species in order to examine the relatedness of species and strains of the genus *Streptomyces*, and found that glucose dehydrogenase, alcohol dehydrogenase, 3-hydroxybutyrate dehydrogenase, phosphoglucose isomerase and esterase exhibited either weak non-reproducible or highly heterogeneous band patterns which were suitable for dissecting the strains within a species and a cluster group.

When observed on solid media, the growth of a *Streptomyces* colony is an example of a primitive multicellular developmental cycle. Early in the life cycle, growth occurs as expansion of hyphal tips. Then, aerial branches develop and secondary metabolism commences. Each aerial hypha grows into a multinucleoid filament which eventually subdivides into haploid spores. Figure 1.1 shows the life cycle of *Streptomyces* (Hopwood, *et al.*, 1986).

Figure 1.1 The life cycle of *Streptomyces* (Hopwood, *et al.*, 1986)


free spore
germination & outgrowth
substrate mycelium
grey pigmentation, maturation & release of spores
whiE
germination ? & outgrowth
bldB
rounding up of immature spores
whiF?
growth of aerial hyphae with fibrous sheath
hyphal fragments
bldA, bldC or bldD absent
bldA
bldC
bldD
aberrant sporulation septation in surface hyphae
whiH
whiA
whiB
(whiG not needed)
growth of prostrate surface hyphae lacking fibrous sheath
aerial hypha
wall thickening & rounding off at junctions of spore compartments
whiD
whiG
initiation of coiling
whiA, whiB, whiI
whiH
sporulation septation
completion of coiling

Streptomyces usually grow in liquid cultures as pellets or hyphal aggregates. (Williams, *et al.*, 1974). The pellets are composed of densely interwoven hyphae and are generally spherical in shape. A proportion of the cells are nutrient-limited and as the pellet increases in size, growth is eventually confined to a shell of limited thickness at the surface of the sphere (Pirt, 1967). This growth pattern leads to many *Streptomyces* cultures containing mycelium of variable physiological states. Hobbs, *et al.*, (1989) reported that the addition of polyanions to *Streptomyces* growth media eliminates most of the problems associated with pellet growth. Daza, *et al.*, (1989) reported that the ratio between Ca^{2+} and phosphate or nucleotides, can play an important role in the induction of sporulation in liquid cultures. They suggested that a high concentration of Ca^{2+} induces the differentiation process.

The sporogenesis of *Streptomyces* involves two basic stages: a) sporulation septum synthesis and b) maturation. The completion of the sporulation septum and the deliniation of the mycelium into spore-sized compartments, mark the beginning of a series of partially known changes resulting in the formation of mature arthrospores.
Cross and Attwell, (1975) proposed that the role of controlled autolysis during arthrospore maturation is very important. Manzanal and Hardisson, (1978) suggested that the regulation of enzymes involved in wall synthesis and lysis play a fundamental role in the entire maturation process of *Streptomyces.*

Streptomyces are well known for their ability to produce a wide variety of antibiotics and extracellular enzymes. Many of the antibiotics produced by *Streptomyces* are in current clinical or agricultural use and include phenolic or macrolide derivatives produced by a sequence of reactions similar to those responsible for fatty acid biosynthesis (Hopwood and Khosla, 1992). Table 1.1 shows some of the recently discovered antibiotics produced by *Streptomyces* species.

Streptomyces often rely on degradation of polymeric materials for their nutrition by secretion of a wide variety of extracellular hydrolytic enzymes. The important function

Table 1.1 Some recently-discovered antibiotics produced by *Streptomyces*

Organisms	Antibiotics	Reference
Streptomyces sp. (DSM 5087)	Landomycins	Henkel, *et al.*, (1990)
Streptomyces spheroides	Lactam	Kojiri, *et al.*, (1992)
Streptomyces sp. (Ni-80)	Urauchimycins	Imamura, *et al.*, (1993)
Streptomyces sp. (MJ147-72F6)	Aldecalmycin	Sawa, *et al.*, (1994)
Streptomyces sp. (K106)	Nisamycin	Hayashi, *et al.*, (1994)
Streptomyces sp. (KO-7888)	Phthoxazolins	Shiomi, *et al.*, (1995)
Streptomyces sp. (DO-114)	Clecarmycins	Fujii, *et al.*, (1995)

of these microorganisms is the degradation of a wide range of organic compounds that are resistant to break down by other microbes. In batch cultures of *Streptomyces*, secretion of extracellular enzymes often coincides temporarily with the onset of antibiotics and pigment biosynthesis and with sporulation (Chater, 1984). Daza, *et al.*, (1990) suggested that the formation of secondary metabolites, extracellular enzyme synthesis and differentiation in *Streptomyces*, respond to common triggering mechanisms. Horinouchi and Beppu, (1990) concluded that there is a close relationship between morphological differentiation and secondary metabolism in *Streptomyces* and they observed that spontaneous loss of production of a certain antibiotic is frequently accompanied by the loss of sporulation in certain *Streptomyces* species.

1.4 Secondary metabolism

Maplestone, *et al.*, (1992) adopted a definition of a secondary metabolite "as a naturally produced substance which does not play an explicit role in the internal economy of the organism that produces it." They suggested that the ability to produce secondary metabolites has evolved as part of the producing organism's strategy for survival. Secondary metabolites are biosynthesised by bacteria, fungi, algae, corals, sponges, plants and lower animals. Secondary metabolites are characterised by: the specificity of their occurrence, their wide variation, by the appearance of chemical groups and structural units which are absent in primary metabolites and the difficulty of recognising a function of the metabolites for the producing agent (Zahner, 1987).

Many secondary metabolites are known to have physiological activities (Barna and Williams, 1984). Despite the large number of physiologically active secondary metabolites known, the activity of an even larger number is not known because they either have no physiological activity or because they have not been subjected to the correct screening assays.

Secondary metabolites have sophisticated and widely varying structures requiring similarly sophisticated and diverse biosynthetic pathways. Hopwood, (1988) has noted, in the case of antibiotic production, that these biosynthetic pathways typically involve approximately 10-30 steps catalysed by a corresponding number of gene products. The genetic complexity involved in the biosynthesis of secondary metabolites is in general much greater than for primary metabolite biosynthesis. In primary metabolite biosynthesis, protein synthesis is a common step, and these materials are frequently the product of one gene. By contrast, the complex pathways of secondary metabolism are expensive both in energetic terms and in genetic terms. Clark, *et al.*, (1995) indicated that oxygen limitation stimulates secondary metabolite production in some cases and inhibits it in others. The complexity of secondary biosynthetic pathways and the clustering of genes necessary for the biosynthesis and regulation of secondary metabolites provides strong evidence that these compounds have been positively selected as a result of their antagonistic functions.

Industrial screening programmes for novel microbial metabolites are often designed so that each isolate under test is grown in a variety of media designed to provide different growth-limiting nutrients (Nisbet, 1982). A wide variety of novel secondary metabolites have been detected. These include: antibacterial, antifungal, antiviral and antitumour compounds, enzyme inhibitors, pharmacologically and immunologically active agents, products useful in agriculture and microbial regulators (Franco and Coutinho, 1991).

The initiation of secondary metabolism in both eukaryotic and prokaryotic microorganisms often accompanies cellular differentiation suggesting that a close relationship may exist among the multiple regulatory systems of these two complex phenomena. The occurrence of secondary metabolites is not common to all microorganisms but restricted to certain taxa or even strains. These metabolites are often synthesized only in a certain period of the life cycle of a given organism mostly

after cessation of vegetative growth (Gröeger, 1992). The formation of microbial secondary metabolites in batch cultures is frequently delayed until the rate of biomass accumulation declines, creating two metabolically distinct phases of culture development: the trophophase when resources are committed to rapid growth and the subsequent idiophase in which secondary metabolites are made (Beppu, 1995). Secondary metabolites can be more readily assayed and isolated in an idiophase, when rapid product synthesis is supported by high enzyme levels (Vining, 1986). To separate trophophase and idiophase activities, the culture medium must support early biomass accumulation at a high rate and then allow a gradual transition to stationary phase under conditions that activate the genetic systems for secondary metabolism (Liao, *et al.*, 1995).

1.4.1 Regulation of metabolism

Microorganisms are able to alter their composition and metabolism in response to environmental changes. The major regulatory mechanisms which confer this flexibility operate at both the level of enzyme synthesis and at the level of enzyme action (Drew and Demain, 1977). A review by Rose, (1979) suggested that some of the most important mechanisms responsible for regulation of primary and secondary metabolism include: a) substrate induction, b) catabolite repression and c) feedback repression.

1.4.1.1 Substrate induction

Some enzymes are produced constitutively, they are always made in substantial amounts when cells are growing. Other enzymes are inducible, they are formed only in the presence of key substrates in the medium. The structural genes of inducible enzymes are normally inactive or operate at a very basal rate in the absence of substrate.

1.4.1.2 Catabolite repression

This phenomenon frequently occurs when the cell is grown in a medium containing more than one utilizable growth substrate. In carbon catabolite repression, enzymes are synthesized which catabolize the best substrate and only after exhaustion of this substrate are enzymes produced to break down the poorer growth substrates. Vining and Doull, (1988) reviewed catabolite repression of secondary metabolism in *Streptomyces* and suggested that carbon, nitrogen and phosphate catabolite repression play an important role in the regulation of secondary metabolism.

1.4.1.3 Feedback repression

While synthesis of catabolite enzymes is often controlled by induction and catabolite repression, biosynthesis of anabolic enzymes may be regulated by feedback or end product repression. In the classical model explaining feedback repression, the regulator gene produces an apo-repressor protein, which when combined with a co-repressor (end product), binds to the operator site and blocks transcription. In the absence of the end-product, no repression takes place (Demain, 1985).

1.4.1.4 Autoregulatory factors

Apart from regulation under environmental changes, autoregulatory factors can play an important role in the regulation of secondary metabolism. Among the several known autoregulators, A-factor has been extensively studied (Khokhlov, *et al.*, 1973). Beppu, (1992) assumed that secondary metabolites play no functional role in their producer organisms but they may work as autoregulators of cellular differentiation in *Streptomyces*. The involvement of diffusible self regulatory factors that positively control differentiation processes has long being recognised in various *Streptomyces* species. Horinouchi and Beppu, (1992) showed that a low molecular weight regulatory substance, A-factor, triggers streptomycin production and aerial mycelium formation in

Streptomyces griseus. A-factor exerts its regulatory role by binding to a specific receptor protein which, in the absence of A-factor, acts as a repressor type regulator for morphological and physiological differentiation. The A-factor signal via the A-factor receptor protein is transferred to downstream genes, such as streptomycin production genes and sporulation genes, through multiple regulatory genes in a complex regulatory cascade (Horinouchi and Beppu, 1994). A-factor analogues, have been identified as self-regulatory factors for secondary metabolism and/or morphological differentiation in a variety of *Streptomyces* species. Recent genetic and biochemical analyses have revealed the involvement of various protein kinases in the control of sporulation and secondary metabolism in various *Streptomyces* species.

1.4.2 Secondary metabolites from *Streptomyces*

Streptomycetes are well known for their ability to produce diverse, bioactive, secondary metabolites and also for their complex morphology which is similar to that of fungi. A number of secondary metabolites from *Streptomyces* have been screened, some of which inhibit the characteristic functions of eukaryotic cells. Desphande, *et al.*, (1988), after performing an extensive research program for isolation and studies on the mechanism of action of secondary metabolites from *Streptomyces*, have documented a wider group of compounds that are not antimicrobial agents. A number of these metabolites are useful as herbicides, insecticides, antiparasitic and hypoglycaemic agents.

Strohl, *et al.*, (1991) showed that hybrid secondary metabolites can be produced as a result of interspecies cloning of antibiotic biosynthesis genes in *Streptomyces*. Õmura, (1992) isolated two novel microbial metabolites from *Streptomyces*: triacsin, an inhibitor of acetyl-CoA synthetase, from *Streptomyces* sp. SK-1894 and phthoxazolin, an inhibitor of cellulose biosynthesis, from *Streptomyces* sp. OM-5714. Members of

the genus *Streptomyces* produce a variety of polyketide products, many of which have applications in medicine and agriculture Hopwood, (1995).

More recently, Obanye, *et al.*, (1996) found that *Streptomyces coelicolor* A3(2) produced methylenomycin as the sole detectable secondary metabolite when grown in a minimal medium. They suggested that a switch in the pattern of carbon metabolism occurred during a period of slower growth in batch culture which immediately preceded entry into the stationary phase. This coincided with the period of methylenomycin production.

1.5 Thermophiles

Microorganisms can be divided into three major groups based on their minimum and maximum temperature for growth: psychrophiles, mesophiles and thermophiles. Bacteria exhibit unique diversity in their ability to grow at different temperatures. The temperature range for a species is generally considered to be a stable character; however, mutants may be isolated that have a minimum and a maximum temperature below or above the parent organism. Some bacteria may also be able to grow at different temperatures by training cultures, through an increase or decrease of temperature (Lindsay, 1995). Williams, (1975) defined thermophilic organisms as those with an optimum growth temperature above 50°C and a maximum of more than 60°C. Bacterial thermophiles include eubacteria, cyanobacteria and archaebacteria.

Interest in the ecology, physiology and evolution of microorganisms adapted to grow at relatively high temperatures has increased enormously in recent years, stimulated by awareness of the potential of thermophilic microbes in biotechnological processes. Habitats known to harbour a considerable variety of thermophilic microorganisms include: solar heated ponds, marine bays, sediments, soils and rocks, habitats that are self heated through microbial activity such as compost heaps and

industrial plants. The number of known anaerobic thermophilic species exceeds the number of aerobic thermophilic species. This reflects the fact that the solubility of oxygen drops dramatically with increasing temperature, thus facilitating the development of anoxic conditions in most high temperature habitats. The taxonomic diversity of thermophilic microorganisms appears to be less than that of the mesophilic organisms. Gottschal and Prins, (1991) reported that all major physiological categories of mesophilic organisms are also found amongst the thermophiles. Table 1.2 shows the grouping of thermophilic microorganisms as described by Edwards, (1990). More recently, Tanner, *et al.*, (1996) concluded that hydrophobic effects allow for discrimination between thermophiles and psychrophiles.

Reizer, *et al.*, (1985) carried out a study on the thermoadaptive mechanisms of the membrane of thermophiles, using *Bacillus stearothermophilus* var. *nondiastaticus* as test organisms. They concluded that unsaturated fatty acids were not produced by cells grown at 65°C. Also, the molecular packing of phospholipids in monolayers was more expanded with phospholipids from 45°C grown cells as compared with cultures grown at 35°C. Respiratory enzymes of thermophiles appear to be functionally similar to the mesophilic enzymes but differ in their thermostability and unusual high turnover rates. Energy coupling at extreme temperatures seems inefficient by the high maintenance coefficients and the high permeability of the cell membrane to protons (Konings, *et al.*, 1992). Driessen, *et al.*, (1996) investigated membrane composition and ion-permeability in thermophiles and suggested that at their growth temperature, the permeability of the cytoplasmic membrane of thermophilic bacteria to protons is high compared with sodium ions. Proton permeability increases with temperature and has a comparable value for most species at their respective optimal growth temperatures. Sodium permeability increases also with temperature but is lipid independent.

Henle, *et al.*, (1982) examined the biochemical mechanisms responsible for thermotolerance and suggested that: 1) thermotolerance results neither from the

Table 1.2 Grouping of thermophilic microorganisms (Edwards, 1990)

Groups	Temperature range (°)	Organisms
Thermotolerant	20-50	*Bacillus subtilis*
Facultative thermophiles	30-60	*Bacillus coagulans*
	25-58	*Streptomyces thermoviolaceus*
	25-50	*Torula thermophilia*
Obligate thermophiles	40-80	*Bacillus stearothermophilus*
	75-79	*Thermus aquaticus*
	37-65	*Thermomonospora chromogena*
	55-74	*Synechococcus lividus*
	40-68	*Clostridium thermocellum*
	35-78	*Thermoanaerobium ethanolicus*
Caldoactive	50-90	*Sulfolobus acidocaldarius*
	55-85	*Thermothrix thioparus*
	50-85	*Desulfovibrio thermophilus*
	50-95	*Methanococcus jannaschii*
Barothermophile	80-110	*Pyrodictium brockii*

synthesis of homologous, inherently heat-resistant macromolecules, nor the rapid resynthesis of denatured native macromolecules and 2) thermal death is the result of heat damage to critical macromolecules. Acquired thermotolerance refers to the enhanced survival of organisms at lethal temperatures after a brief exposure to near lethal temperatures. This response correlates with the synthesis of a small number of proteins known as heat-shock proteins which has led to the hypothesis that thermotolerance depends on one or more of these specific proteins (Trent, *et al.*, 1994). Lindsay, (1995) suggested that enzyme thermostability appeared to be associated with an increased use of hydrophobic amino acids.

Recently, the microbiology of thermophiles has been dominated by the isolation and characterisation of thermophilic *Archaea* species, many of which grow at extreme temperatures (in excess of 100°C). Many new thermophilic eubacterial species have also been isolated from a wide range of thermal environments. The majority of the thermophilic Gram-positive bacteria that have been described belong to the genera *Bacillus, Alicyclobacillus* and *Clostridium* (Carreto, *et al.*, 1996).

1.5.1 Use of thermophilic organisms

The use of thermophilic organisms for industrial applications has increased significantly. Thermophiles can be used as sources of thermostable proteins and enzymes, as biocatalysts for conversion of biomass into fuel-related compounds and feedstock chemicals, in leaching processes and waste management. Some of the advantages for the use of thermophiles in biotechnological processes are summarised by Edwards, (1990):

1) Faster reaction times.
2) Reduced risks of contamination.
3) Reduced cooling costs of operating large fermentations.
4) Thermophilic treatment of sewage wastes will kill off pathogenic bacteria and viruses.

5) Decreased viscosity of the growth media.

Thermophiles are expected to produce qualitatively new biocatalysts. Today the most promising thermophilic biocatalysts are thermophilic enzymes not only because of their enhanced thermostability but also because they are more resistant to denaturing agents and more tolerant to higher solute (reactant) concentrations. The variety of thermostable enzymes has been steadily increasing for use in industrial applications, mainly as replacements for thermolabile enzymes (Zamost, *et al.*, 1991). The application of enzymes from isolated extreme thermophiles in biotechnological processes is hampered by their unconventional fermentation conditions (Morana, *et al.*, 1995). The expression of genes encoding for thermophilic proteins in mesophilic hosts, permits the production of enzymes in high yield by using conventional fermentation plants.

1.6 Thermophilic actinomycetes

Greiner-Mai, *et al.*, (1987) suggested that any actinomycete that grows at 50°C or at higher temperatures can be considered thermophilic. Amner, *et al.*, (1993) concluded that thermophilic actinomycetes, with few exceptions, thrive best at temperatures between 45 and 60°C. The identification of thermophilic actinomycetes is based primarily upon morphological and biochemical criteria (Kurup and Fink, 1975).

Thermophilic actinomycetes are common in naturally high temperature habitats such as leaf and compost heaps and in over-heated stores of plant materials such as hay, grain and bagasse. The resistant spores are disseminated widely in soils and accumulate in fresh water and marine sediments (Goodfellow and Cross, 1984).

Recovery and isolation of thermophilic actinomycetes is best at 50°C (Edwards, 1993). The use of traditional microbiological techniques for isolation of thermophilic actinomycetes from overheated substrates, resulted in poor recovery and limited

diversity of actinomycetes colonies due to excessive growth of thermophilic bacteria on isolation plates (Amner, *et al.*, 1988). Amner, *et al.*, (1989) designed an improved medium (R8) for the selective isolation of *Saccharomonospora viridis*. This medium allowed the growth of other thermophilic actinomycete genera as well and reduced the number of bacilli. Kurtböke, *et al.*, (1992) suggested that phage may be used for the selective isolation of actinomycetes. Based on these results, Kurtböke, *et al.*, (1993) concluded that phage susceptibility of thermophilic bacteria proved a selective means of reducing their numbers and thus facilitate the detection and isolation of thermophilic actinomycetes.

Thermophilic actinomycetes have received considerable attention for their potential production of antibiotics. Table 1.3 shows a list of initial antibiotics produced by thermophilic actinomycetes. Lacey, (1988) found that these bacteria play an important role in the decomposition of organic materials at elevated temperatures. As a result, thermophilic actimomycetes have been investigated for the production of a variety of hydrolytic enzymes. Several reports have appeared which indicate that determination of enzymatic profiles of thermophilic actinomycetes can be useful for identification purposes (Waitkins, *et al.*, 1980; Hofstad, 1980). Table 1.4 shows enzymes produced by thermophilic actinomycetes and their properties.

1.7 Thermophilic *Streptomyces*

Early studies, which focused on the ability of a number of *Streptomyces* species to grow at higher temperatures, caused some controversy. Opinions differed as to whether these organisms should be assigned a distinct taxonomy (Craveri and Pagani, 1962) or whether they were merely thermotolerant variants of established mesophilic *Streptomyces* (Corbaz, *et al.*, 1963).

Table 1.3 List of initial antibiotics produced by thermophilic actinomycetes

Organisms	Antibiotics	Reference
Saccharomonospora viridis	Thermoviridin	Schuurmans, *et al.*, (1956)
Strain T-12/3	Antibiotic T-12/3	Kosmachev, (1965)
Streptomyces thermophilus	Thermomycin	Schone, (1951)
Streptomyces thermoviolaceus	Granaticin	Desai and Dhala, (1970)
Thermonospora sp.	T-SA-125	Dewedar, *et al.*, (1979)

Table 1.4 Enzymes produced by thermophilic actinomycetes and their properties

Organisms	Growth Topt (°)	Enzymes	Thermostability	Reference
Streptomyces sp.	55	Protease	up to 60°C	Nakanishi, *et al.*, (1974)
T. vulgaris (A60)	50	Protease	60% at 80°C	Desai and Dhala, (1969)
Thermoactinomyces sp.	50	Carboxypeptidase	40% at 60°C	Osterman, *et al.*, (1984)
Streptomyces thermoviolaceus	57	Protease	up to 50°C	James, (1990)
Streptomyces thermoviolaceus	57	α-amylase	55°C	Goldberg and Edwards, (1990)
Streptomyces EC22	50	Cellulase	65°C	Ball, *et al.*, (1992)
Streptomyces thermoviolaceus	57	Xylanase	up to 70°C	Tsujibo, *et al.*, (1992)

Topt= optimum temperature for growth

Studies by Henssen and Schnepf, (1967) revealed the ability of some *Streptomyces* species to grow above 45°C with optimum temperature for growth between 45 and 65°C. Lacey, (1973) reported that thermophilic *Streptomyces* are widely distributed in nature, particularly in compost heaps and decaying vegetable matter, where temperatures of at least 65°C occur. An extensive numerical phenetic survey of the type strains of *Streptomyces* species, carried out by Williams, *et al.*, (1983), has revealed that many *Streptomyces* are able to grow at 45°C. Goodfellow, *et al.*, (1987) clarified the taxonomy of thermophilic *Streptomyces* by comparing phenetic data from representative strains with corresponding results obtained from marker mesophilic strains examined by Williams, *et. al.*, (1983). These studies proved that thermophilic *Streptomyces* were not merely variants of established mesophilic species but were distinct on the basis of morphology and biochemical properties. Thermophilic *Streptomyces* contrasted with the mesophilic strains in that none produced melanin pigments, they seldom exhibited activity against chitin, guanine, hypoxanthine, testosterone, urea and xanthine, and all failed to grow in the presence of 7% (w/v) NaCl. Also, few thermophilic strains grew at 10°C or used compounds such as D-melibiose, raffinose, salicin and xylitol as sole carbon sources. More recently, Kim, *et al.*, (1996) analysed thermophilic clades within the genus *Streptomyces* by 16S ribosomal DNA sequence comparisons and suggested that the thermophilic streptomycetes do not merit recognition as a distinct taxon within the genus *Streptomyces*. It was suggested that thermophilic *Streptomyces* form at least two phyletic lines which can be distinguished from clades composed of mesophilic streptomycetes. Members of the *Streptomyces thermodiastaticus* clade showed a closer relationship to some of the mesophilic streptomycetes. It is likely, that this species and allied mesophilic strains form a monophyletic group.

1.7.1 *Streptomyces thermonitrificans*

Deshmukh, *et al.*, (1993) reported that a strain of *Streptomyces thermonitrificans* produced a high activity of intracellular glucose isomerase when grown in a medium containing xylose, supplemented with sorbitol as the second carbon source. They found that the addition of Mg^{2+} enhanced enzyme production whereas Co^{2+} had no effect. Subsequent work by Deshmukh, *et al.*, (1994) revealed that partially purified glucose isomerase from *Streptomyces thermonitrificans,* when coupled to glutaraldehyde-activated Indion 48-R, retained 30-40% activity of the soluble enzyme. However, an approximately twofold increase in the activity could be achieved by binding the enzyme in the presence of glucose.

1.7.2 *Streptomyces thermovulgaris*

Revina, *et al.*, (1989) isolated a proteinase from *Streptomyces thermovulgaris* strain T-54 and by testing the substrate specificity of the enzyme against insulin B-chain, ACTH, pike paralbumin TTT and several synthetic peptides, they found that the enzyme was an endopeptidase which cleaves the peptide bonds formed by the alpha-carboxyl group of a glutamic acid residue.

Studies by Khaidarova, *et al.*, (1990) revealed that culture filtrates of *Streptomyces thermovulgaris* contained a proteinase which was active towards azocasein. They reported that the proteinase was completely inactivated by PMSF and DEP inhibitors and by thiol reagents. The enzyme had a pH activity optimum of 7.8-8.2 and a temperature optimum of 55°C.

Yeoman and Edwards, (1994) tested the ability of a number of actinomycetes to produce enzymes when grown on rapemeal derived media. It was found that *Streptomyces thermovulgaris* produced the highest activity of protease. By performing fermentation experiments it was demonstrated that growth was biphasic and protease was produced during the second slower phase. By analysing the protease as

azocaseinase activity, they revealed that the presence of calcium enhanced the thermostability. Additionally, they suggested that *Streptomyces thermovulgaris* produced more than one kind of protease. Subsequent work by Yeoman and Edwards, (1997) attributed the main protease activity to two types of serine protease. A metallo protease component and an azocaseinase component were also present. They concluded that when cell-free supernatant fluid was concentrated using ultrafiltration, rather than acetone precipitation, a higher percentage and a greater range of proteases were recovered. Also, a more diverse range of proteases were produced on rapemeal-derived medium when compared with yeast extract medium.

1.7.3 *Streptomyces thermoviolaceus*

James and Edwards, (1988) reported that *Streptomyces thermoviolaceus* synthesized the antibiotic granaticin as a secondary metabolite. They discovered that growth and granaticin production were supported by different carbon sources. Additional work by James and Edwards, (1989) revealed that the yield of granaticin was greatest at 45°C, whereas the rate of synthesis was most rapid at 37°C.

James, (1990) found that *Streptomyces thermoviolaceus* produced extracellular protease along with granaticin as a secondary metabolite. The amount of each secondary metabolite synthesised was highly dependent on growth rate, pH and temperature. Later work by James, *et al.*, (1991) showed that protease activity was greatest at 50°C and that zinc and manganese could partially reactivate the enzyme activity.

Goldberg and Edwards, (1990) purified and characterised an extracellular α-amylase from *Streptomyces thermoviolaceus* subsp. *apingens*. They reported that the enzyme activity was optimal at a pH of 7.2 and a temperature of 55°C. They also showed that EDTA inhibited enzyme activity and that the enzyme required Ca^{2+} for both activity and thermostability.

Culture filtrate of *Streptomyces thermoviolaceus* OPC-520 was found to secrete two types of xylanases (Tsujibo, *et al.*, 1992). They reported that the optimum pH levels for the enzyme activity was 7.0, whereas the optimum temperature was between 60 and 70°C. Later studies by Tsujibo, *et al.*, (1993) lead to purification of chitinase from *Streptomyces thermoviolaceus* and showed that the enzyme had a high optimum temperature, pH level and heat stability.

Bahri and Ward, (1993) have sequenced an α-amylase encoding gene from *Streptomyces thermoviolaceus* CUB74 cloned in *Streptomyces lividans*. The activity of the α-amylase was strongly inhibited by tendamistat, a potent inhibitor of mammalian α-amylase. The enzyme was stable at 70°C when $CaCl_2$ was present.

Featherstone and Edwards, (1993) used *Streptomyces thermoviolaceus* in order to study the survival of streptomycetes released into mushroom compost. Production of granaticin was used as a marker for identification. Compost amended with different nitrogen sources resulted in reduced recovery rates of released *Streptomyces thermoviolaceus* compared with untreated controls.

Iqbal, *et al.*, (1994) reported that *Streptomyces thermoviolaceus* produced large amounts of extracellular peroxidase activity when grown on xylan. The activity was due to multiple isoforms of peroxidase which were found to be haemoproteins. Finally, they demonstrated that peroxidase activity was optimal at pH close to neutrality and that enzymes exhibited activity at elevated temperatures.

More recently, Brabban and Edwards, (1996) characterised the growth and product formation by *Streptomyces thermoviolaceus*, grown in a glutamate salts medium. By assessing the applicability of a number of intracellular dehydrogenases as indicators for growth in a particulate rapemeal medium, it was suggested that energy metabolism is an important factor in defining the onset of secondary metabolism. They concluded that particulate rapemeal medium also resulted in high yields of commercially important extracellular enzymes.

1.8 *Streptomyces* molecular biology

The study of the molecular genetics of *Streptomyces* has been enhanced by the recent development of methodologies that permit the application of DNA cloning techniques to these organisms (Suarez and Chater, 1980b; Thompson, *et al.*, 1980). *Streptomyces* are quite versatile genetically. Many strains have natural systems of gene exchange through conjugation, a process which requires cell contact and results in the transfer of large segments of chromosome from a donor to a recipient. Generalised transduction is not available for these strains, although Stuttard, (1979) has demonstrated transduction in *Streptomyces venezuelae*. A natural system of competence for transformation is also lacking although artificially promoted recombination through the use of protoplasts is very efficient. Lomovskaya, *et al.*, (1980) concluded that restriction modification systems are widespread in the genus *Streptomyces* and could limit any interspecific gene transfer. Lilly, *et al.*, (1995) established new methods for a single-stranded DNA mediated gene transfer system via transformation, fusion or transduction of *Streptomyces* using a variety of vectors. They found that plasmid shuttle vectors are particularly useful as single-stranded vectors and increase the efficiency of gene transfer into highly restrictive host cell systems.

The isolation and characterisation of DNA regions that regulate gene expression in *Streptomyces* is of particular interest since these organisms contain DNA having an extremely high G+C content. The isolation of transcriptional control signals in *Streptomyces* can provide an opportunity for analysis of regulation of gene expression in an organism that undergoes both morphological differentiation and regulation of secondary metabolism. Positive regulatory genes affecting enzyme synthesis might be cloned by searching for increased secretion of extracellular enzymes in a poor producer such as *Streptomyces lividans*, which is used extensively as a host for cloning and

expressing genes from other *Streptomyces* species (Bahri and Ward, 1993). For efficient transformation, protoplasts and a suitable vector are required.

1.8.1 Protoplasts

Several investigators have examined the formation of *Streptomyces* protoplasts (Bradley, 1959; Kochkina and Rautenshtein, 1968). Sagara, *et al.*, (1971) concluded that mycelia from *Streptomyces griseoflavus*, grown in a high glycine containing medium, were sensitive to lysozyme action and that the protoplasts formed lost mesosomes. Okanishi, *et al.*, (1974) reported the cultural conditions for preparing stable *Streptomyces* protoplasts and for reverting them to the filamentous state at high frequency on the surface of synthetic agar plates. They suggested that effective transformation and stabilisation of the protoplasts was accomplished by using a hypertonic medium containing $MgCl_2$ and $CaCl_2$. A high reversion rate was obtained by incubating the protoplasts on a hypertonic agar medium containing $MgCl_2$, $CaCl_2$, phosphate and casaminoacids. Bibb, *et al.*, (1978) reported that polyethylene glycol (PEG) enhanced the transformation frequency of protoplasts. More recently, Anné, *et al.*, (1990) during investigations using *Streptomyces venezuelae* established the required conditions for efficient transformation of *Streptomyces* species. They concluded that factors such as culture age, medium composition, temperature, lysozyme and glycine concentration and type of PEG can be critical for efficient formation, transformation and regeneration of *Streptomyces* protoplasts.

1.8.2 Vectors

Work by various workers (Okanishi, 1979; Chater, 1979; Hopwood, 1978) lead to the identification of plasmids in many *Streptomyces* species which were implicated in the control of a range of phenotypic properties. A distinctive property of many *Streptomyces* plasmids is their capacity to give rise to a visible reaction in plate

cultures when a strain, bearing a plasmid, grows in contact with a strain lacking the corresponding plasmid and transfers its plasmid to the recipient strain.

Bibb, *et al.*, (1978) reported the development of a plasmid transformation system for *Streptomyces* which allowed the cloning of any DNA sequence into these organisms. The system involved the uptake of covalently closed circular DNA by protoplasts and the visual detection of transformants, at high resolution, after regeneration of the protoplasts. Bibb, *et al.*, (1980) reported that two groups of plasmids were potentially suitable for the construction of cloning vectors for *Streptomyces*: a) SCP(2), first isolated from *Streptomyces coelicolor* A3(2) and b) the SLP1 series of plasmids isolated from *Streptomyces lividans.* Bibb and Cohen, (1982) developed a series of promoter-probe plasmid vectors in order to study gene expression in *Streptomyces.* The use of these vectors have indicated considerable promoter heterogeneity (Janssen, *et al.*, 1985). Ward, *et al.*, (1986) constructed and characterised a series of multi-copy, promoter-probe plasmid vectors for *Streptomyces.* These plasmids derived from a broad host range plasmid, pIJ101 that replicates in most *Streptomyces* species tested (Kieser, *et al.*, 1982), and have a wide application. The availability of such versatile vectors permit the rapid isolation and characterisation of promoter sequences from a range of *Streptomyces* genes and species.

Cloning and analysis of transcriptional signals is greatly facilitated by the availability of promoter-probe vectors and the subsequent ability to isolate and characterise regions of DNA involved in the initiation of transcription. There are a few promoter-probe plasmids which have been developed for the study of gene expression in *Streptomyces.* Asturias, *et al.*, (1990) developed a bifunctional *Streptomyces-E. coli* promoter-probe vector, pULJA30, in order to isolate and characterise nucleotide sequences involved in transcription initiation and regulation. Important features of this new vector include: wide *Streptomyces* host range and a high plasmid copy number.

Plasmid DNA can be introduced into *Streptomyces* cells by polyethylene-glycol (PEG) assisted transformation of protoplasts. In many studies the conditions and procedure as described for *Streptomyces lividans* (Hopwood, *et al.*, 1981) have been applied. Parameters that increase transformation efficiency were adjusted to generate reproducibly a population of cloned genes likely to represent the entire genome. Thompson, *et al.*, (1982) concluded that factors which influence the recovery of variable transformants include: growth phase of the mycelium, ionic and osmotic characteristics of the medium during protoplast formation and transformation and moisture content and protoplast density during regeneration.

Plasmid transformants can be detected by selecting for the expression of plasmid phenotype expression. *Streptomyces* plasmids such as pIJ702 and pIJ486 (Katz, *et al.*, 1983; Ward, *et al.*, 1986) carry the resistance gene for thiostrepton which has been extensively used as selective resistance marker. van-Wezel and Bibb, (1996) described an *Escherichia coli* plasmid, pIJ2581, that can be used for the efficient construction of stable gene disruptants and of gene deletions in *Streptomyces.* Integration of pIJ2581 derivatives carrying chromosomal sequences is achieved by selecting for plasmid-encoded thiostrepton resistance. Wehmeier, (1995) constructed four new shuttle vectors for *Escherichia coli* and *Streptomyces,* pUWL218, pUWL219, pUWL-SK and pUWL-KS, which permit recognition of recombinant plasmids on XGal plates in *Escherichia coli.* These vectors possess multiple cloning sites with a number of unique restriction sites and allow direct sequencing of re-derivatives using the pUC sequencing primers.

Dyson and Evans, (1996) have constructed a new *Streptomyces-Escherichia coli* shuttle vector, pUCS75. In contrast to other commonly used shuttle vectors, pUCS75 retains the primary site for second-strand synthesis of the parental replicon, pIJ101. This sequence can not only enhance structural stability of the plasmid, but also confers on it an elevated copy number when replicated in *Streptomyces.*

Most of the cloning vectors for the genetic analysis of *Streptomyces* are plasmids but several are derived from temperate bacteriophages (Harris, *et al.*, 1983). Those derived from φC31 have so far found most use (Rodicio, *et al.*, 1985). φC31 forms plaques on a wide range of *Streptomyces* many of which it also lysogenises. Because of mycelial growth which results in culture containing cells of diverse morphological and physiological state, some unfavourable for phage multiplication, *Streptomyces* phages usually lyse only a part of a culture before phenotypically resistant cells arise (Hopwood, *et al.*, 1973). However, true lysogeny has been demonstrated in many *Streptomyces* (Lomovskaya, *et al.*, 1972). Many *Streptomyces* bacteriophages display a broad host specificity that is limited primarily by the expression of restriction barriers in particular hosts (Hahn, *et al.*, 1990). *Streptomyces* that propagate a high percentage of *Streptomyces* bacteriophages tend to be much less restricting for plasmid transformation than those that propagate a smaller percentage of bacteriophages (Matsushima and Baltz, 1996).

1.9 Hydrolytic enzymes

One of the major advantages of Gram-positive microorganisms is their ability to secrete proteins into the culture medium. In most cases secretion into the culture medium significantly simplifies the purification of products (Aretz, *et al.*, 1989). Goldberg and Dice, (1974) showed that some enzymes synthesized by bacterial cells are subjected to turnover in the same way as mammalian cell proteins. As a result, Guijarro, *et al.*, (1983) concluded that bacterial proteins are not completely stable in growing microorganisms. Protein degradation is dependent on the activity of proteolytic enzymes. Laluce and Molinari, (1977) suggested that the secretion of proteolytic enzymes is an active process associated with all phases of microbial growth and especially active during the logarithmic phase.

1.9.1 Hydrolytic enzymes from *Streptomyces*

The actinomycetes are well known producers of a variety of extracellular enzymes including proteases, xylanases, cellulases, amylases, lipases, nucleases and phosphatases. Williams, *et al.*, (1983) reviewed the ability of *Streptomyces* species to produce a range of industrially important metabolites and extracellular proteins. Several genes of *Streptomyces* have been cloned which encode extracellular enzymes. These include agarase from *Streptomyces coelicolor* (Bibb, *et al.*, 1987), endoglucosidase H from *Streptomyces plicatus* (Robbins, *et al.*, 1981), xylanase from *Streptomyces lividans* (Mondou, *et al.*, 1986), α-amylase from *Streptomyces hygroscopicus* (Hoshiko, *et al.*, 1987), β-galactosidase from *Streptomyces lividans* (Eckhardt, *et al.*, 1987) and β-lactamases from *Streptomyces cacaoi, badius* and *fradiae* (Jaurin, *et al.*, 1988). Expression of genes encoding extracellular enzymes such as agarase and chitinase, is glucose repressible at the transcriptional level (Virolle and Gagnat, 1994).

Among the enzymes produced by *Streptomyces*, proteases have been the subject of much research (Zlotnik, *et al.*, 1984). Gibb, *et al.*, (1989) reported that *Streptomyces* C5-A13 produce an extracellular protease activity during the stationary phase which is an effect related to growth rate. Carbonate anions were found to stimulate the production of extracellular protease activity. The optimal concentration of soluble carbonate was 60-80mM and the stimulation by carbonate was shown not to be due to a pH effect. Renko, *et al.*, (1989) isolated a trypsin-like proteinase from *Streptomyces rimosus* culture filtrates. The enzyme was stable from pH 4.5 to 9.0 and up to 40°C, and contained three disulphide bridges, three histidines and three methionines per molecule. Kang, *et al.*, (1995) identified three proteases, a chymotrypsin-like (CTP), a trypsin-like (TLP) and a metalloprotease (MTP) in cultures of *Streptomyces albidoflavus* SMF301. The dynamics of protease production were determined and the roles of the proteases in morphological differentiation were deduced as follows: CTP is

essential for hydrolysing the proteinaceous nitrogen source for mycelium growth; TLP plays a role in the formation of aerial mycelium in solid culture and MTP may participate in the maturation of the spores. Nasser and Foda, (1995) screened the grey series of *Streptomyces* species for their ability to produce extracellular xylanases and found that enzyme biosynthesis was favoured in media with initial pH values around neutrality. They also revealed that high levels of xylanase activity were formed in the presence of different organic and inorganic nitrogen sources and polysaccharides such as lactose, galactose and mannose. More recently, Mori, *et al.*, (1996) reported the detection and preliminary characterisation of microbial proline 3-hydroxylase activities, in whole cells of *Streptomyces* sp. strain TH1, which hydroxylate free L-proline to free cis-3-hydroxy-L-proline. The reaction was inhibited by Co^{2+}, Zn^{2+}, and Cu^{2+} and accelerated by L-Ascorbic acid.

1.9.2 Hydrolytic enzymes from thermophilic organisms

Lauwers, *et al.*, (1981) concluded that in obligate thermophiles the stability of enzymes is independent of the growth temperature within the range of 60 to 100°C. Facultative thermophiles were found to produce heat sensitive enzymes when growing in the mesophilic region but thermostable enzymes at higher temperatures. Heinen and Lauwers, (1983) examined the thermostability of several enzymes from a facultative thermophilic actinomycete *Streptomyces* sp. and suggested a correlation between the growth temperature of the cultures and the heat stability of the enzymes.

Ball, *et al.*, (1992) investigated cellulase-related activities of ten actinomycete strains against *p*-nitrophenyl β-D-cellobioside (pNPC) and found the activity detected in the thermophilic *Streptomyces* EC22 was the highest. Culture supernatant studies revealed that pNPC activity was optimal at 65°C and between pH 6.0 and 8.0.

Takami, *et al.*, (1989) found that alkalophilic *Bacillus* sp. produced an extremely thermostable alkaline protease which was stable to 10 mins incubation at 60°C. The

enzyme was stabilised with the addition of calcium ions especially at higher temperatures. In addition, the protease was completely inactivated by PMSF, but little affected by EDTA and SDS.

There are examples of enzymes purified from organisms that grow near and above 100°C. These include: proteases, amylolytic-type enzymes, hydrogenases, redox proteins, various ferredoxin-linked oxidoreductases, dehydrogenases and DNA polymerases, some of which are active up to 140°C (Adams, 1993). Hei and Clark, (1994) suggested that pressure stabilisation of an enzyme may be related to its thermophilicity and concluded that hydrophobic interactions, which have been implicated in the stabilisation of many thermophilic proteins, contribute to the pressure stabilisation of enzymes from thermophiles.

1.10 Lipases

Lipases are defined as glycerol ester hydrolases (EC 3.1.1.3) hydrolyzing tri, di and monoglycerides present at an oil-water interface (Vorderwülbecke, *et al.*, 1992). Studies by Jensen, *et al.*, (1983) on the specificity of lipases, showed that these enzymes vary in their catalytic properties and substrate specificity and enzymes from various sources can be used to catalyse specific reactions. Lipases are widely distributed in vertebrates (Gargouri, *et al.*, 1989), plants (Huang, 1990) and microorganisms (Sugiura, 1984). They hydrolyse oils and fats to glycerol and free fatty acids, carbon and energy sources that can easily be utilised by many organisms. Jaeger, *et al.*, (1994) concluded that the kinetics of lipase reactions do not follow the classical Michaelis-Menten model, since there is a sharp increase in lipase activity observed when the substrate starts to form an emulsion. An important feature of lipases is that they are able to catalyse a heterogeneous reaction in which the water-soluble enzyme interacts with the insoluble triglyceride substrate at the interface of the aggregated

substrate and water (Gowland, *et al.*, 1987). A number of assays to determine lipase activity have been developed. A summary of currently used methods is given in Table 1.5 (Jaeger, *et al.*, 1994).

There is an increased interest in lipases from different sources (microorganisms, animals and plants) due to their potential applications in industry and in medicine. Microbial and mammalian lipases have been purified to homogeneity, allowing the successful determination of their primary amino acid sequence and three dimensional structure (Taipa, *et al.*, 1992).

1.10.1 Microbial lipases

Microbial lipases are mostly extracellular in nature and in some cases are inducible by the inclusion of lipid substrates in the growth media (Jaeger, *et al.*, 1994). Filamentous fungi are also well exploited for production of lipases, since they produce many extracellular enzymes. A review by Godtfredsen, (1990), suggested that the genera *Rhizopus, Rhizomucor, Mucor, Geotrichum, Aspergillus and Penicillium* are the most productive species. Lipases from several *Penicillium* strains have been purified and characterised (Oi, *et al.*, 1967; Maliszewska and Mastalerz, 1992). Stöcklein, *et al.*, (1993), purified an extracellular lipase from *Penicillium expansum* DMS 1994, when grown in a medium containing 0.1% olive oil, and identified it as a glycoprotein with a molecular weight of 25KDa. The enzyme formed active dimers and showed maximum activity at pH 9.0 and 45°C. The lipase was also able to hydrolyse various oils, as well as mono, 1,2 and 1,3 diolein and triolein. Nakano, *et al.*, (1995) esterified glycosides by a mono and diacylglycerol lipase from *Penicillium camembertii.* The lipases formed two monoesters; a major monoester whose trimethylolpropane moiety was esterified and a minor monoester bearing an oleic acid group at the glucose moity. Phillips and Pretorius, (1991), purified and characterised an extracellular lipase from *Galactomyces geotrichum* with a relative molecular weight of 57KDa and an optimal

Table 1.5 Lipase activity assay methods (Jaeger, *et al.*, 1994)

Assays	Reaction product	Method
Plate	Free fatty acids	Coloured indicators
Spectroscopic	Mostly free fatty acids	Enzymatic conversion
Fluorescence	Free fatty acids or aggregated substrate	Complex formation or fluorescence shift
Titrimetric	Free fatty acids	pH determination
Surface pressure	Free fatty acids	Measurement of drop in volume or decrease in surface tension

activity at pH 7.5 and 30°C. Papaparaskevas, *et al.*, (1992) suggested that production of extracellular lipase by the yeast *Rhodotorula glutinis* was substantially enhanced when the type and concentration of carbon and nitrogen source, the initial pH of culture medium and the growth temperature were consecutively optimised. El-Sawah, *et al.*, (1995) studied some properties of an extracellular lipase produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* and found that maximum enzyme activity was observed against olive oil and butter oil as enzyme substrates. Maximum lipase production was reached in the presence of glucose as sole carbon source and olive oil as sole lipid source. More recently, Macris, *et al.*, (1996) studied an extracellular lipase from *Aspergillus niger*. The enzyme was produced at elevated activity levels under optimum growth conditions. Biosynthesis of lipase occurred only in the presence of lipids and was completely repressed by glucose and glycerol.

A review by Jaeger, *et al.*, (1994) on bacterial lipases revealed that the synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors such as ions, carbon sources or presence of non-metabolizable polysaccharides. It also showed that a comparison of different bacterial lipases, on the basis of primary structure, revealed only a very limited sequence homology. Finally, it has been demonstrated that with only few exceptions, bacterial lipases are able to completely hydrolyse a triacylglycerol substrate although a certain preference for primary ester bond has been observed.

Gilbert, *et al.*, (1991) purified an extracellular lipase from a Tween-80-limited continuous culture of *Pseudomonas aeruginosa* EF2. They found that the lipase was composed of a single subunit which exhibited both lipase and esterase activity. They also showed that lipase activity was not inhibited by the chelating agent EDTA.

Brune and Götz, (1992) reviewed the biochemical properties of lipases produced by *Staphylococcus aureus* and *Staphylococcus hyicus*. These lipases have a broad substrate specificity, including the ability to hydrolyse water-soluble substrates. The

Staphylococcus hyicus enzyme also exhibited phospholipase activity. Talon, *et al.*, (1995) purified an extracellular lipase from *Staphylococcus warneri* which was secreted as a protein with a molecular mass of 45 KDa. Purified lipase had an optimum pH of 9.0 and an optimum temperature of 25°C and correlated with growth. Lipase activity was strongly inhibited by cobalt, zinc and EDTA, whereas calcium and manganese enhanced it. The results of inhibition studies were consistent with the view that lipases possess a serine residue at the catalytic site.

1.10.2 Lipases of Streptomyces origin

Peczynska-Czoch and Mordarski, (1988) suggested that more attention should be paid to lipases from actinomycetes and *Streptomyces* since these microorganisms are known for their capacity to produce various secondary metabolites and hence provide a potential source of enzymes of atypical substrate specificity. Sztajer, *et al.*, (1988) demonstrated that some *Streptomyces* species can be highly lipolytic. Perez, *et al.*, (1993) managed to clone a gene encoding an extracellular lipase from *Streptomyces* sp.M11 using the high-copy-number vector pIJ486 and *Streptomyces lividans* 66 as host and found that the gene is most likely monocistronic. The lipase gene promoter was found to be similar to other *Streptomyces* vegetative promoters. Cruz, *et al.*, (1994) cloned and sequenced an extracellular lipase encoding gene from *Streptomyces albus* G. The sequence revealed amino acid substitutions which might be responsible for the greater thermal stability of the *Streptomyces* lipases.

1.10.3 Lipase-producing thermophilic bacteria

Gowland, *et al.*, (1987) isolated a number of lipase-producing thermophilic bacteria from natural habitats. One of the isolates, a highly thermophilic *Bacillus* sp. produced the maximum level of lipase. They concluded that lipase synthesis was inducible and subject to catabolite repression. Sigurgisladottir, *et al.*, (1993) examined the lipase

activity of thermophilic bacteria from Icelandic hot springs and found that activity was higher at 40°C and 60°C than at 80°C. It was concluded that the thermophile lipase is more thermostable than the commercial lipases derived from mesophilic sources. Salleh, *et al.*, (1993) investigated the parameters affecting the production of extra and intracellular lipases from a thermophilic *Rhizopus oryzae* and found that all carbon sources tested, with the exception of sucrose, inhibited the production of extracellular lipase but enhanced the production of intracellular lipase. The temperature optima were 45 and 37°C for extra and intracellular lipases respectively. Handelsman and Shoham, (1994) isolated and characterised a thermostable lipase from a thermophilic *Bacillus* sp. and found that the enzyme had a molecular weight of 20KDa and was most active at pH 7.0 at 70°C. The lipase had no apparent requirement for cofactors and its activity was completely inhibited in the presence of 1mM HgCl sub(2). Ushio, *et al.*, (1996) discovered a new thermostable extracellular lipase from *Pseudomonas* sp. NT-163 and suggested that additives such as stearyl, palmityl and oleyl alcohols were highly effective as lipase inducers. More recently, Khyami-Horani, (1996) found that a thermotolerant variant of *Bacillus licheniformis* strain H1 (isolated from Jordan valley soil) was highly active in degrading macromolecules and possessed a lipase activity with half life of 30 mins at 70°C. The activity was produced during exponential growth.

While considerable data on proteases, carbohydrases and other enzymes of unusual properties have accumulated, there is little information regarding the properties of lipases in thermophilic *Streptomyces* species.

1.10.4 Application of lipases

The economic importance and application of lipases has increased in recent years. Each application requires specific properties of the lipases with respect to substrate specificity, stability, temperature and pH-dependence of their ability to catalyse ester

synthesis reactions in organic solvents and in superficial fluids (Stöcklein, *et al.*, 1993). The potential of lipases for industrial application has been reviewed by Harwood, (1989). These applications include the synthesis of food ingredients, pharmaceuticals, agrochemicals, fragrances and flavours and their use as additives to detergents. Winkler, *et al.*, (1990) suggested that lipases can also be subject to structural studies and protein design. Recently, many new potential applications of lipases have been proposed and since the methods for lipase production have been improved, some of these new uses will be economically viable. A review by Björkling, *et al.*, (1991) showed that lipases can be used in: a) modification of fats and oils, b) detergent industry, c) paper manufacturing, d) organic chemical processing, e) new products which are environmentally more acceptable due to a very rapid biodegradation and low toxicity. Some of the biotechnological applications of bacterial lipases are shown in Table 1.6.

The importance of heat stable lipases in biotechnology is growing rapidly and at the present time a large number of lipases are commercially available (Ison, *et al.*, 1990). An important factor for increasing the rate of reaction of the various applications where lipases are involved is temperature. This requires the use of enzymes from thermophilic bacteria which are expected to produce more heat stable enzymes (Gowland, *et al.*, 1987). The availability of commercial lipases from thermophilic bacteria is of great importance since they are more tolerant to higher substrate (reactant) concentrations and more resistant to denaturing agents.

1.11 Esterases

Esterases are enzymes which catalyse the hydrolysis of a large number of aliphatic and aromatic esters and are widely distributed in various living organisms. Esterases belong to the large enzyme family of serine-hydrolases, comprising various proteases,

Table 1.6 Bacteriological applications of bacterial lipases

Type of reaction	Origin of lipase	Product (application)	Reference
Glycerolysis of fats and oils	*Pseudomonas*	Monoacylglycerols	Yang, *et al.*, (1993)
Acylation of sugar alcohols	*Chromobacterium viscosum*	Sugar monoacylesters	Chopineau, *et al.*, (1988)
Resolution of racemic alcohols	*Arthrobacter* sp.	Building blocks for chiral drugs	Margolin, (1993)
Intramolecular esterification	*Pseudomonas* sp.	Macrocyclic lactones	Makita, *et al.*, (1987)
Polytransesterification of diesters	*Chromobacterium* sp.	Oligomers	Margolin, *et al.*, (1987)
Esterification to glycerol	*Chromobacterium viscosum*	----------------------------	Osada, *et al.*, (1990)

lipases, esterases and other hydrolases. They are characterised by the presence of a catalytic triad in their active site (Brady, *et al.*, 1990) responsible for the hydrolysis of ester or amide bonds. Ollis, *et al.*, (1992) identified the presence of a serine consensus motif in esterases and suggested that this enzyme is a typical serine hydrolase which performs hydrolysis of ester bonds after nucleophilic attack of the hydroxyl group from the active serine residue. The economic importance and application of esterases has increased in recent years (Janssen, *et al.*, 1994). These enzymes vary in their catalytic properties and substrate specificities and enzymes from various sources can be used to catalyse specific reactions (Macrae, *et al.*, 1983). Esterases have generally be shown to have dimeric or monomeric structures. Studies on different microorganisms have shown that multiple forms of esterases are usually present and that esters are normally split not by one but several enzymes (Lambrechts, *et al.*, 1995).

1.11.1 Esterases of microbial origin

Only a few studies have been made into the properties of isolated esterases of microbial origin (Parkkinen, 1980; Lee and Lee, 1989; Tsakalidou and Kalantzopoulos, 1992). There are cases where esterases produced by microorganisms such as baker's yeast (Toshimitsu, *et al.*, 1986) are considered as intracellular enzymes whereas there are microorganisms which produce extracellular enzymes (Kugimiya, 1988). The specific activities of esterases indicate that the electrophoretic variations of these enzymes in bacterial populations are the result of allelic variations at specific gene loci. Esterases can distinguish bacteria at the species or subspecies level, both by their biochemical properties and by their electrophoretic differences (Goullet and Picard, 1995). Hespell and O'Bryan-Shah, (1988) examined esterase activities in *Butyrivibrio fibrisolvens* strains and found that the activity was predominantly cell associated and increased with cell growth until the stationary growth phase was reached, after which the activity remained stable. Also, it was concluded that the addition of magnesium and

manganese chloride generally had no effect on the activity whereas, when sodium EDTA was added, a loss of esterase activity was observed.

Kugimiya, *et al.*, (1992) expressed an esterase gene from a thermophilic bacterium, *Bacillus stearothermophilus* IFO12550, in *Bacillus brevis* and found that the esterase was characterised by high thermostability and a preference for triglycerides with short-chain fatty acids rather than long-chain ones. Shao and Wiegel, (1995) purified and characterised two thermostable acetyl xylan esterases from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an anaerobic thermophilic endospore former, and found that the esterase had molecular masses of 195 and 106 KDa respectively. Their optimal activity occurred at pH 7.0 and 80°C and at pH 7.5 and 84°C respectively. Donaghy and McKay, (1995) determined extracellular esterase production by *Penicillium expansum, Penicillium brevicompactum* and *Aspergillus niger* in both liquid and solid-state culture. Extracted enzyme for each organism showed activity in the presence of methyl ferulate and methyl coumarate. More recently, Tsuboi, *et al.*, (1996) found an extracellular esterase from *Candida albicans* A-714 which was induced in a medium containing yeast nitrogen base and Tween 80. The induced esterase was heat labile and had maximum activity at pH 5.5. Enzyme activity was stimulated by the addition of sodium taurocholate, an activator of lipase.

1.11.2 Esterases of *Streptomyces* origin

Streptomyces species have been found to produce extracellular esterases. Lin and Kolattukudy, (1980) reported on an esterase of *Streptomyces scabies* which acted as a cutinase. MacKenzie, *et al.*, (1987) have reported the presence of an esterase that releases ferulic acid from wheat bran in *Streptomyces olivochromogenes.* McQueen and Schottel, (1987) purified and characterised an extracellular esterase produced by *Streptomyces scabies* that was involved in the breaking down of suberin. The characteristics of the esterase produced were heat stability and extracellular location. In

addition, esterase production was induced by the presence of Zn^{2+}. Deobald and Crawford, (1987) have shown that *Streptomyces viridosporus* produces an extracellular coumarate ester esterase that was involved in hydrolysing esterified acids from lignin. Donnelly and Crawford, (1988) reported that this enzyme was unstable above 40°C but retained activity when stored at 4 or -20°C. They concluded that the enzyme was a component of the lignocellulose-degrading enzyme system of *Streptomyces viridosporus* and that esterase activity resulted from activities of several isoenzymes. Magnuson and Crawford, (1992) screened peroxidase-deficient mutants of *Steptomyces viridosporus* T7A, for their production of acid precipitable polymeric lignin, extracellular peroxidase and esterases against a polyclonal antibody. Their findings indicated a possible role of esterases in lignin solubilization and degradation.

Hale, *et al.*, (1992) cloned and expressed an esterase gene from *Streptomyces scabies* FL1 in *Streptomyces lividans* using plasmids pIJ486 and pIJ702. They found that the esterase gene was expressed during the later stages of growth and was regulated by zinc. Nishimura and Sugiyama, (1994) found that *Streptomyces lavendulae* H646-SY2 produced cholesterol esterase extracellularly. By cloning and sequencing a gene encoding the cholesterol esterase, a putative signal sequence for secretion was found.

1.12 Protein biosynthesis and secretion by *Streptomyces*

Early investigations of protein turnover in bacteria suggested that bacterial proteins were very stable or turned over extremely slowly (Guijarro, et al., 1983). Studies on protein secretion in bacteria showed that the intracellular precursors of the exported proteins contain a N-terminal peptide chain called signal sequence which is essential in the translocation of the protein. While these mechanisms of secretion have been well established in various organisms, they are not as well documented in *Streptomyces*.

In the last few years, secretion of both homologous as well as heterologous proteins from *Streptomyces* altered by genetic engineering techniques has been investigated in several laboratories. In Gram-positive bacteria such as *Streptomyces*, proteins are secreted directly into the surrounding environment, whereas in Gram-negative organisms they are generally retained in the periplasmic space of the cell walls (Kluepfel, 1991). The advantages in producing proteins in their secreted form are significant. It allows easy recovery of the enzymes and separation from all intracellular proteins. In *Streptomyces thermoviolaceus* protein secretion appears to be linked to, or a component of, secondary metabolism (James, *et al.*, 1991). Their data provided strong support that protein secretion is not occurring through some cultural perturbation and may be linked to antibiotic production.

1.13 Aims of the project

• To test the ability of thermophilic *Streptomyces* species to grow on a range of lipid based media.

• To study the activity of lipase from these cultures and identify their properties with respect to thermotolerance, sensitivity to inhibitors, sensitivity to a series of divalent metals and pH.

• To obtain detailed information concerning growth patterns and enzyme production by performing a series of fermentation experiments.

• To purify the lipase produced.

• To clone the lipase genes into a mesophilic recipient and purify the cloned gene product.

2.1 Organisms used

Five thermophilic Streptomyces species, from the University of Liverpool, School of Biological Sciences, stock collection, were used in preliminary experiments: *Streptomyces thermovulgaris, S.thermoflavus, S.thermonitrificans, S.thermoviolaceus* and *S.thermodiastaticus. Streptomyces lividans,* a mesophilic streptomycete, was also used in some experiments.

2.1.1 Maintenance of *Streptomyces species*

Streptomyces species were maintained on complex solid media that contained (g/l): glucose, 4; malt extract, 10; yeast extract, 4. All the above chemicals were obtained from DIFCO. Trace element solution was added at 1ml/l and 1 litre of this contained 5g of citric acid and 1g of each of the following: $MnCl_2$, $ZnCl_2$ and $FeSO_4$. Citric acid was included in the trace element solution in order to chelate the metals and prevent precipitation. The pH was adjusted to 7.2 with 20% (w/v) NaOH and 20g of agar (Lab M No.2) added for preparation of solid media.

After 48-72 hours incubation on agar at 50°C, mature spores were resuspended in 30% (v/v) glycerol and stored at -70°C. Every 3-4 weeks further stock cultures were raised from stock spore suspensions.

2.1.2 Growth of *Streptomyces species*

The *Streptomyces* species were grown in defined salts media which contained (g/l): MOPS (3-[N-morpholino] propanesulphonic acid), 5.23; $(NH_4)_2SO_4$, 2; $MgSO_4$ $7H_2O$, 1; K_2HPO_4, 1; $CaCl_2$ $2H_2O$, 0.05 and 17.5g of an appropriate carbon source (olive oil, vegetable oil, Tween 80, Tween 40 or Tween 60). Basic grade, Tesco own brand olive oil

and vegetable oil were obtained from Tesco Store Ltd., UK. 1ml/l trace element solution that contained 1g/l of: $MnCl_2$ $4H_2O$, $ZnSO_4$ $7H_2O$, $FeSO_4$ $7H_2O$ and 5g/l citric acid was added (James and Edwards, 1989). MOPS was used in the solution to act as a buffer in media to restrict pH changes during growth. The resultant solution was adjusted to pH 7.0 prior to autoclaving. Inocula were raised from spores by adding 1ml of spore suspension to 100ml of salts medium in 250ml flasks which were incubated for 48h at 50°C and 200rpm in an orbital shaker (New Brunswick Scientific, Edison, N.J., USA). In the case of *Streptomyces lividans,* incubation was at 30°C.

2.1.3 Growth in fermenter

Large scale batch culture was carried out in a LH500 series fermenter with a 2L vessel with a working volume of 1500ml. The temperature was maintained at 30 or 50°C by using a heating/cooling finger through which heated water from a water bath was circulated. Temperature control used a thermistor linked to a temperature control unit and a 100W electrical heater within the medium. The pH was controlled to 8.0 by using a pH probe connected to a pH control unit which regulated acid (0.1M HCl) and alkali (0.1M NaOH) addition (Mercer, 1994). 1ml of spore suspension was used to inoculate the defined salts medium in flasks (100ml in 250ml) which, after growth at 30°C for 20h or 50°C for 16h with shaking, was used to inoculate the fermenter at the appropriate temperature. In order to minimise wall growth by attached mycelium: 1) the internal vessel surfaces were coated with dichloromethylsilane and 2) a magnetic follower was included inside the vessel and periodically moved around by a powerful magnet held on the external surface to scrap off any attached mycelia (James, *et al.*, 1991).

Throughout the fermentation, samples were removed by means of a stainless steel sampling hood. This could be connected to a wide array of sample vessels via a universal

screw thread. A side arm on the hood allowed fitting to a 100ml syringe. Sampling was carried out by opening the clipped sampling line and drawing the required volume of sample into the connected bottle by using the syringe. The line was then flushed with air and the sample bottle changed aseptically.

2.2 Growth measurement

Growth was monitored by estimating dry weights in culture samples (35ml) that were harvested and pelleted by centrifugation at 1800 *g* for 10 mins. The pellet was washed with water, resuspended in 5ml of distilled water and placed in a pre-dried, pre-weighed foil cap and dried to constant mass at 105°C in an oven.

2.3 Qualitative assay of lipid hydrolysis using agar-plates

Hydrolytic activity of streptomycetes was measured using agar plates that contained: 0.5M phosphate buffer, 10ml/l; $CaCl_2$, 2.8g/l; agar, 20g/l and olive oil, 20g/l. The agar was poured into petri dishes and, after cooling, wells (0.5cm in diameter) were cut into which culture supernatant samples were loaded. Precipitation of free fatty acids with calcium (white zones) was used as an indicator of hydrolytic activity after 2 hours at 70°C (Sigurgisladottir, *et al.*, 1993) which was detectable as clear zones around the well perimeter.

2.4 Lipase assay

Lipase activity was detected spectrophotometrically (Stöcklein, *et al.*, 1993) using p-nitrophenyl palmitate as the substrate (Winkler and Stuckmann, 1979). The assay contained: isopropanol, 4ml; p-nitrophenyl palmitate, 12mg; 0.05M phosphate buffer (pH 8.0), 36ml and sodium deoxycholate, 828mg. Aliquots of this freshly prepared medium (800μl) were prewarmed at 37°C. Cell free supernatant fluid from *Streptomyces* cultures were pre-heated at 60, 70, 80 and 90°C for a period of 80 minutes and 50μl samples were removed at time intervals of 5 minutes, stored on ice and used to assay lipase activity. The reaction was stopped by adding 100μl of ethanol and 50μl of 2M NaOH was added to develop the colour. Lipase activity was measured at OD_{410}. An enzyme free assay mix treated in the same way was used as control. One enzyme unit is defined as 1nmol of p-nitrophenol enzymatically released from the substrate/ml/min. Under the conditions described the extinction coefficient of p-nitrophenol is E_{410}: 15000cm^2/mg.

2.5 pH profile

In order to find the optimum pH for lipase activity the assay was performed at various pH values ranging from 2 to 10. To obtain the required pH values the following buffers (50mM) were used: sodium citrate-HCl (pH 2-3), acetic acid-NaOH (pH 4-5), potassium dihydrogen phosphate-NaOH (pH 6-7), Tris-HCl (pH 8-9) and glycine-NaOH (pH 10).

2.6 Protein estimation

Protein was measured by the method of Lowry, *et al.*, (1951) using Folin-Ciocalteau phenol reagent (BDH). Bovine serum albumin (BSA) was used as standard at concentrations of 0 to 200µg/ml.

2.7 Intracellular lipases

Mycelia of *Streptomyces thermoviolaceus* and *thermodiastaticus* grown at 50°C for 24 and 48 hours, were washed and resuspended in 50mM Tris-HCl pH 8.0 buffer. Intracellular lipases were released by disruption of the cells with an MSE sonicator using three 1 min bursts at an amplitude of 16um peak to peak with cooling between each application for 1 min on ice. Broken cells were pelleted at 12000 *g* for 10 mins at 4°C and supernatant was collected. Lipase activity was determined using the p-nitrophenyl palmitate assay as described before.

2.8 Effect of metals and inhibitors

In order to determine the effects of divalent metals and inhibitors on lipase activity, various experiments were performed in which metals such as Ca, Mg, Cu, Zn, Mn and Fe (in the form of chloride salts and at a final assay concentration of 4mM) and inhibitors such as EDTA, o-phenanthroline, PMSF, TLCK, PCMB and Leupeptin (all at a final assay concentration of 5mM) were added to cell free culture supernatant samples before assaying lipase activity using the spectrophotometric assay described above.

2.9 Concentration of lipase activity from cell-free culture supernatants

The following methods were used to achieve concentration of lipase activity from cell free culture supernatants:

2.9.1 Acetone precipitation

A mixture of culture supernatant and acetone was kept at -20°C for 5h. The resulting precipitate was centrifuged at 12000 *g* for 15 mins at 4°C and the pellet was resuspended in 50mM Tris-HCl buffer (pH 7.5) to one fifth of the starting volume. Lipase activity was measured by using the spectrophotometric assay at 37°C. In order to test the efficiency of lipase concentration using this method, different ratios of supernatant versus acetone were used.

2.9.2 Polyethylene glycol (PEG) precipitation

A 50% (v/v) solution of PEG 4000 or 6000 in 50mM Tris-HCl (pH 7.5) was prepared. The solution was mixed and vortexed with culture supernatant to give a final PEG concentration of 20% (v/v). The resulting solution was kept on ice for 16h and centrifuged at 12000 *g* for 25 mins at 4°C. After discarding the supernatant the resulting pellet was resuspended in 50mM Tris-HCl buffer (pH 7.0) to a tenfold concentration and lipase activity was measured by using the spectrophotometric assay at 37°C.

2.10 Separation of lipases

2.10.1 SDS-PAGE electrophoresis

Concentrated culture supernatant samples were analysed by SDS-PAGE electrophoresis. Polyacrylamide gels were constructed by the method of Laemmli, (1970). Concentrated samples were added to an equal volume of double strength SDS-sample buffer (2xSDS-SB) prior to electrophoresis. Protein molecular mass standards (SIGMA, low molecular weight standard mixture) were heated for 5 mins at 100°C, to denature the proteins, in 1xSDS-SB before loading on the gel. Samples heated for 10 mins at various temperatures, were loaded onto the acrylamide gel prepared by using a 10% acrylamide resolving solution and a 5% acrylamide stacking solution. Electrophoresis took place in running buffer {0.1% SDS, 0.303% Tris and 1.44% (w/v) glycine}. Gels were run using a mini-Protean II electrophoresis cell (Bio-Rad) according to the manufacturers instructions.

Acrylamide resolving solution: A 10% solution contained (ml): water, 4; 30% (w/v) acrylamide mix, 3.3; 1.5M Tris (pH 8.8), 2.5; 10% (w/v) SDS, 0.1; 10% (w/v) ammonium persulfate, 0.1 and TEMED, 0.004.

Acrylamide stacking solution: A 5% solution contained (ml): water, 2.7; 30% (w/v) acrylamide mix, 0.67; 1.0M Tris (pH 6.8), 0.5; 10% (w/v) SDS, 0.04; 10% (w/v) ammonium persulfate, 0.04 and TEMED, 0.004.

2xSDS-SB: This contained: 0.125M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) 2-mercaptoethanol and 0.1% (w/v) bromophenol blue.

Tris glycine electrophoresis buffer: This contained: 25mM Tris, 250mM glycine (electrophoresis grade) (pH 8.3) and 0.1% (w/v) SDS.

2.10.2 Development of gels

In order to develop the gel the following techniques were used:

2.10.3 Staining with Coomassie Brilliant Blue

0.25g of Coomassie Brilliant Blue R250 was dissolved in 90ml of methanol:water (1:1 v/v) and 10ml glacial acetic acid. The resulting solution was filtered through a Whatman No1 filter in order to remove any particulate matter. The gel was immersed in 4 volumes of the staining solution for 4h, followed by destaining in the same methanol/acetic acid solution, without the dye, for 4h (Iqbal, *et al.*, 1994). The destaining solution was changed three times. Proteins stained as blue bands were photographed as required.

2.10.4 Staining with silver salts

The gel was incubated for 6h at room temperature in 6 gel volumes of a solution of ethanol:glacial acetic acid:water (30:10:60). The fixing solution was discarded and 6 gel volumes of 30% (v/v) ethanol were added followed by incubation at room temperature for 30 mins. After discarding the ethanol, 10 gel volumes of deionized water were added and the gel was incubated at room temperature for 10 mins. After discarding the solution, 5 gel volumes of a 0.1% (w/v) solution of $AgNO_3$ were added and the gel was incubated for 30 mins at room temperature. After discarding the $AgNO_3$ solution and washing with deionized water, 5 gel volumes of a freshly made solution of 2.5% (w/v) sodium carbonate and 0.02% (v/v) formaldehyde were added. The gel was incubated at room temperature and washed until stained bands appeared. Finally the gel was washed in 1% (v/v) acetic acid, followed by several changes of deionized water.

2.11 Protoplast preparation

This method is based on the method described by Hopwood, *et al.*, (1985).

A 0.2ml spore suspension of *Streptomyces lividans* was grown in 25ml YEME medium with 0.5% (w/v) glycine for 40h at 30°C and 100rpm (New Brunswick Scientific, Edison, N.J., USA). The culture was centrifuged at 3000 *g* for 10 mins so that sedimentation of mycelia took place. The supernatant was discarded and the pellet was resuspended in 15ml of 10.3% (w/v) sucrose solution. The mixture was centrifuged at 3000 *g* for 10 mins. After repeating the washing step, the resulting mycelia were resuspended in 4ml lysozyme solution and incubated at 30°C for 50 mins. 5ml P buffer was added and the mixture filtered through a cotton wool filter to remove any remaining mycelia. The protoplasts were centrifuged at 3000 *g* for 7 mins and resuspended in 10ml of P buffer. A haemocytometer was used to count a tenfold dilution of the protoplasts and the number of protoplasts was determined by using the following equation:

$$X \times 4 \times 10^{6} \times 10 \text{ count/ml}$$

X is the mean number of protoplasts in the smallest square of the haemocytometer. In order to find the true number of protoplasts, samples were diluted in P buffer and in detergent (0.01%, w/v SDS) and plated on R2YE plates. Any colonies arising were subtracted from the total number of protoplasts since they have arisen from non-protoplasted units. The regeneration frequency was found to be: 7.69% ± 0.56.

Lysozyme solution: This contained 1mg/ml lysozyme in P buffer

R2YE medium: This contained (g/l): yeast extract, 5; sucrose, 103; K_2SO_4, 0.25; $MgCl_2$ $6H_2O$, 10.12; glucose, 10; TES, 5.73; Difco Casaminoacids, 0.1; H_2O to 1000ml. To 100ml aliquots, 2.2g agar was added and autoclaved. At the time of use the following solutions were added after autoclaved separately: 20% (w/v) L-proline, 1.5ml; 0.5% (w/v)

KH_2PO_4, 0.25ml; 1M NaOH, 0.7ml; 5M $CaCl_2$ $2H_2O$, 0.4ml and 0.2ml of trace element solution.

2.12 DNA analysis

2.12.1 Isolation of chromosomal DNA

The following two methods were used:

2.12.2 Maxiprep protocol-Alkali lysis method

A culture of *Streptomyces* species was grown at 50°C for 20h in half strength YEME medium (in the case of *Streptomyces lividans* the culture was grown at 30°C for 48h). The culture was centrifuged at 12000 *g* for 15 mins. The supernatant was decanted and the pellet washed twice in 0.3M sucrose. The resulting mycelia were resuspended in 2.5ml of lysis buffer and 0.5ml lysozyme solution was added. The resulting solution was mixed and incubated for 30 mins at 37°C, 0.4ml of 10% (w/v) sarkosyl was then added and the lysate was dissolved in 1ml of 0.25M EDTA, 5ml TE buffer, 9g caesium chloride and 0.6ml of 10mg/ml ethidium bromide. After mixing by inversion, the solution was pipetted into a 3.9ml Quick-Seal tube and sealed. After balancing the tube against another tube, containing caesium chloride solution of the same density, centrifugation took place at 273940 *g* for 20h at 20°C in a Sorval ultracentrifuge. An 18-gauge needle was used to withdraw the chromosomal DNA and UV light was used to visualise the DNA bands. EtBr was removed from the DNA by adding isoamyl alcohol. This step was repeated 3 times until a colourless bottom layer chromosomal DNA was obtained. CsCl was removed from the DNA by adding 1 volume of 1M ammonium acetate plus 2 volumes of ethanol. The DNA was left at room temperature for 15 mins and centrifuged for 10 mins at 20°C. The

supernatant was aspirated off and the pellet was dried by vacuum desiccation. The DNA pellet was resuspended in 100μl of sterilised distilled water and kept at 4°C.

Half strength YEME medium: This contained (g/l): yeast extract, 1.5; malt extract, 1.5; Bacto-peptone, 2.5; glucose, 5 and 1ml of 2.5M $MgCl_2$ $6H_2O$ autoclaved separately.

Lysis buffer: This contained: 25mM Tris-HCl (pH 8.0), 10mM EDTA and 50mM sucrose.

Lysozyme solution: This contained: 50mg/ml lysozyme in 0.01 Tris-HCl (pH 8.0) and 20μl RNAse (10mg/ml heated for 10 mins at 90°C).

TE buffer: A 10x solution contained: 0.1M Tris-HCl (pH 8.0) and 10mM sodium EDTA (pH 8.0). When required, this solution was diluted ten-fold with sterilised distilled water.

2.12.3 Rapid extraction with guanidium thiocyanate

This method is based on the method of Pitcher, *et al.*, (1989). 1ml of overnight culture was centrifuged in 1.5ml Eppendorf tube at 12000 *g* for 10 mins and the supernatant was discarded. The cells were resuspended in 100μl of fresh lysozyme solution. The suspensions were incubated at 37°C for 30 mins. Cells were lysed with 500μl of GES reagent and cell suspensions were vortexed and left at room temperature for 10 mins. The lysates were cooled on ice and 250μl of 7.5M ice cold ammonium acetate added with mixing followed by further incubation on ice for 15 mins. 500μl of chloroform and 2-pentanol (24:1) was added and after vortexing the mixture was centrifuged at 13000 *g* for 10 mins. The aqueous (top) layer was retained and the process was repeated until the protein interface disappeared. An equal volume of ice cold isopropanol was added to the supernatant fluid and after inversion for 1 min, to mix the solutions, the tube was left on ice for 10 mins. Fibrous DNA precipitate was deposited by centrifugation at 13000 *g* for 10 mins and the resulting pellet was washed twice in 70% (v/v) ethanol and dried under

vacuum. DNA samples were redissolved overnight at 4°C in 100μl of sterilised deionized water.

GES reagent: This was prepared as follows: Guanidium thiocyanate (60g), 0.5mol/l EDTA at pH 8.0 (20ml) and deionized water (20ml) were heated at 65°C with mixing until dissolved. 5ml of 10% (v/v) sarkosyl was added after cooling. The solution was made up to 100ml with deionized water, filtered through a 0.45μm Nalgene filter (BDH LTD) and stored at room temperature.

Lysozyme solution: This contained: 50mg/ml lysozyme in TE buffer (pH 8.0).

2.12.4 Quantitation of DNA using the spectrophotometric method

DNA suspensions were placed in quartz cuvettes and absorbance readings were taken at 260 and 280nm using the Perkin-Elmer Spectrophotometer. An optical density reading of 1.0 at 260nm corresponds to 50μg/ml of DNA, 20μg/ml of oligonucleotides and 40μ g/ml of RNA. The ratio of OD_{260} : OD_{280} gives a value of 1.8 for pure DNA.

2.12.5 Agarose gel electrophoresis

In order to characterise and purify DNA fragments agarose gel electrophoresis experiments were performed, according to details given in "Molecular Cloning a Laboratory Manual" (Sambrook, *et. al.*, 1989). To make a permanent record of the gels, photographs were taken during transillumination under UV light (310nm).

2.12.6 Partial digestion of chromosomal DNA

For the partial digestion of chromosomal DNA a modification of the Hopwood, *et. al.*, (1985) method was used. An aliquot of chromosomal DNA (200-500μg) was partially digested with 1unit of *Bam*H1 and 1μl of 10x restriction enzyme buffer. The reaction was

incubated at 37°C for 1h. Digestion was analysed by electrophoresis using a 0.7% (w/v) agarose gel. The reaction was stopped by heating at 65°C for 15 mins. The DNA sample was extracted with phenol/chloroform and the DNA was precipitated with 2 volumes of ethanol and washed with 70% (v/v) ethanol in order to remove salt. The DNA was recovered by centrifugation at 13000 *g* for 10 mins, redissolved in 90µl of TE buffer and kept at 4°C.

Restriction enzyme buffer: This contained (final concentration in mmol/l, 1/10 diluted): Tris-HCl, 50; $MgCl_2$, 10; NaCl, 100 and dithioerythritol, 1.

2.12.7 Isolation of plasmid pIJ702

The following two methods were used:

2.12.8 Mini plasmid preparation

This preparation is based on the method described by Hopwood, *et. al.*, (1985).

The plasmid-containing strain of *Streptomyces lividans* was grown in 250ml flasks containing 25ml of YEME-sucrose medium at 30°C for 48h. Mycelia were harvested by centrifugation at 12000 *g* and washed twice in 0.3M sucrose solution. 500µl of lysozyme solution was used to resuspend the pellet and the suspension was introduced into an Eppendorf tube which was incubated at 37°C for 30 mins. 250µl alkaline SDS was added and after vortexing, the solution was incubated at 70°C for 15 mins. After cooling at room temperature, 105µl of phenol/chloroform was added and mixed so that one layer was formed. The homogenised mixture was centrifuged at 12000 *g* and the aqueous phase was transferred to another tube. 70µl of 3M sodium acetate and 700µl of isopropanol were added and the tube was left at room temperature for 5 mins. After centrifuging at 12000 *g* for 5 mins the supernatant was discarded and the pellet was dried, redissolved in 50µl TE

buffer (pH 8.0) and left overnight at 4°C. The next day, 5µl of 3M sodium acetate and 25µl neutral phenol/chloroform were added, the solution vortexed followed by centrifugation at 12000 *g* for 10 mins. The top phase was removed to another tube and after adding 700µl of ethanol the plasmid DNA was precipitated by centrifugation at 12000 *g* for 10 mins. The resulting pellet was dried and resuspended in 50µl TE buffer and kept at 4°C.

YEME-sucrose medium: This contained (g/l): yeast extract, 3; malt extract, 3; Bacto-peptone, 5; glycose, 10; sucrose, 340. The pH was adjusted to 7.2. At the time of use, 2ml of $MgCl_2$ $6H_2O$ (2.5M) was added after autoclaved separately.

Lysozyme solution: This contained: 1mg/ml lysozyme in TE buffer containing 0.3M sucrose.

2.12.9 Lysis by alkali

This method is a modification of the method described by Ish-Horowicz and Burke, (1981). A plasmid containing strain of *Streptomyces lividans* was grown in 250ml flasks containing 25ml YEME-sucrose medium at 30°C for 48h. The medium was supplemented with 50µg/ml thiostrepton to fully induce the plasmid. The culture was centrifuged at 12000 *g* for 10 mins at 4°C and the supernatant was removed by aspiration, leaving the pellet as dry as possible. The pellet was resuspended in 100µl of an ice cold solution containing: 50mM sucrose, 25mM Tris-HCl (pH 8.0) and 10mM EDTA (pH 8.0). 500µl lysozyme solution was added and mixed. 200µl of freshly prepared solution containing: 0.2N NaOH and 1% (w/v) SDS was added and the contents were mixed by inverting the tube rapidly 5 times. 150µl of an ice cold solution containing: 5M sodium acetate (60ml), glacial acetic acid (11.5ml) and water (28.5ml) were added. The tube was vortexed in an inversed position for 5 seconds and stored on ice for 3 mins prior to centrifugation at 12000 *g* for 5 mins at 4°C. The supernatant was transferred to another tube and an equal

volume of phenol/chloroform was added and mixed by vortexing. The solution was centrifuged at 12000 *g* for 2 mins at 4°C and the supernatant again transferred to another tube. The DNA was precipitated with 2 volumes of ethanol and after mixing by vortexing, the mixture was allowed to stand for 2 mins at room temperature. After centrifugation at 12000 *g* for 5 mins at 4°C, the supernatant was removed and the pellet was rinsed with 1ml of 30% (v/v) ethanol at 4°C. The supernatant was removed and the pellet was left to dry in the air for 10 mins. The nucleic acid was redissolved in 50μl of TE buffer (pH 8.0) containing DNAse free pancreatic RNAse (20μg/ml) and stored at -20°C.

2.12.10 Digestion of plasmid pIJ702

1μl of the plasmid DNA solution was added to an Eppendorf tube containing 8μl of water. 1μl of 10x restriction enzyme buffer and 1 unit of *BglII* restriction enzyme was added. The reaction was incubated for 1.5h at 37°C. An aliquot was removed and the extent of digestion was analysed by electrophoresis through a 0.7% (w/v) agarose gel. When the digestion was completed, the sample was extracted with phenol/chloroform and the DNA was precipitated with 2 volumes of ethanol for 15 mins at 0°C. The DNA was recovered by centrifugation at 12000 *g* for 10 mins at 4°C and redissolved in 90μl of 10mM Tris-HCl (pH 8.3).

Restriction enzyme buffer: This contained (final concentration in mmol/l, 1/10 diluted): Tris-HCl, 10; $MgCl_2$, 5; NaCl, 100 and 2-mercaptoethanol, 1.

2.12.11 Removal of 5' phosphate group from linearised plasmid DNA

To an aliquot of linearised plasmid DNA, 0.5U calf intestine alkaline phosphatase (CIAP) and 10μl of 10x CIAP dephosphorylation buffer were added. The mixture was incubated at 37°C for 30 mins. Another aliquot of CIAP was added and the incubation was

continued for a further 45 mins at 55°C. SDS and EDTA (pH 8.0), to final concentrations of 0.5% (w/v) and 5mM respectively, were added and after mixing well, proteinase K was added to a final concentration of 100µg/ml. The reaction was cooled to room temperature and the mixture was extracted once with phenol and once with phenol/chloroform. 0.1 volume of 3M sodium acetate (pH 7.0) was added and after mixing two volumes of ethanol were added. The solution was kept at 0°C for 15 mins and the DNA was recovered by centrifugation at 12000 *g* for 10 mins at 4°C. The pellet was washed with 70% (v/v) ethanol and the precipitated DNA was redissolved in TE (pH 7.6) at a concentration of 100µg/ml and stored in aliquots at -20°C.

10x CIAP dephosphorylation buffer: This contained: 10mM $ZnCl_2$, 10mM $MgCl_2$, and 100mM Tris-HCl (pH 8.3).

2.12.12 Ligation of DNA

Chromosomal DNA and plasmid dephosphorylated DNA were mixed in a ratio of 30µg/ml : 10µg/ml respectively. 7.5µl of water was added and the solution was warmed at 55°C for 10 mins to melt any cohesive termini that have reannealed. The mixture was chilled at 0°C and the following reagents were added: 1µl of 10x T4 DNA ligase buffer, 1u T4 DNA ligase and 1µl of 5mM ATP. The reaction was incubated at 16°C for 4h followed by phenol/chloroform treatment and ethanol precipitation. The DNA was redissolved in up to 20µl TE buffer.

10x T4 ligase buffer: This contained: 200mM Tris-HCl (pH 7.6), 50mM $MgCl_2$, 50mM dithiothreitol and 500µg/ml bovine serum albumin.

2.13 Transformation of protoplasts by plasmid DNA

An aliquot contained 4×10^9 of freshly prepared protoplasts/ml was centrifuged at 3000 *g* for 7 mins. The supernatant was removed and 1μg of the ligation mixture was added. 0.5ml of 25% (v/v) PEG 1000 in T buffer was added, followed by 5ml P buffer and the solution was centrifuged at 3000 *g* for 7 mins. The resulting pellet was resuspended in 1ml P buffer. 0.1ml of the suspension was plated on R2YE agar and incubated at 30°C.

T buffer: This contained: 10.3% (w/v) sucrose, 25ml; distilled water, 75ml; trace element solution, 0.2ml; K_2SO_4 (2.5%, w/v), 1ml. To 9.3ml of this solution the following reagents were added: 0.2ml $CaCl_2\,H_2O$ (5M) and 0.5ml of 1M Tris-maleic acid buffer (pH 8.0).

P buffer: This contained (g/l): sucrose, 103; $MgCl_2\,6H_2O$, 2.02; K_2SO_4, 0.25. 2ml of trace element solution was added and distilled water to 800ml. The solution was dispersed to 80ml aliquots and autoclaved. At the time of use, 1ml KH_2PO_4 (0.5%, w/v), 10ml $CaCl_2\,2H_2O$ (3.68%, w/v) and 10ml TES buffer (5.73%, v/v) were added after autoclaved separately.

TES buffer: This contained: 1mM EDTA (pH 8.0), 10mM Tris-HCl (pH 8.0) and 2.92g NaCl.

2.14 Detection of transformants

Transformation plates were incubated at 30°C for 17h, and 1.25ml of a sucrose solution (0.3M) containing 300μg/ml thiostrepton in DMSO (dimethylsulfoxide) was used to overlay the plates which were reincubated until sporulation (Iqbal, *et al.*, 1994). Non melanin producing colonies were considered as true transformants.

2.15 Screening for the production of lipases

Non melanin producing colonies were transferred onto an olive oil agar plate and incubated at 30°C for 5 days. Clear zones around the colonies provided an indication of lipase production.

Olive oil agar plates: This contained (g/l): yeast extract, 3; malt extract, 3; peptone, 5; olive oil, 3; agar, 18. The pH was adjusted to 7.0.

2.16 Statistical determination

For most experiments performed, 3 to 6 measurements were recorded and the standard error of the mean was calculated from the standard deviation of the samples:

$$SD = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

$$SE = \frac{SD}{\sqrt{n}}$$

Where : SD=standard deviation

x=Mean value

n=number of treatments

SE=Standard error

t-test

This test was used in order to determine whether sample means were statistically significantly different:

$$SEdiff = \sqrt{\frac{(n1-1)SD1^2 + (n2-1)SD2^2}{(n1+n2-2)} \frac{n1+n2}{n1n2}}$$

$$t_s = \frac{(x1-x2)}{SEdiff}$$

Where: SEdiff=Standard error of the difference

n1 and n2=Sample size of samples 1 and 2

SD1 and SD2=Standard deviation of samples

ts=t-test statistic

x1 and x2=sample means of samples

ts was compared with the critical value of t from table of t-distribution.

Chapter 3

Preliminary characterisation of lipase production from thermophilic *Streptomyces*

3.1 Introduction

The use of thermophilic *Streptomyces* as a source of enzymes stable at high temperature and in adverse chemical environments, has been highlighted in numerous publications (Ball, *et al.*, 1992; Bahri and Ward, 1993; Iqbal, *et al.*, 1994). There is a general relationship between enzyme thermostability and the thermotolerance of the host bacterium; enzymes become increasingly thermostable as one moves up the temperature scale through psychrophiles, mesophiles and thermophiles (Edwards, 1993). This is of some significance to those bacteria such as *Streptomyces* which are involved in biodegradation of polymeric substances during composting processes, via extracellular hydrolases. The enzymes must be sufficiently thermotolerant to be able to withstand the fluctuating temperatures found in such environments (Goodfellow, *et al.*, 1987).

Most of the studies on lipases have been reported for mesophilic organisms (Stöcklein, *et al.*, 1993) and those on bacterial lipases aimed to define conditions for optimum lipase production (Jaeger, *et al.*, 1994). Although, it is possible to produce substantial amounts of lipase protein from both Gram-positive and Gram-negative bacteria, no clear picture has emerged so far from the large amount of experimental data concerning the physiology of lipase biosynthesis and its regulation. A variety of conditions have been described which stimulate or repress the production of lipases by bacteria. These include the influence of environmental factors such as ions, inhibitors, carbon sources or presence of non-metabolizable polysaccharides. Previous work by Perez, *et al.*, (1993) suggested that the lipase from *Streptomyces* species is a small protein of molecular mass 27.9 KDa which may require a second gene product for high level expression. Sequence analysis of the *Streptomyces albus* G lipase-encoding gene revealed amino acid substitutions which may be responsible for the observed greater thermal stability of *Streptomyces* lipases (Cruz, *et al.*, 1994). Olukoshi and Packter,

(1994) suggested that the relationship between growth of different species of *Streptomyces* and the synthesis of lipases was fairly reproducible under various cultural conditions. Minimal formation of lipases was found during the exponential phase of growth but they were invariably accumulated during stationary phase.

Lipases are currently derived from mesophilic sources and their optimum temperature for commercial applications is in most cases approximately 35-40°C. However, thermophilic *Streptomyces* have an optimum growth temperature of 45-65°C, which makes lipases isolated from such strains good candidates for lipid modifications. While considerable data on proteases, carbohydrases and other enzymes of unusual properties have accumulated, there is little information regarding the properties of lipases from thermophilic *Streptomyces* species.

This chapter describes preliminary flask culture experiments aimed to define conditions for optimum lipase production from a range of thermophilic *Streptomyces* species.

3.2 Effect of carbon source on growth and lipase activity

Initially a range of lipid carbon sources (olive oil, vegetable oil,Tween 80, Tween 40 and Tween 60) were selected to determine their effect on growth and lipase production. Five thermophilic *Streptomyces* species were also selected and grown in defined salts media, containing 17.5g/l lipid source at 50°C, in flask cultures which were analysed after 48h incubation. The biomass was separated by centrifugation at 1800 *g* for 10 mins in order to determine dry weight. The cell-free culture supernatants were used to determine the amount of secreted extracellular lipase using p-nitrophenyl palmitate as the enzyme substrate. Results are presented in Tables 3.1 and 3.2. The greatest biomass production, for each organism, was recorded in olive oil followed by Tween 80 and vegetable oil. *Streptomyces thermoviolaceus* and *S. thermodiastaticus*

Table 3.1 Estimation of growth after 48 hours incubation at 50°C.

Dry weights (g/l) [mean±SD; n=6]

Organism	Olive oil	Vegetable oil	Tween 80	Tween 40	Tween 60
S. thermoviolaceus	2.232±0.018	2.071±0.023	2.142±0.013	1.951±0.023	1.865±0.016
S. thermodiastaticus	2.257±0.011	2.077±0.016	2.131±0.016	1.917±0.028	1.841±0.011
S. thermoflavus	2.085±0.025	2.040±0.021	2.068±0.015	1.890±0.027	1.821±0.023
S. thermovulgaris	1.886±0.019	1.789±0.024	1.813±0.026	1.656±0.017	1.597±0.025
S. thermonitrificans	1.776±0.027	1.576±0.025	1.624±0.012	1.460±0.015	1.407±0.022

Table 3.2 Estimation of extracellular lipase activity after 48 hours incubation at 50°C.

Lipase activity (U/100ml supernatant/h) [mean±SD; n=6]

Organism	Olive oil	Vegetable oil	Tween 80	Tween 40	Tween 60
S. thermoviolaceus	6750±4.93	6378±5.00	6450±5.03	5565±4.89	5135±5.05
S. thermodiastaticus	6150±4.87	5878±4.89	5925±4.91	4832±5.04	4349±4.89
S. thermoflavus	5860±4.96	5561±4.93	5747±5.02	4596±4.97	4213±5.02
S. thermovulgaris	5487±5.01	5297±4.98	5349±4.99	4378±4.98	4013±5.03
S. thermonitrificans	5235±4.97	5089±4.96	5167±4.95	4206±4.88	3855±4.99

showed the best growth yield in these media. The same pattern was observed for lipase activities, where olive oil was again the best carbon source for enzyme activity followed by Tween 80.

3.3 Effect of growth phase on lipase activity and extracellular protein production

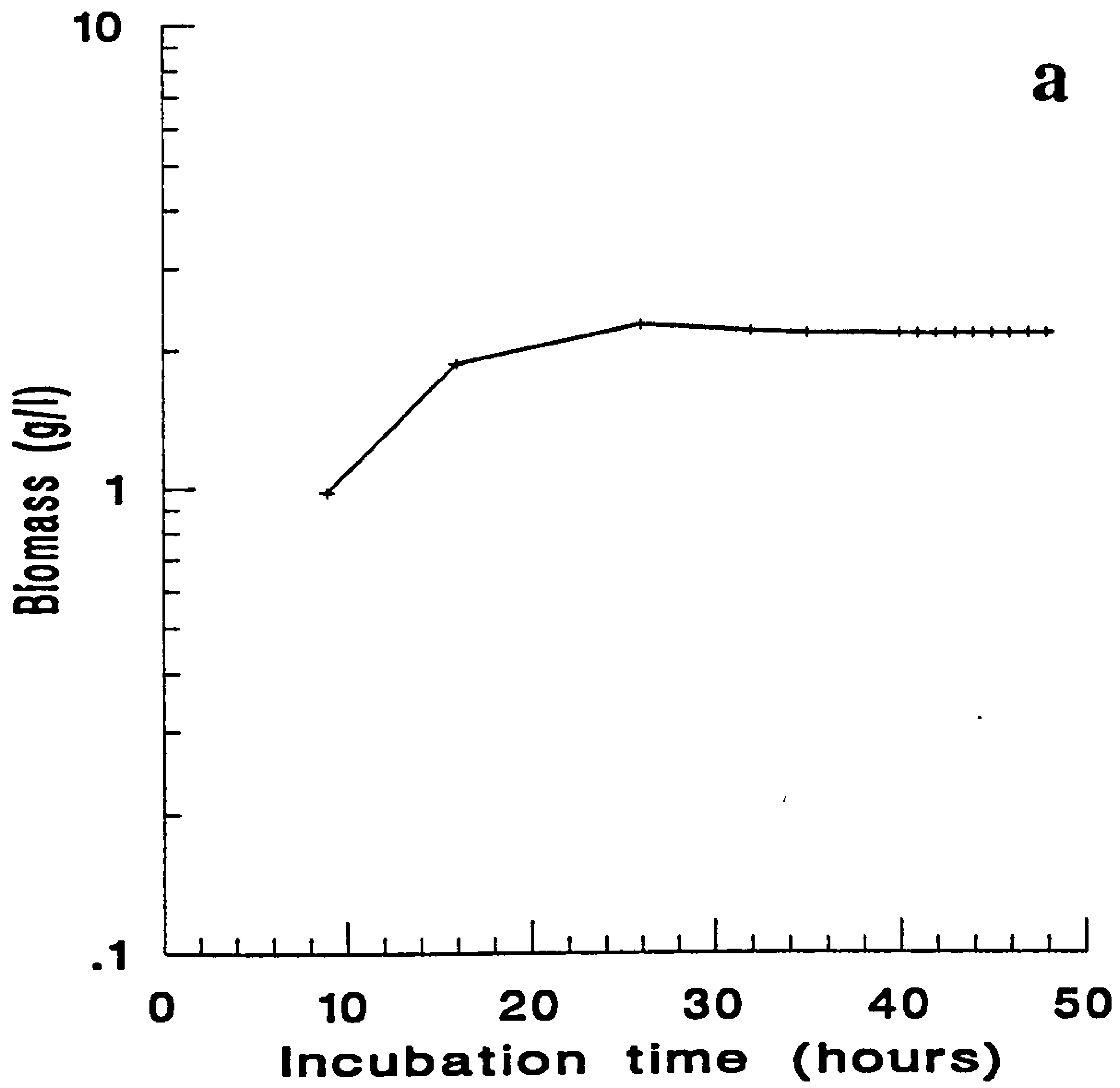
In order to assess the effect of growth phase on lipase activity and extracellular protein production, *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown in the presence of olive oil or Tween 80 at 50°C. The results from *Streptomyces thermoviolaceus* shown in Figures 3.1 and 3.2 indicated that lipase activity and extracellular protein secretion were detected in cell-free culture supernatants during exponential and stationary growth phases for cultures growing with olive oil or Tween 80. For both lipase activity and protein secretion peak values were detected at the end of the exponential and during the stationary growth phases when the biomass production was at its maximum level. Similar results are shown in Figures 3.3 and 3.4 where *Streptomyces thermodiastaticus* was used as the test organism. These results revealed that an increase in biomass production led to an increase in the secretion of lipase and extracellular protein.

3.4 Intracellular lipases

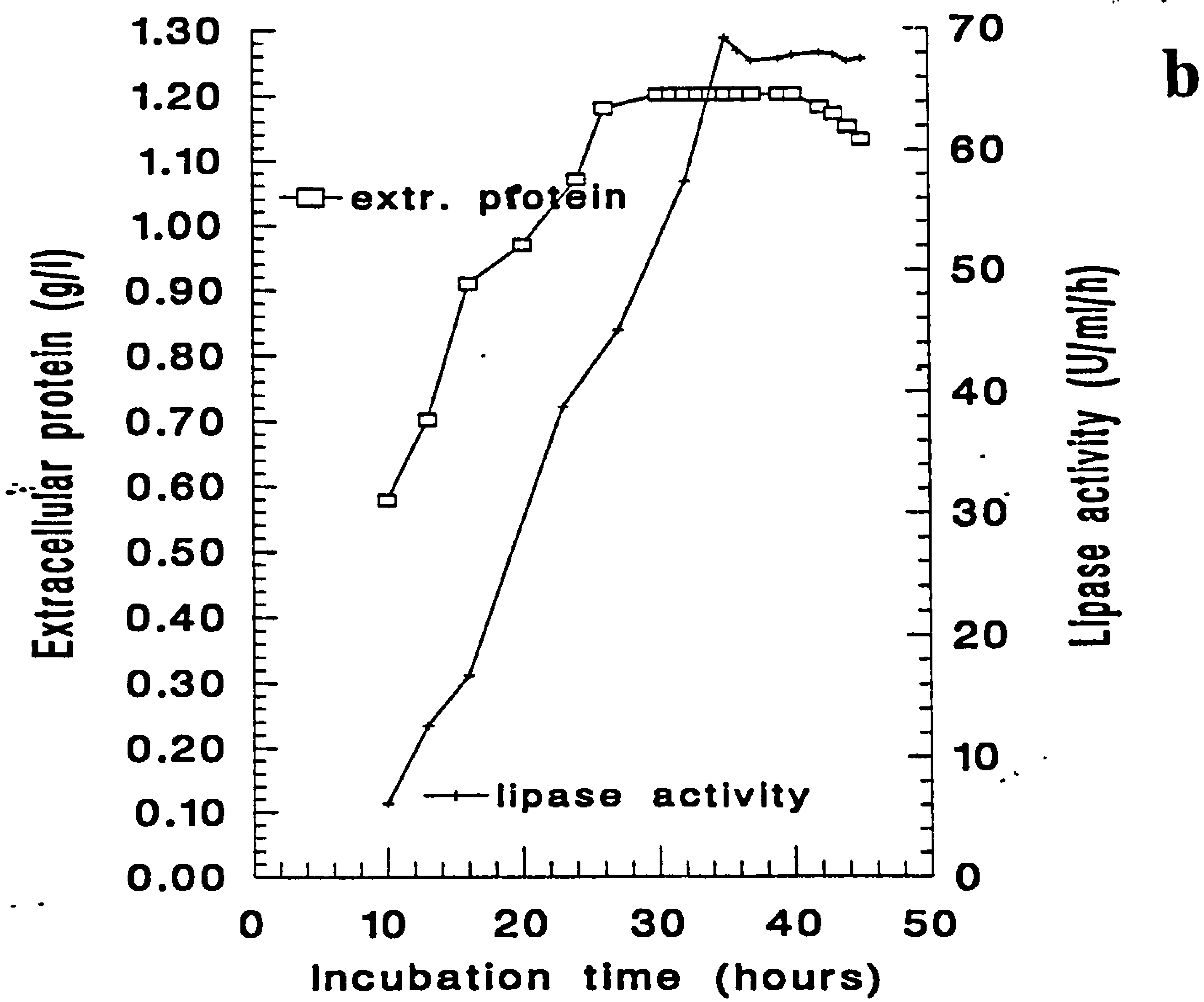
In order to find whether extracellular lipases were secreted by the mycelia or arose as a result of lysis of the mycelia, preliminary characterisation of any intracellular lipase activity took place. *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown at 50°C in the presence of olive oil or Tween 80 as sole carbon source for 24 or 48h. Results are shown in Table 3.3 a) and b). After 24h of growth, some intracellular

Figure 3.1

Graph showing a) the production of biomass and b) the secretion of extracellular protein and lipase activity by *Streptomyces thermoviolaceus* grown in the presence of olive oil at 50°C. Data are representative of at least 3 separate replicates.



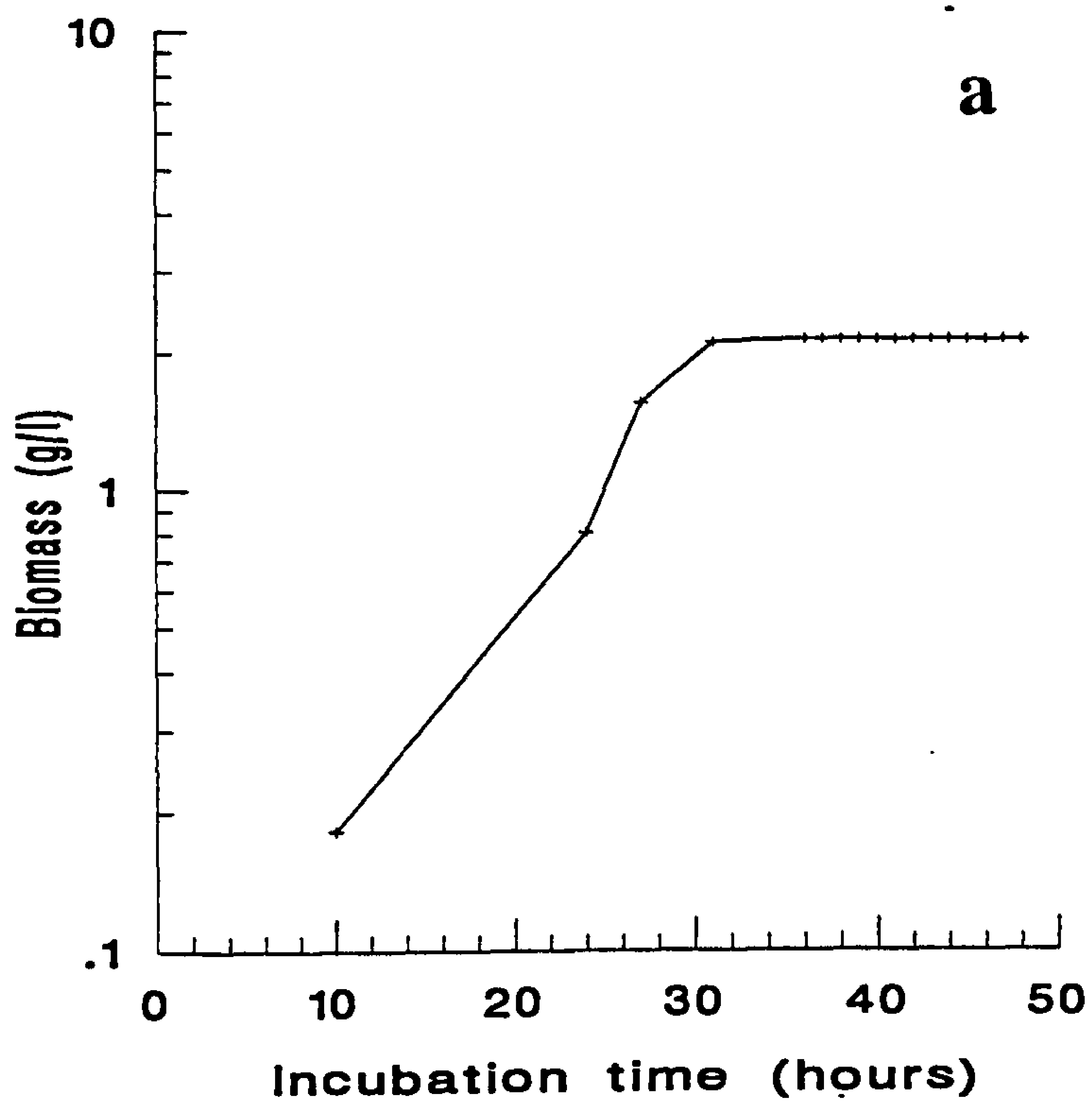
a
Biomass (g/l)
10
1
.1
0
10
20
30
40
50
Incubation time (hours)



b
Extracellular protein (g/l)
Lipase activity (U/ml/h)
extr. protein
lipase activity
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
70
60
50
40
30
20
10
0
10
20
30
40
50
Incubation time (hours)

Figure 3.2

Graph showing a) the production of biomass and b) the secretion of extracellular protein and lipase activity by *Streptomyces thermoviolaceus* grown in the presence of Tween 80 at 50°C. Data are representative of at least 3 separate replicates.


a
10
1
.1
Biomass (g/l)
0
10
20
30
40
50
Incubation time (hours)

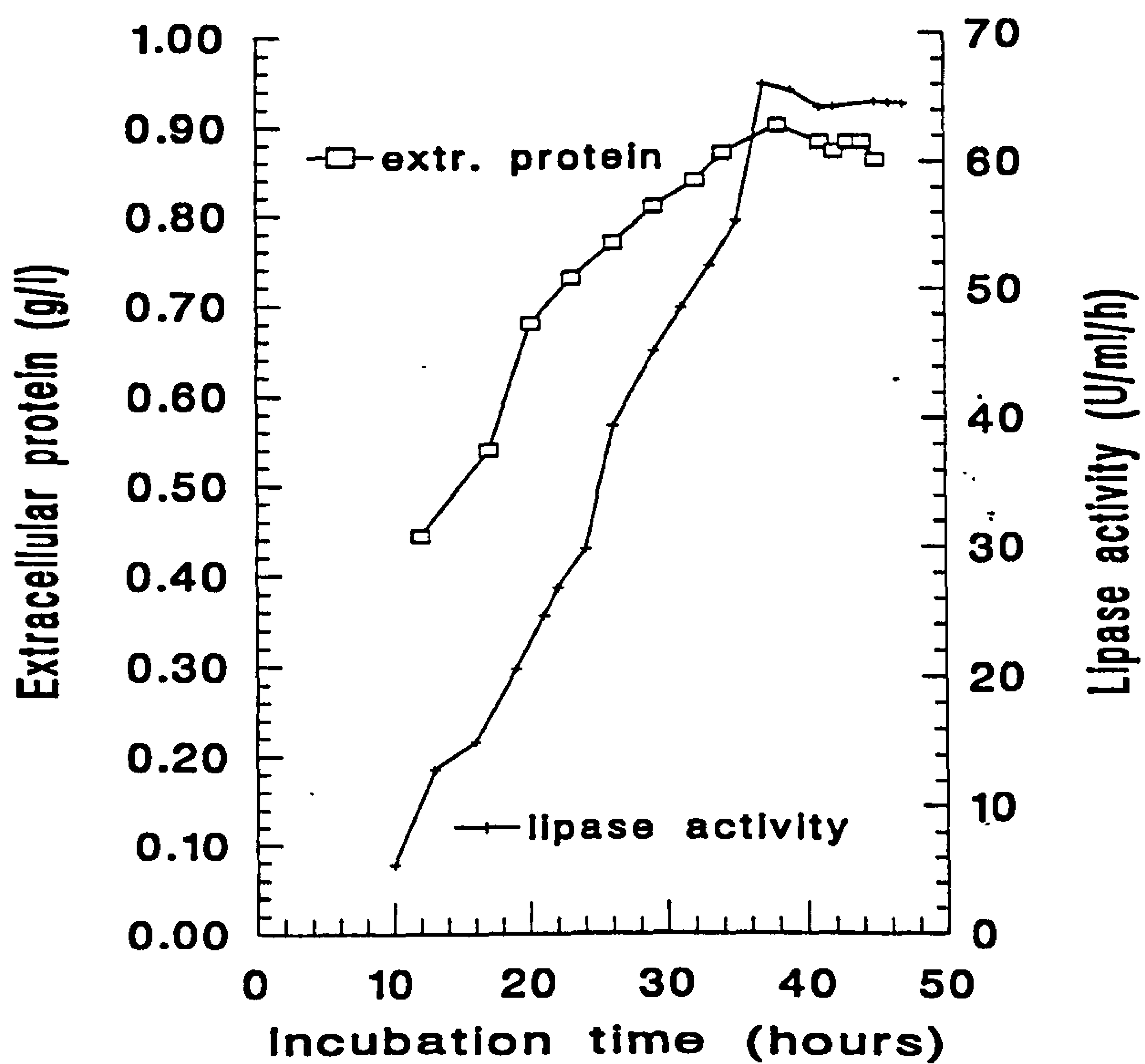
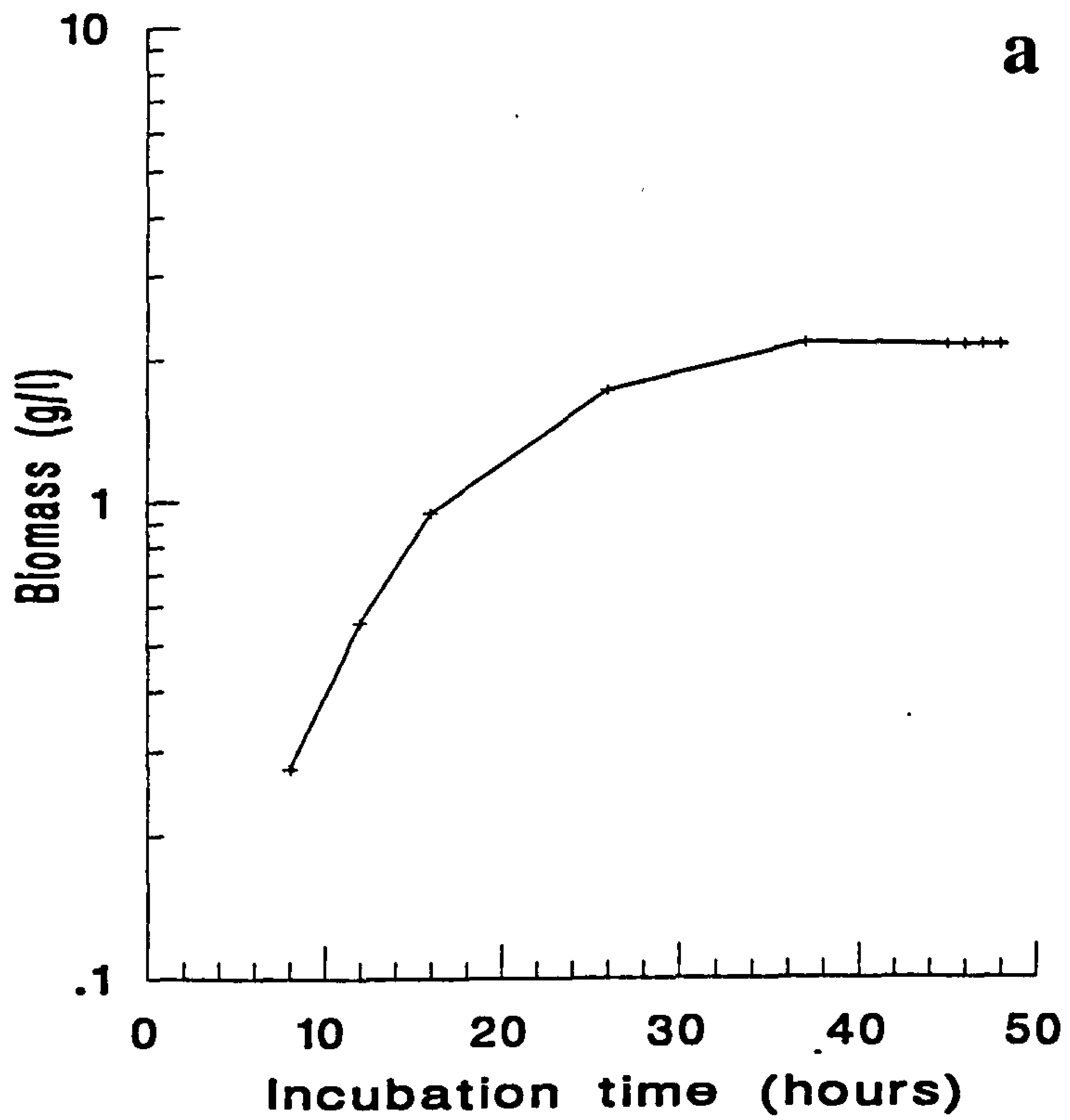
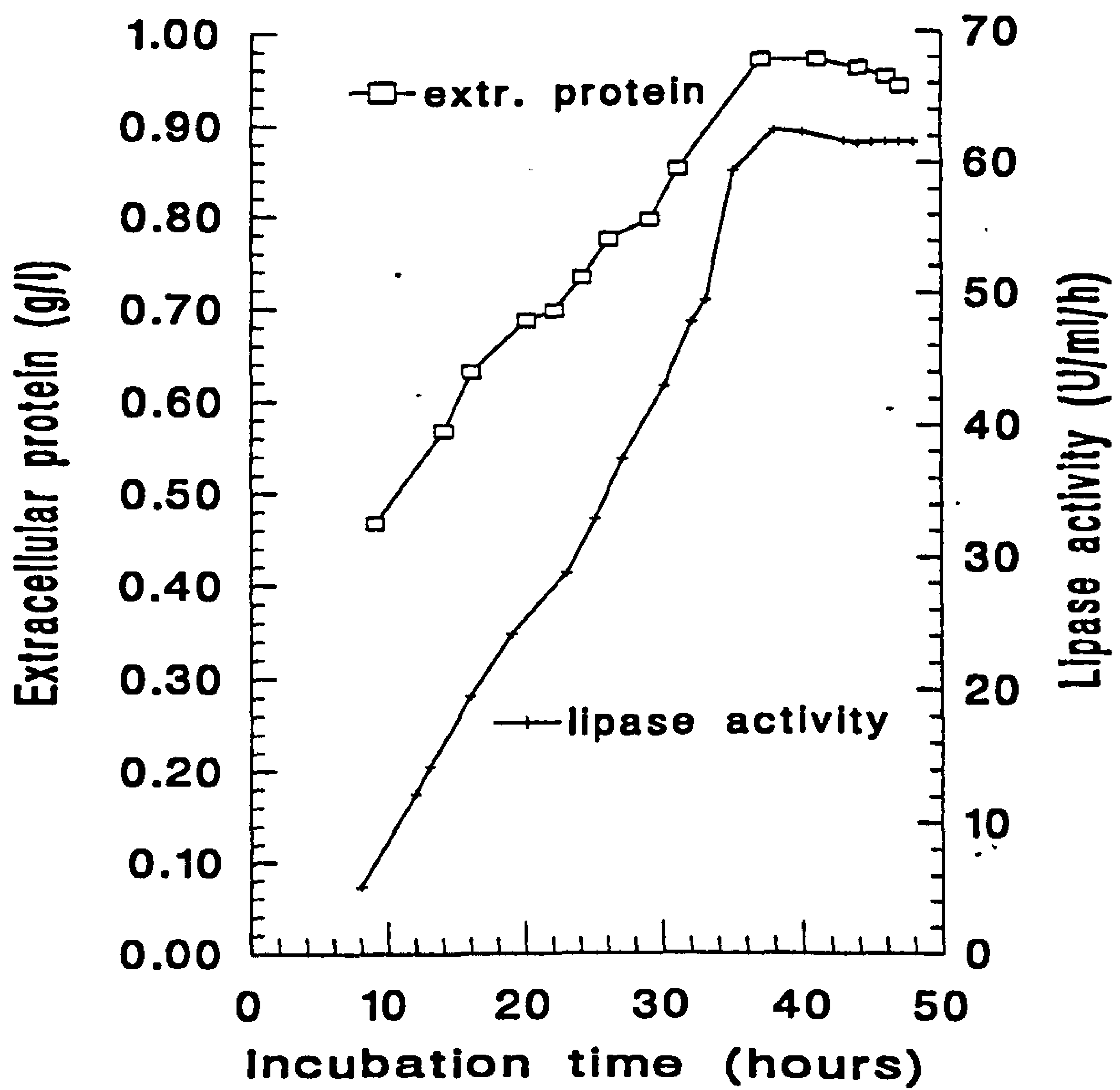

b
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Extracellular protein (g/l)
70
60
50
40
30
20
10
0
Lipase activity (U/ml/h)
extr. protein
lipase activity
0
10
20
30
40
50
Incubation time (hours)

Figure 3.3

Graph showing a) the production of biomass and b) the secretion of extracellular protein and lipase activity by *Streptomyces thermodiastaticus* grown in the presence of olive oil at 50°C. Data are representative of at least 3 separate replicates.



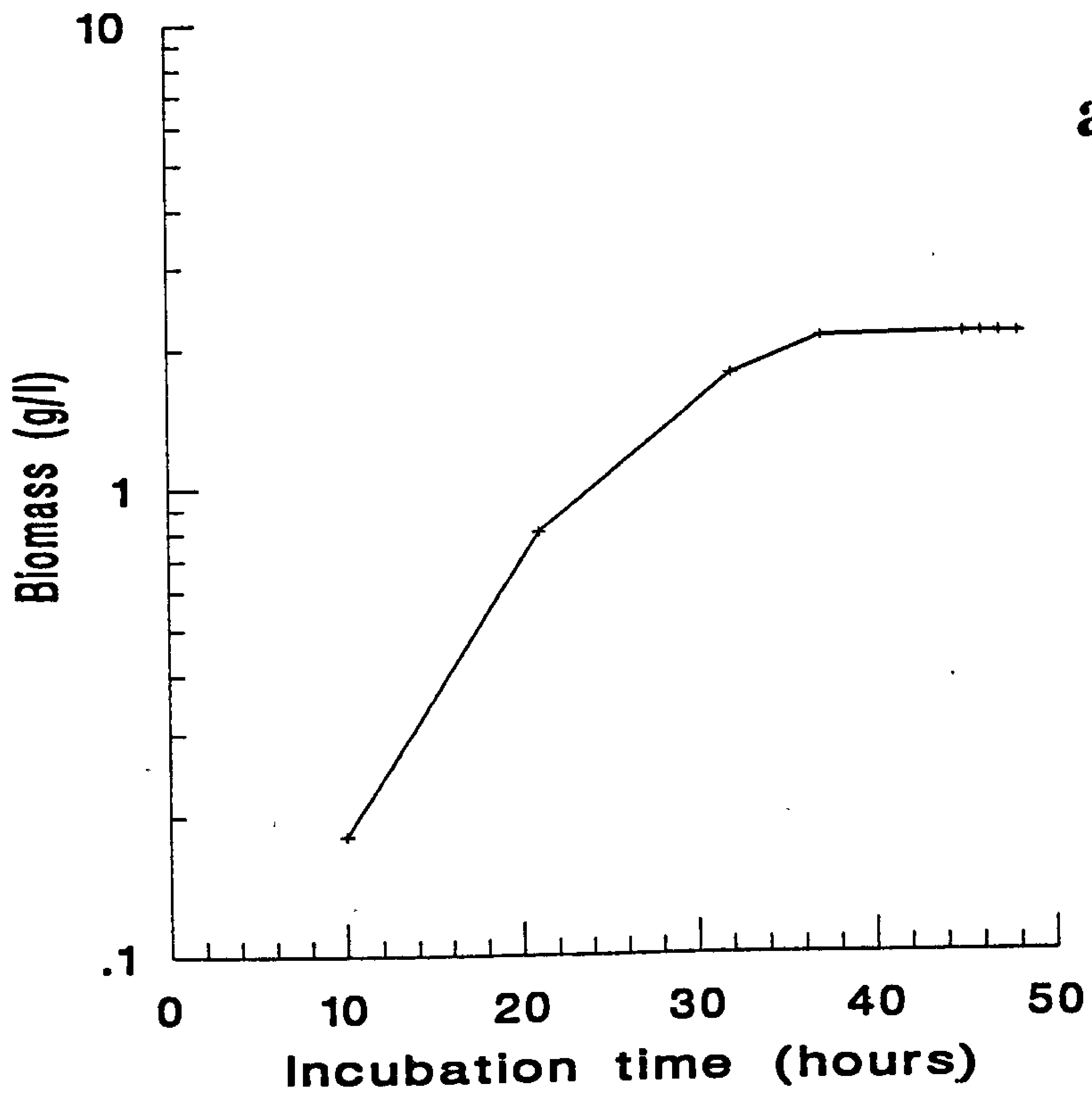
a
Biomass (g/l)
10
1
.1
0
10
20
30
40
50
Incubation time (hours)



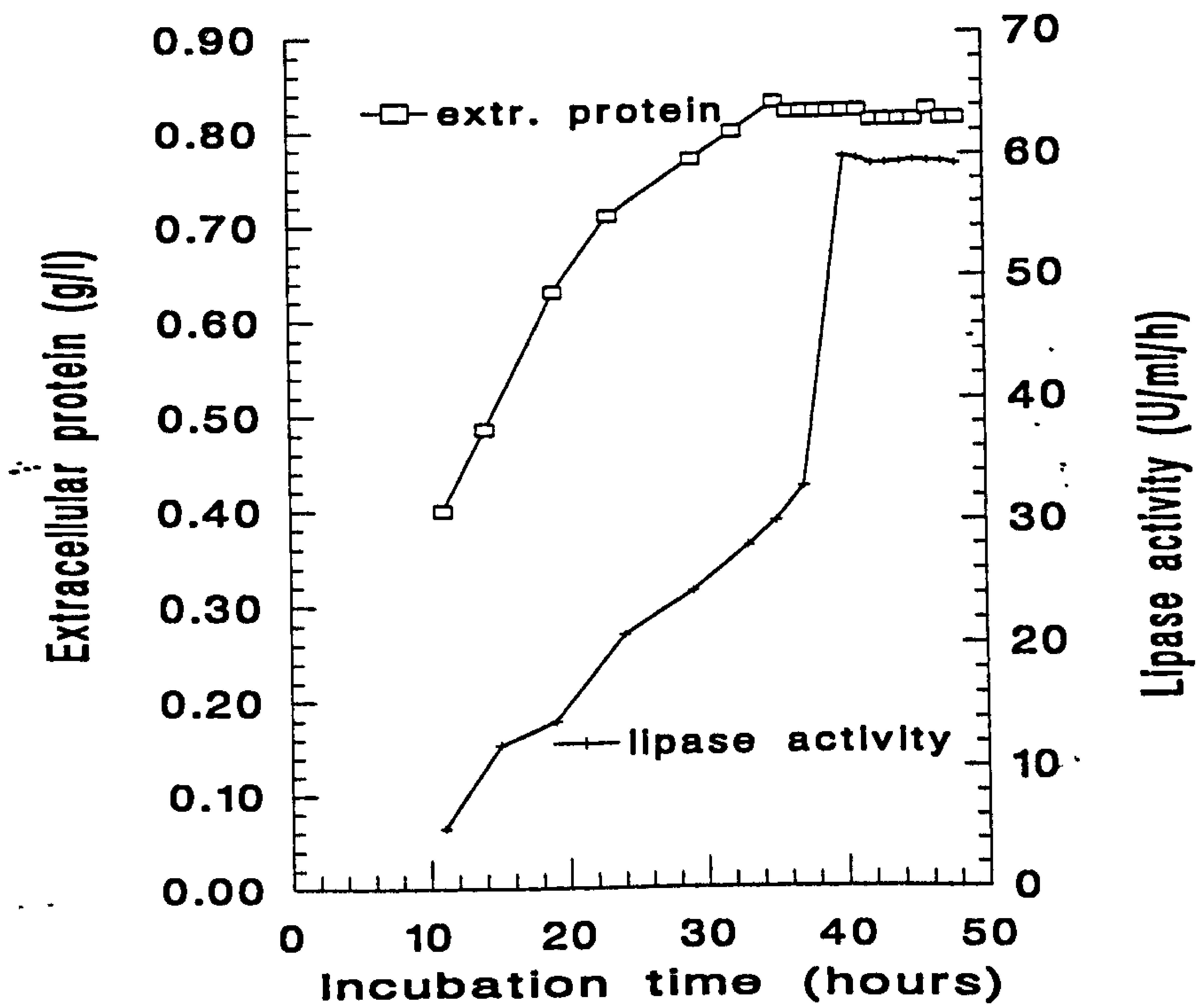
b
extr. protein
lipase activity
Extracellular protein (g/l)
Lipase activity (U/ml/h)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
70
60
50
40
30
20
10
0
0
10
20
30
40
50
Incubation time (hours)

Figure 3.4

Graph showing a) the production of biomass and b) the secretion of extracellular protein and lipase activity by *Streptomyces thermodiastaticus* grown in the presence of Tween 80 at 50°C. Data are representative of at least 3 separate replicates.



a
10
1
.1
Biomass (g/l)
0
10
20
30
40
50
Incubation time (hours)



b
extr. protein
lipase activity
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Extracellular protein (g/l)
70
60
50
40
30
20
10
0
Lipase activity (U/ml/h)
0
10
20
30
40
50
Incubation time (hours)

Table 3.3 a) and b) showing intracellular and extracellular lipase activity from *Streptomyces thermoviolaceus* and *S. thermodiastaticus* after 24 or 48 hours incubation at 50°C. Data are representative of at least 6 separate determinants (mean±SD; n=6).

a) ***Streptomyces thermoviolaceus***

Lipase activity

Lipid source	I/C (U/100ml cell-mass/h) Incubation time		E/C (U/100ml supernatant/h) Incubation time	
	24h	48h	24h	48h
olive oil	90±0.025	450±0.056	2700±1.18	6750±4.93
Tween 80	86±0.029	430±0.061	2580±1.25	6450±5.03

b) ***Streptomyces thermodiastaticus***

Lipase activity

Lipid source	I/C (U/100ml cell-mass/h) Incubation time		E/C (U/100ml supernatant/h) Incubation time	
	24h	48h	24h	48h
olive oil	72.40±0.031	362±0.049	2460±1.25	6150±4.87
Tween 80	69.70±0.035	348±0.055	2370±1.29	5925±4.91

Abbreviations

I/C Intracellular

E/C Extracellular

lipase activity was detected in both organisms which increased 5 fold after incubation for 48h. This was attributed to an increase in biomass. For *Streptomyces thermoviolaceus* the extracellular lipase activity was 30 fold of the intracellular activity after 24h and 15 fold after 48h incubation. For *Streptomyces thermodiastaticus* the values were 34 fold and 17 fold respectively. From these results it was concluded that the higher extracellular lipase activity was a result of secretion by the mycelial cells rather than a result of breakdown of the cells.

3.5 Stability of lipases on storage

In order to investigate the stability of lipases on storage, cell-free supernatant fluids from *Streptomyces thermoviolaceus* and *S. thermodiastaticus*, grown in the presence of olive oil or Tween 80 as the sole carbon source, were kept at 4 and -20°C for a period of 30 days. In order to check the effect of freezing and thawing on enzyme activity, supernatant fluids were kept at -20°C and subjected to a single freeze/thaw cycle daily. Figures 3.5 and 3.6 show the results obtained for *Streptomyces thermoviolaceus*. It was concluded that at 4°C, 92% of the initial activity was retained after 24h irrespective of the carbon source but after 30 days of incubation 82 or 70% of the initial activity remained for olive oil or Tween 80 cultures respectively. At -20°C, 85% of the original activity remained after one month irrespective of the carbon source. During freezing and thawing 26% of the initial activity was lost from cultures grown with olive oil while 32% was lost from cultures grown with Tween 80 after one month. Similar results were obtained for *Streptomyces thermodiastaticus* as shown in Figures 3.7 and 3.8. At 4°C, 77 or 68% of the initial activity remained after 30 days for olive oil or Tween 80 cultures respectively. At -20°C, 81% of the original activity remained after one month. During freezing and thawing the values of the % loss of the initial activity were 31 or 35% respectively. From the results it was concluded that cell-

Figure 3.5

Effect of storage on stability of lipase activity in cell-free culture supernatants of *Streptomyces thermoviolaceus* at 4, -20 and at -20°C after freezing and thawing on every test day. The cultures were grown in the presence of olive oil at 50°C for 48h. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.6

Effect of storage on stability of lipase activity in cell-free culture supernatants of *Streptomyces thermoviolaceus* at 4, -20 and at -20°C after freezing and thawing on every test day. The cultures were grown in the presence of Tween 80 at 50°C for 48h. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.5

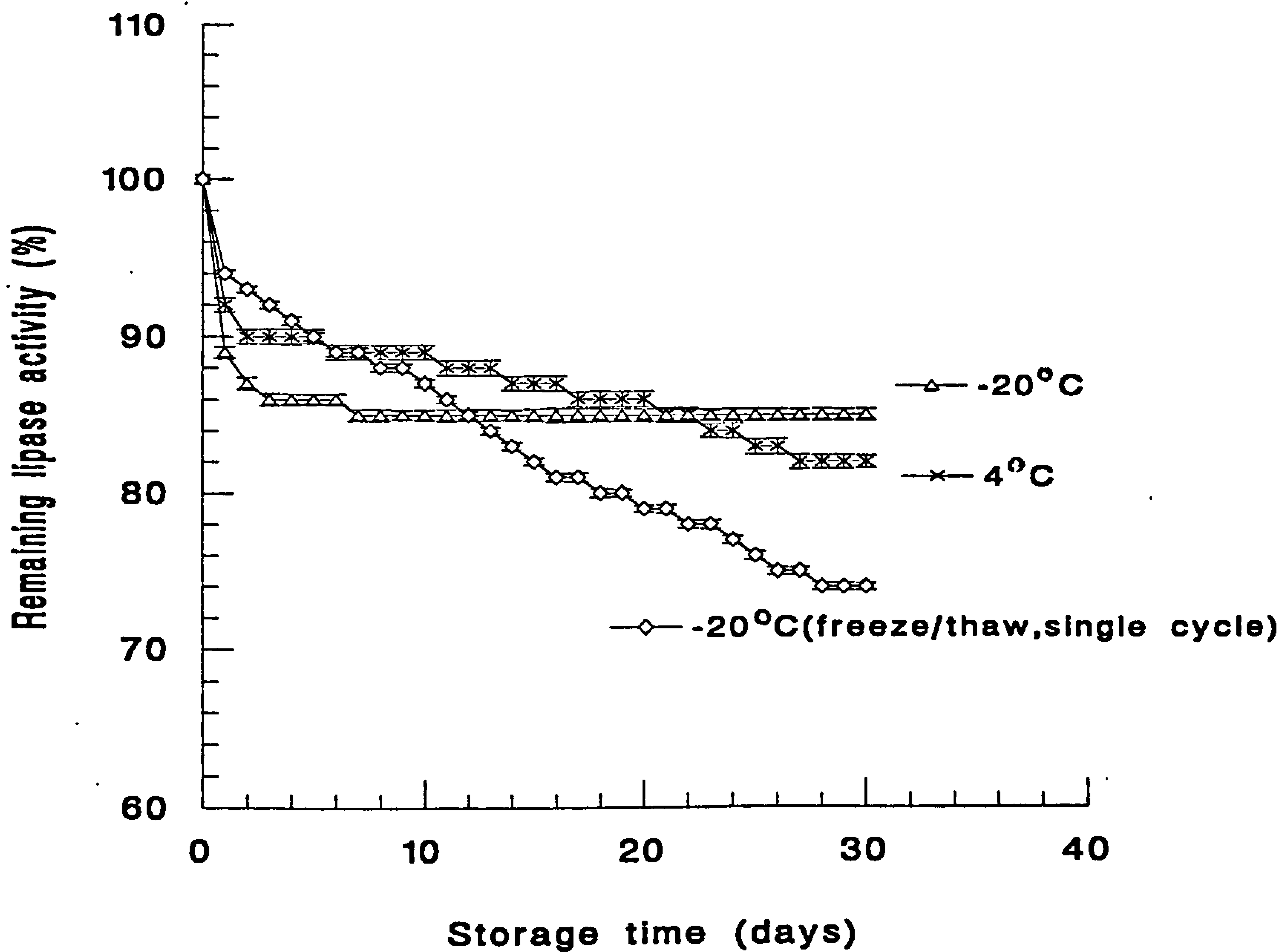


Figure 3.6

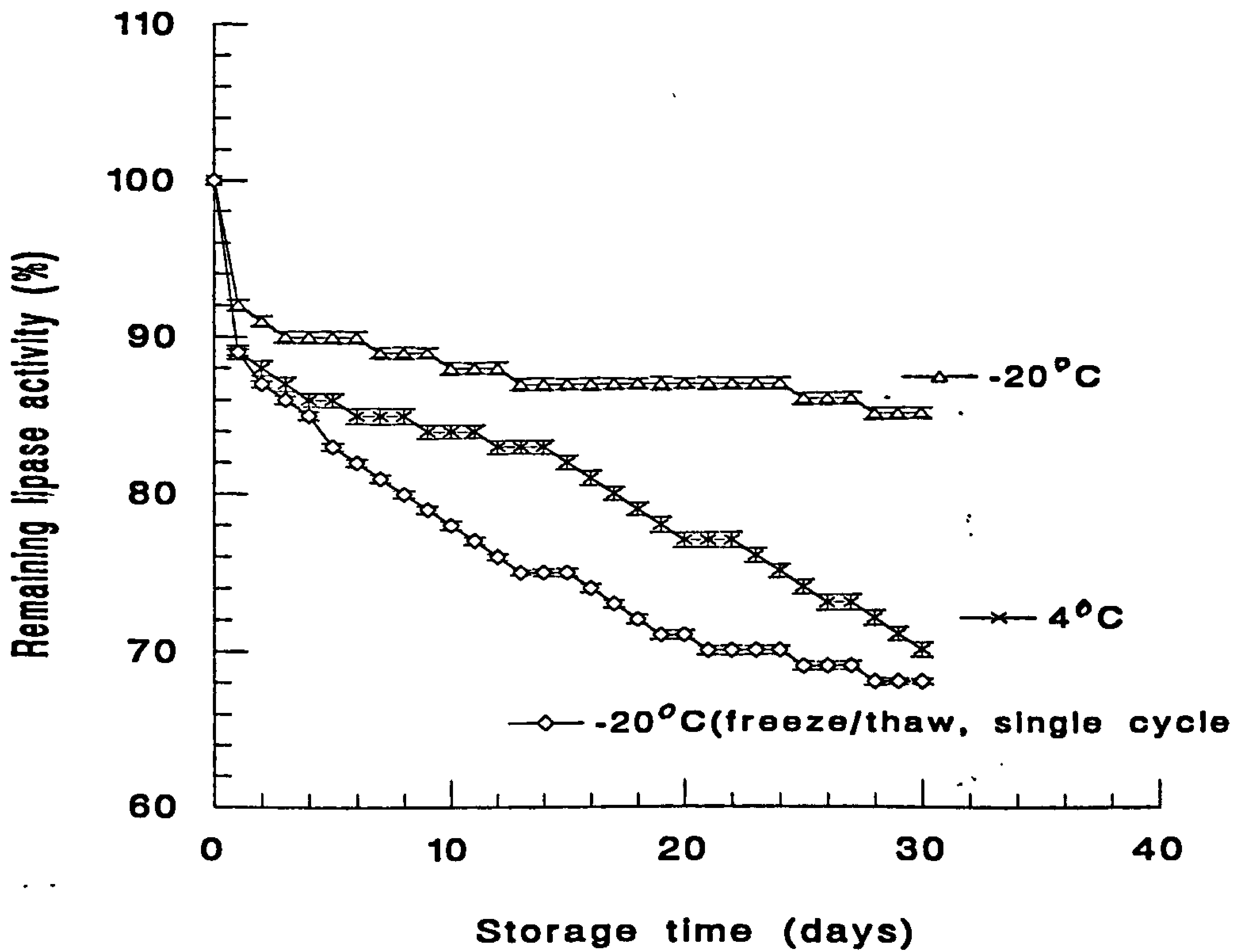


Figure 3.7

Effect of storage on stability of lipase activity in cell-free culture supernatants of *Streptomyces thermodiastaticus* at 4, -20 and at -20°C after freezing and thawing on every test day. The cultures were grown in the presence of olive oil at 50°C for 48h. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.8

Effect of storage on stability of lipase activity in cell-free culture supernatants of *Streptomyces thermodiastaticus* at 4, -20 and at -20°C after freezing and thawing on every test day. The cultures were grown in the presence of Tween 80 at 50°C for 48h. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.7

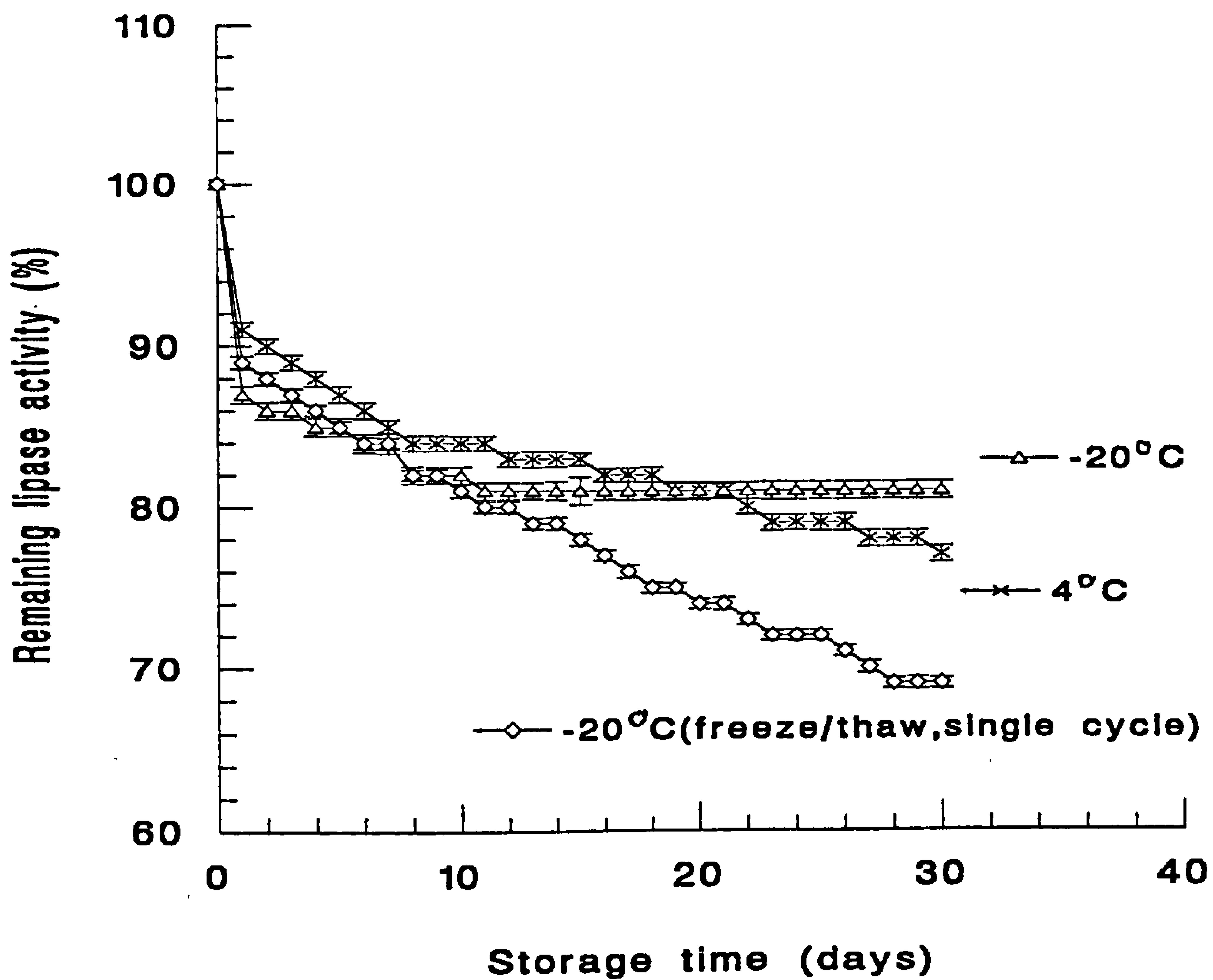
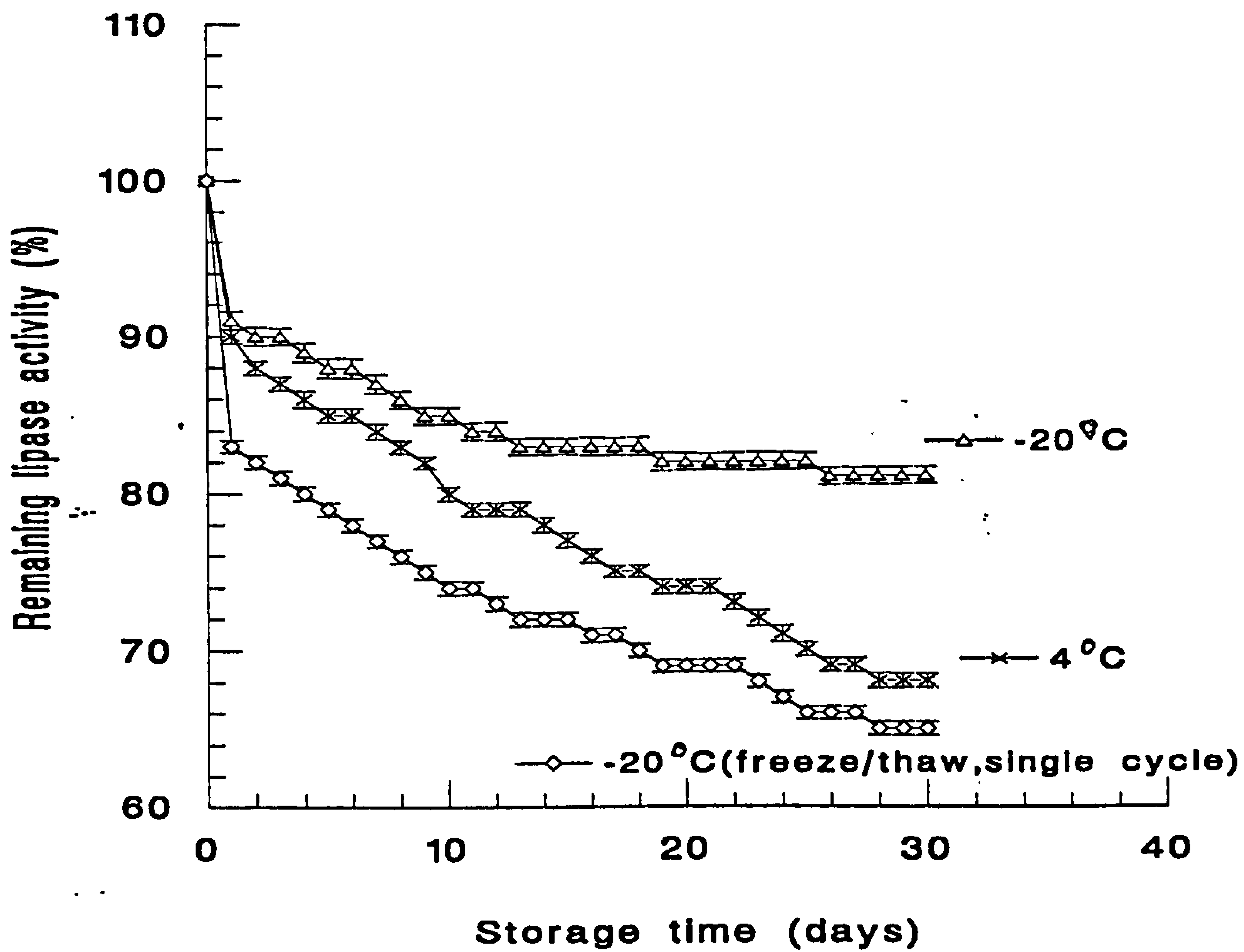


Figure 3.8



free culture supernatants could be stored for long periods at -20°C with minimal loss of activity.

3.6 Effect of pH on lipase activity

In order to examine the effect of pH on lipase activity, cell-free culture supernatants from the five *Streptomyces* species under investigation, were assayed for lipase activity, at pH values ranging from 2 to 10 obtained by using the buffers described before. The results obtained when olive oil or Tween 80 were used as the carbon source are shown in Figures 3.9 and 3.10 respectively and indicate that for every organism, irrespective of the lipid carbon source used for growth, the optimum pH for lipase activity is between 7.0 and 9.0 with the peak value being at pH 8.0.

3.7 Thermostability of lipase activity

The activity of thermophilic lipases is less affected by temperature than the activity of common commercial lipases which indicates that the thermophile lipases are more thermostable than the commercial lipases derived from mesophilic sources (Sigurgisladottir, *et al.*, 1993).

Lipases from culture supernatants of the *Streptomyces* species, grown in defined salts medium containing either olive oil, vegetable oil or Tween 80 as sole carbon source, were heated at 60, 70, 80 and 90°C for 5 minute time intervals up to 80 minutes and stored on ice. Heated samples were then assayed in order to determine lipase activity. Activity was found to be more stable at 70°C although, this was not significantly different from the activity plots obtained at 60 and 80°C. A similar pattern was observed for every organism tested. Figures 3.11 and 3.12 showed the difference in stability between 70°C and 90°C for *Streptomyces thermoviolaceus* and *S.*

Figure 3.9

Graph showing the pH optimum of lipase activity of cell-free culture supernatants from olive oil grown cultures of thermophilic *Streptomyces* species. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.10

Graph showing the pH optimum of lipase activity of cell-free culture supernatants from Tween 80 grown cultures of thermophilic *Streptomyces* species. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.9

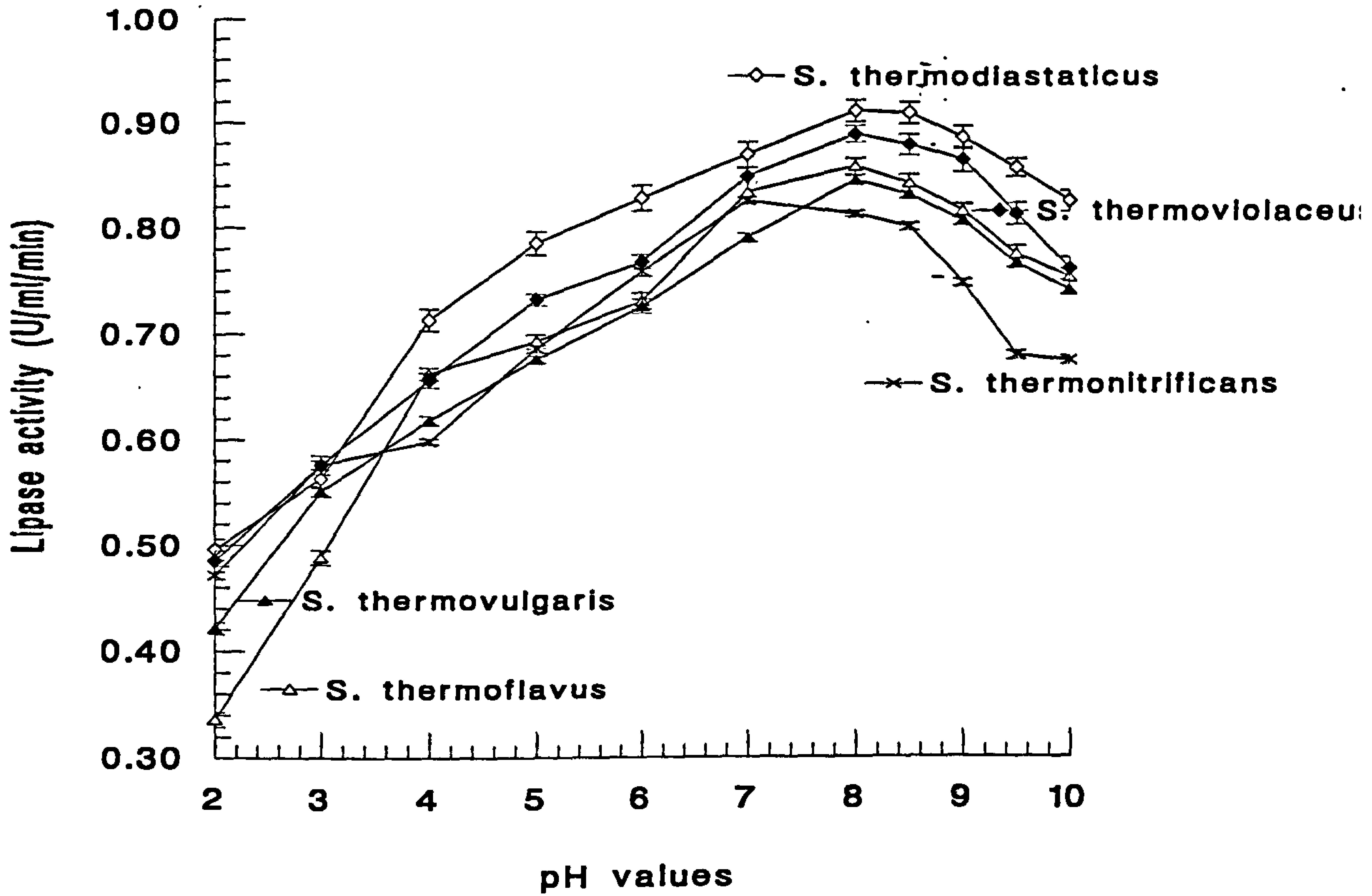


Figure 3.10

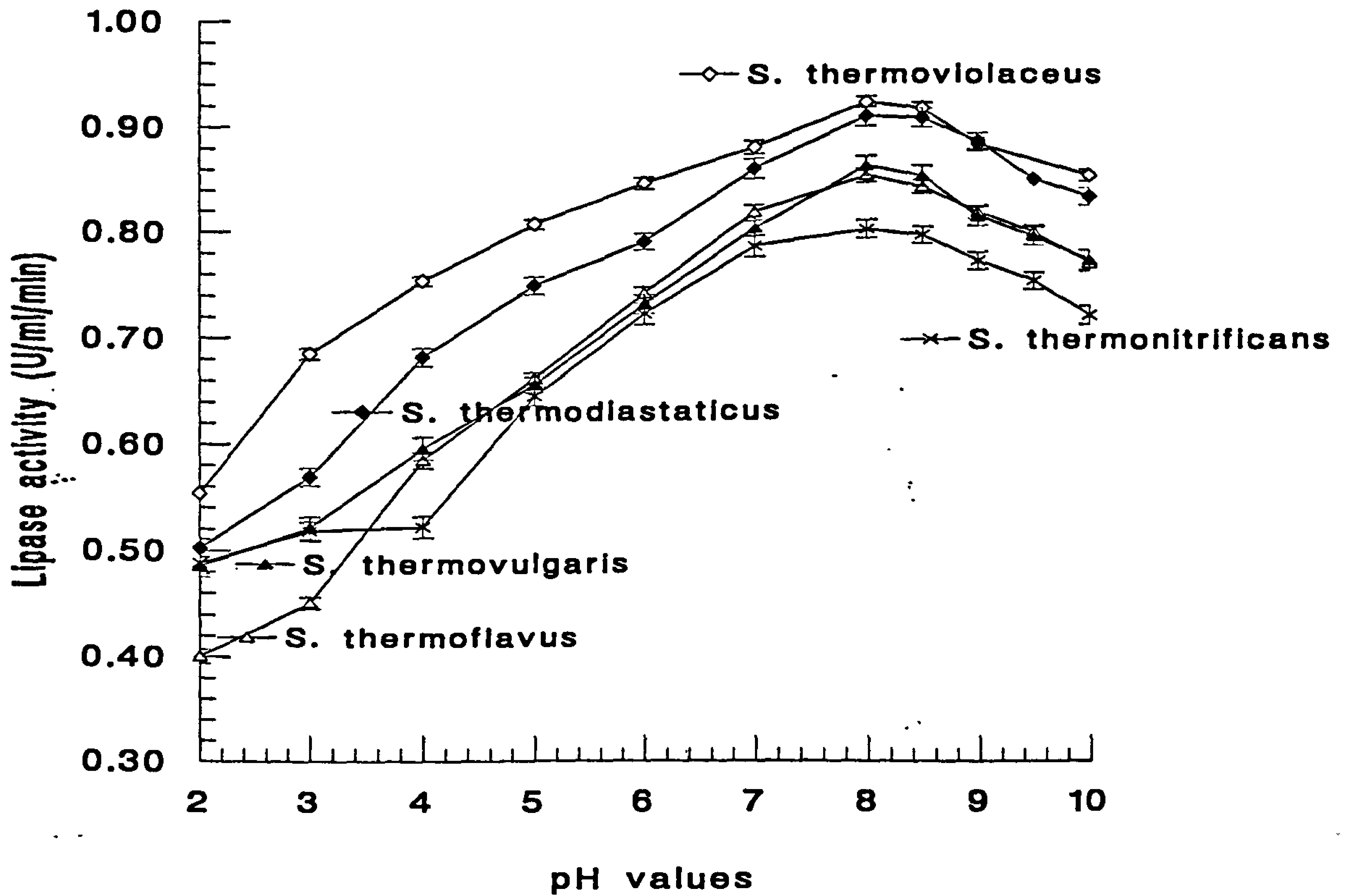
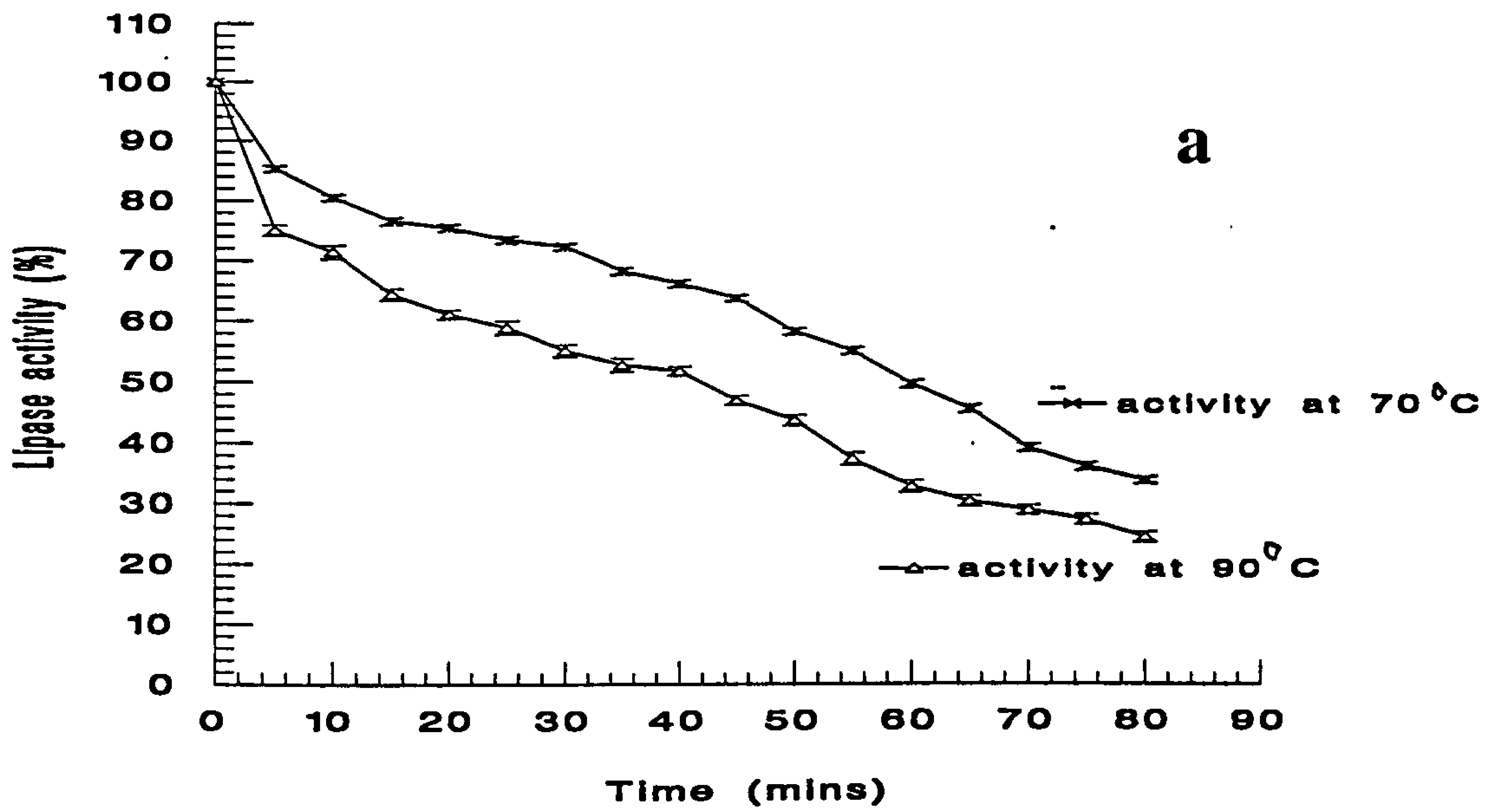
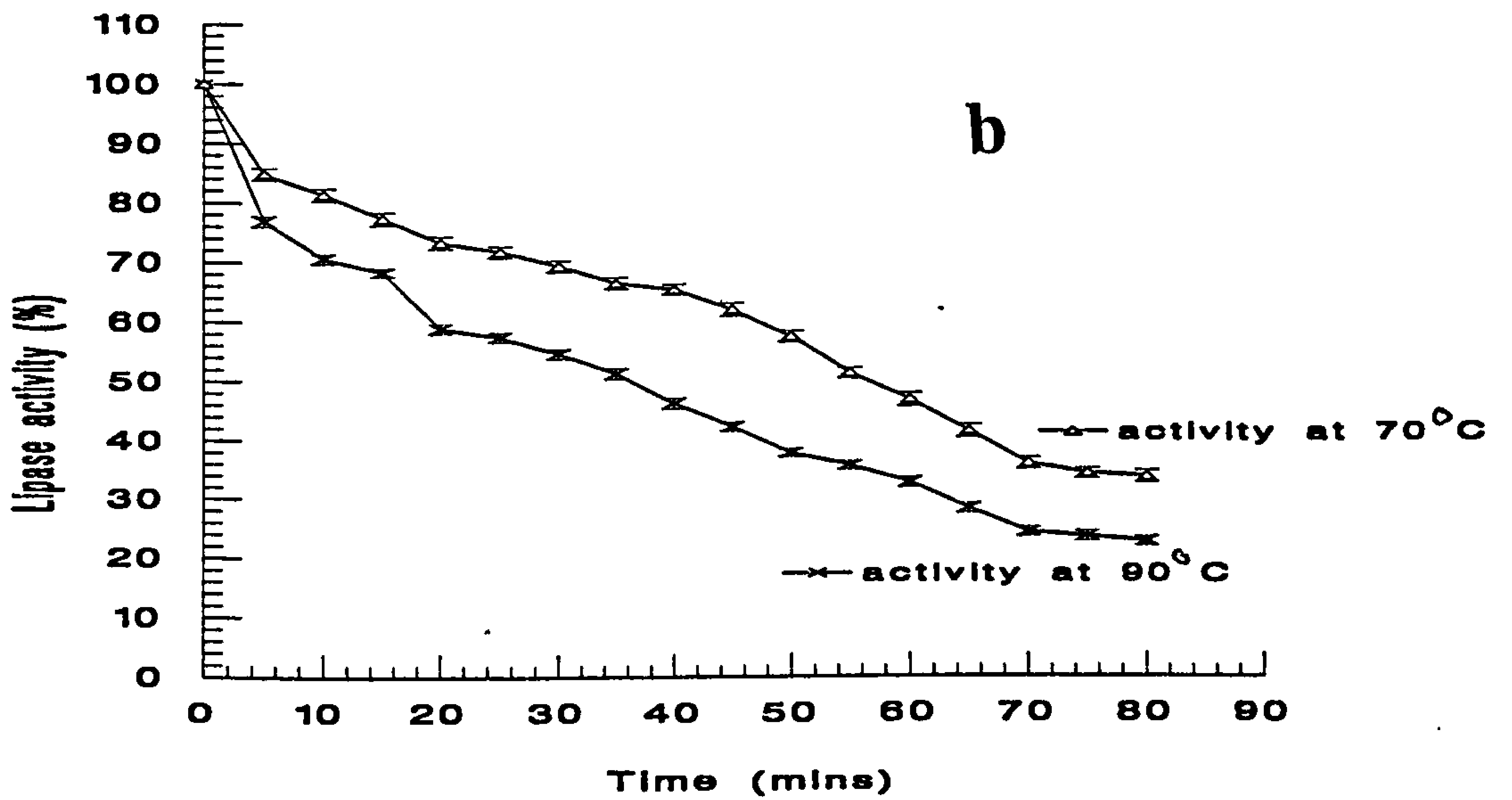


Figure 3.11

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermoviolaceus* from cultures grown at 50°C in the presence of a) olive oil, b) Tween 80 or c) vegetable oil and heated at 70 or 90°C. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.


a
Lipase activity (%)
Time (mins)
activity at 70 °C
activity at 90 °C


b
Lipase activity (%)
Time (mins)
activity at 70 °C
activity at 90 °C

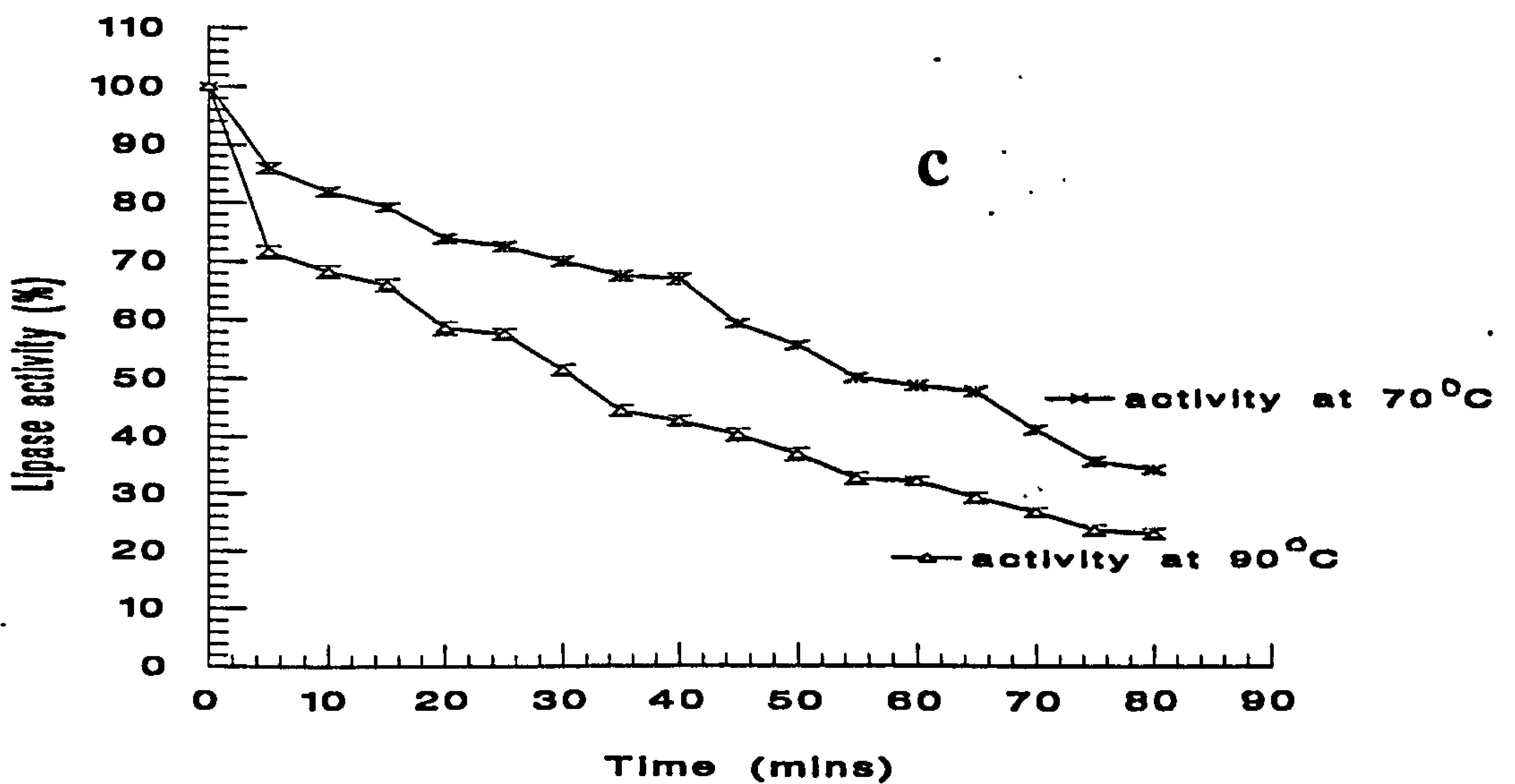
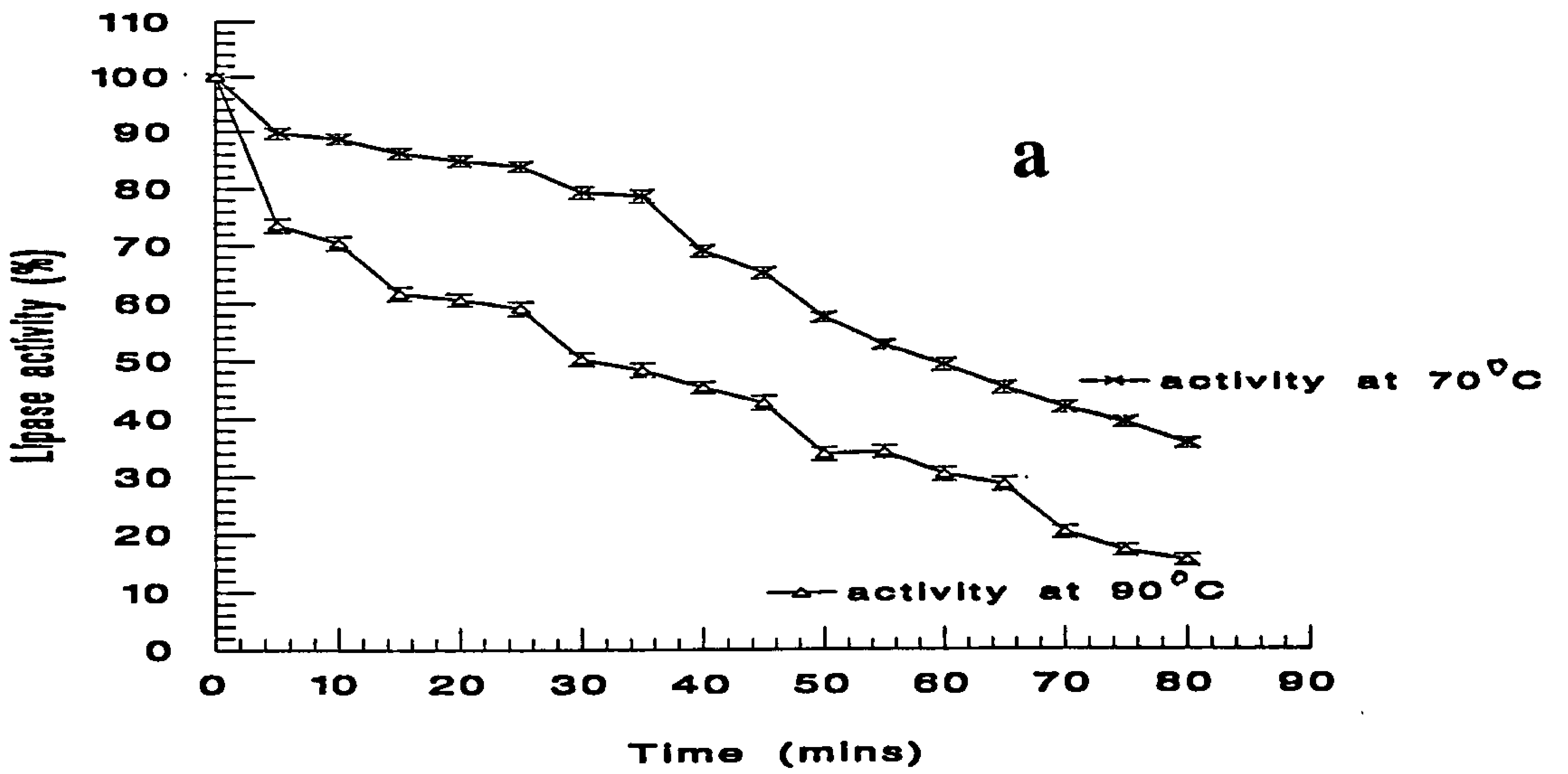
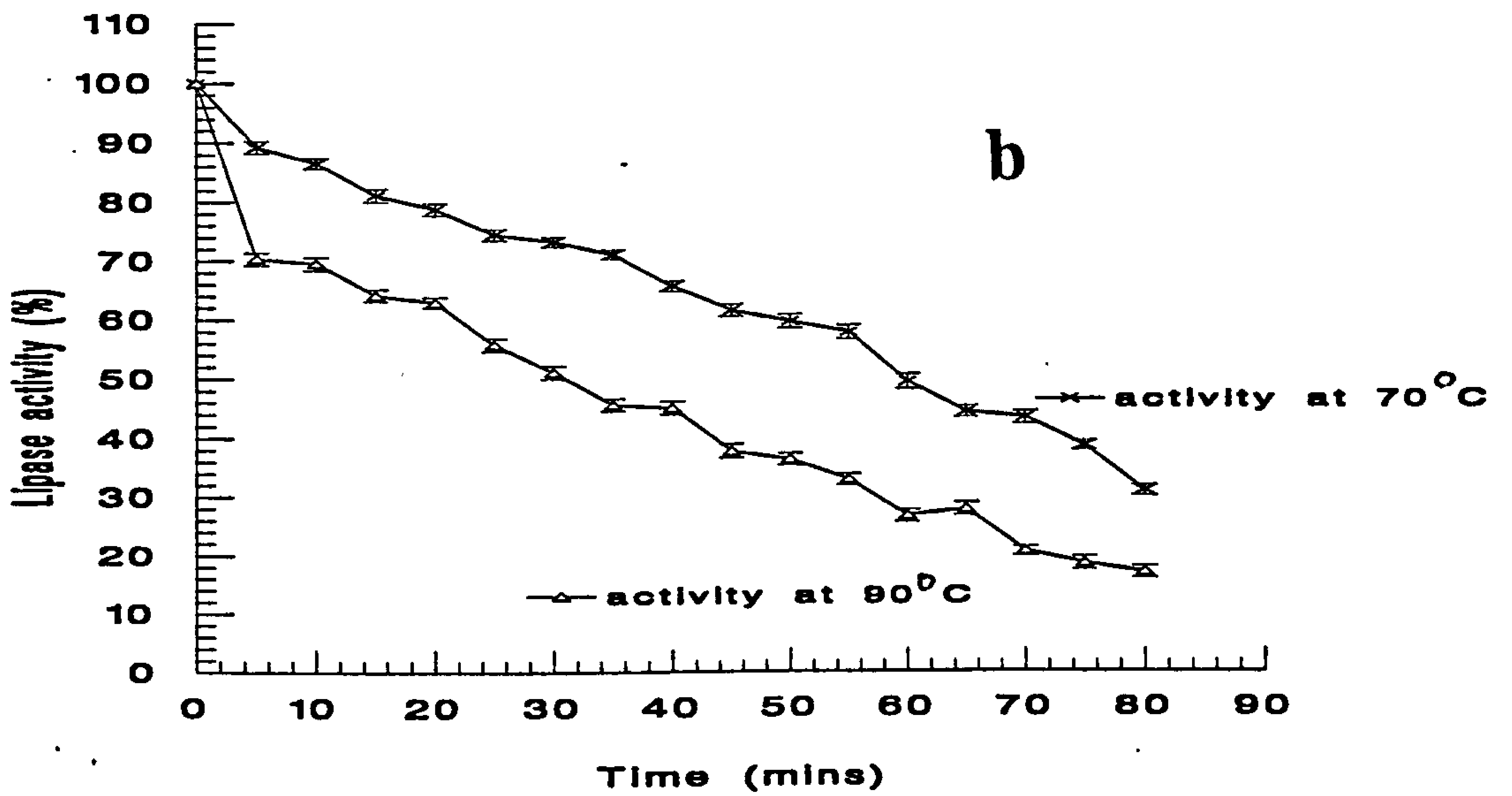
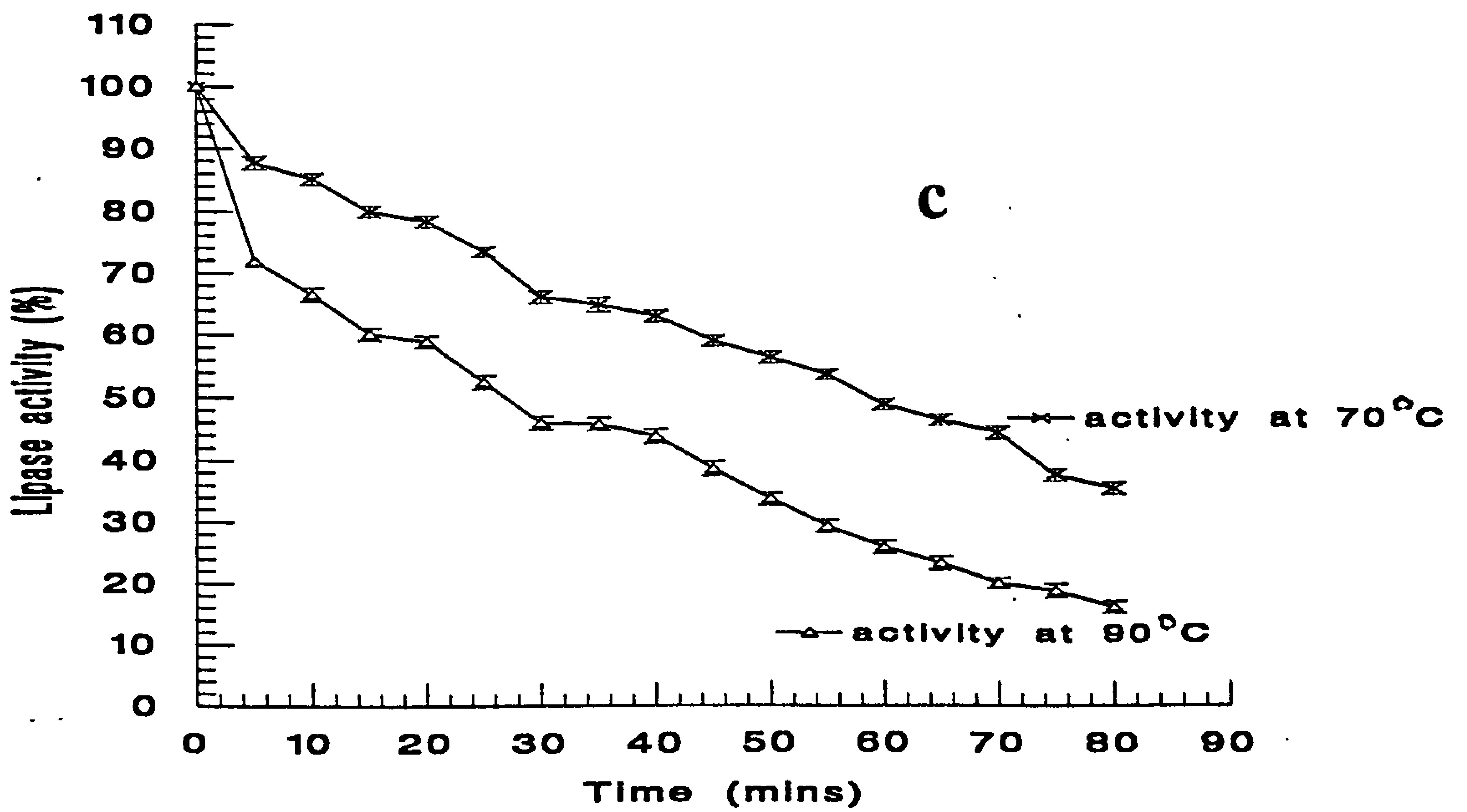

c
Lipase activity (%)
Time (mins)
activity at 70 °C
activity at 90 °C

Figure 3.12

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermodiastaticus* from cultures grown at 50°C in the presence of a) olive oil, b) Tween 80 or c) vegetable oil and heated at 70 or 90°C. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.


a
Lipase activity (%)
Time (mins)
activity at 70°C
activity at 90°C


b
Lipase activity (%)
Time (mins)
activity at 70°C
activity at 90°C


c
Lipase activity (%)
Time (mins)
activity at 70°C
activity at 90°C

thermodiastaticus grown with different lipid sources. Table 3.4 shows the time taken for the loss of 50% of the total activity (T_{50}) from *Streptomyces* species grown with olive oil. It was concluded that lipase activity was more stable at 70°C since T_{50} values were higher at that temperature. As a result, a temperature of 70°C was chosen for subsequent lipase activity assays.

From the results shown in figures 3.13 and 3.14, when *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were examined, it was concluded that lipase activities from olive oil containing media were more thermostable, over an assay period of 80 mins, when compared to Tween 80 or vegetable oil containing media. The same pattern was observed for the other *Streptomyces* species examined.

3.8 Effect of potential inhibitors on lipase activity

By using p-nitrophenyl palmitate as the enzyme substrate, extracellular lipase activity of cell-free supernatants from cultures growing with olive oil or Tween 80, was measured after pre incubation with different inhibitors. The assay temperature was 37°C. The results obtained for *Streptomyces thermoviolaceus* are shown in Table 3.5. TLCK, pCMB, and Leupeptin were all without effect. EDTA and o-phenanthroline (metallo-enzyme inhibitors) showed greatest inhibition in the range of 61 to 70% for olive oil and 62 to 71% for Tween 80 cultures. PMSF (serine, involved in active site, inhibitor) exerted a 29.5 and 30.15% inhibition for olive oil and Tween 80 cultures respectively. Addition of EDTA or o-phenanthroline together with PMSF resulted in 89.30 to 92.20% inhibition. This suggested the presence of two types of lipase activity, one metallo and one serine.

Table 3.4 Time taken for the loss of 50% of the total lipase activity (T_{50}) from thermophilic *Streptomyces* species assayed at 70 or 90°C. Olive oil was used as the carbon source (mean±SD; n=3).

Organism	T_{50} at 70°C (mins)	T_{50} at 90°C (mins)
Streptomyces thermoviolaceus	62±0.98	38±0.87
Streptomyces thermodiastaticus	58±0.95	32±0.84
Streptomyces thermoflavus	54±0.97	29±0.93
Streptomyces thermovulgaris	51±0.96	26±0.86
Streptomyces thermonitrificans	47±0.89	23±0.87

Figure 3.13

Effect of lipid source on the thermostability of lipase activity of *Streptomyces thermoviolaceus* from cultures grown at 50°C in the presence of olive oil, Tween 80 or vegetable oil and heated at 70°C. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.14

Effect of lipid source on the thermostability of lipase activity of *Streptomyces thermodiastaticus* from cultures grown at 50°C in the presence of olive oil, Tween 80 or vegetable oil and heated at 70°C. Data are representative of at least 3 separate replicates. Standard error bars represent the standard errors of the obtained data.

Figure 3.14

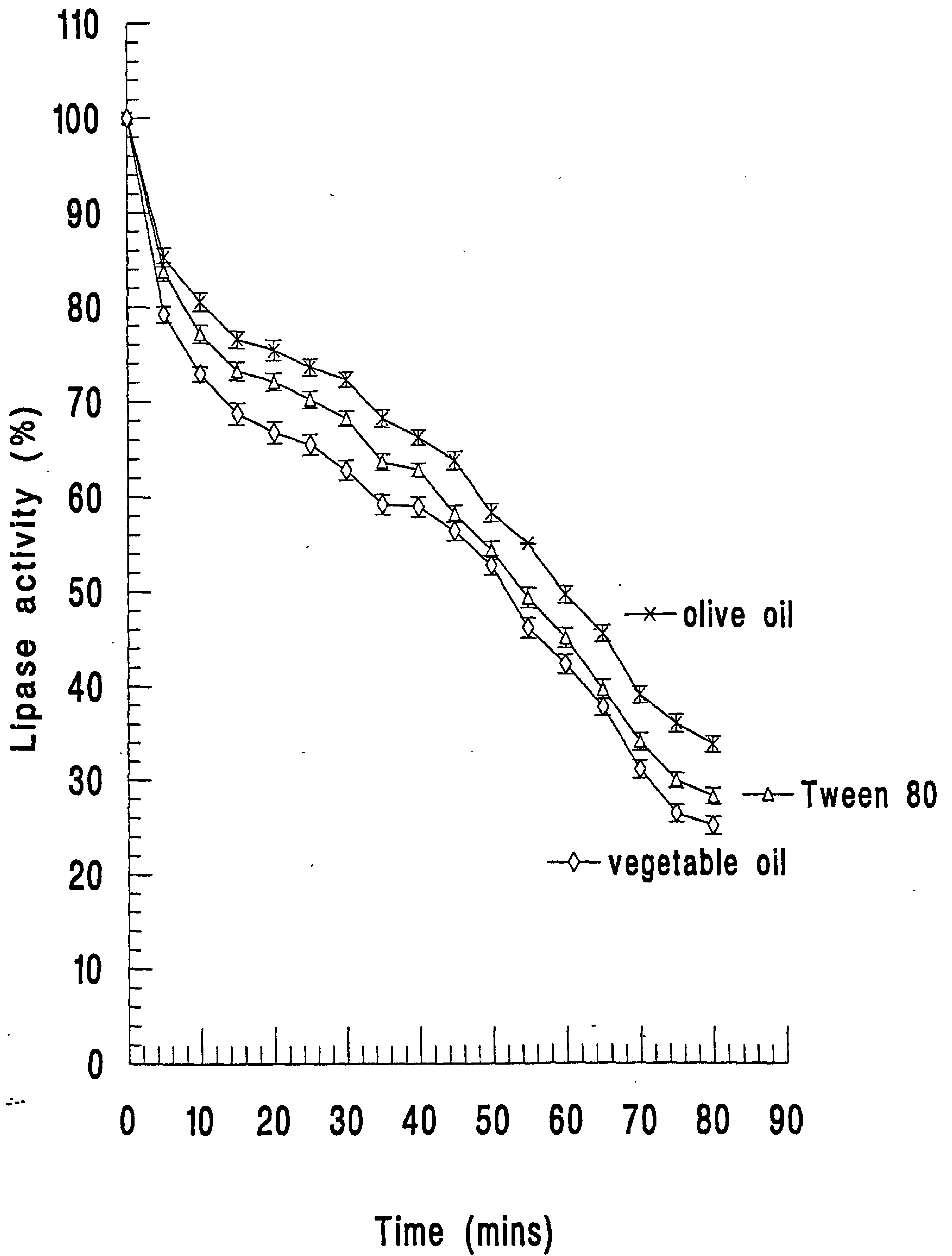


Table 3.5 Effect of various inhibitors on lipase activity from *Streptomyces thermoviolaceus*.

Data are representative of 4 separate determinations (mean±SD; n=4).

Streptomyces thermoviolaceus

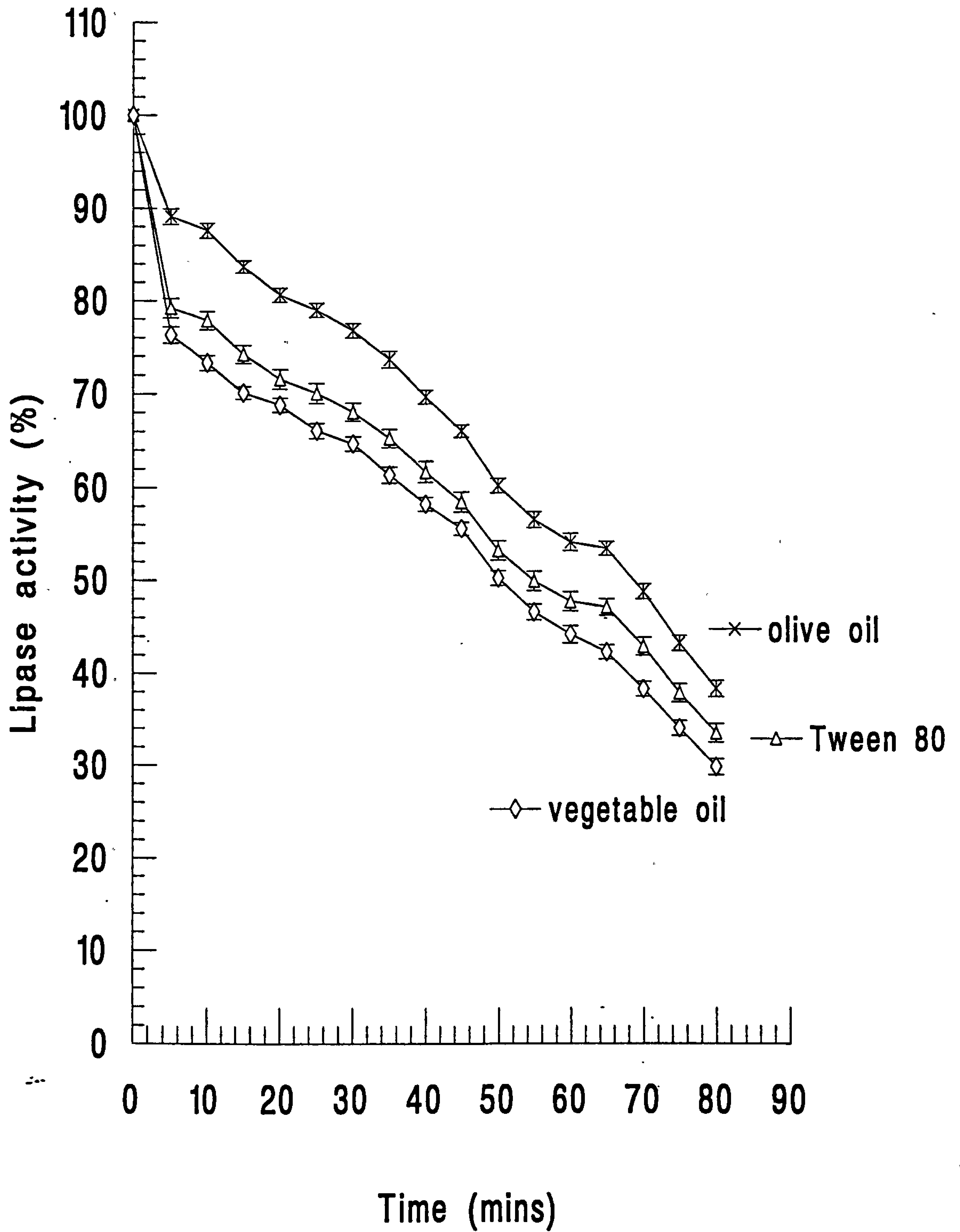
Inhibitors	**% Inhibition**	**% Inhibion**
(5mM)	**olive oil**	**Tween 80**
EDTA	70.08±0.023	71.19±0.011
o-phenanthroline	61.15±0.024	62.09±0.019
PMSF	29.50±0.019	30.15±0.013
TLCK	0	0
pCMB	0	0
Leupeptin	0	0
EDTA+PMSF	91.19±0.027	92.20±0.027
o-phenanthroline+PMSF	89.30±0.014	90.05±0.026

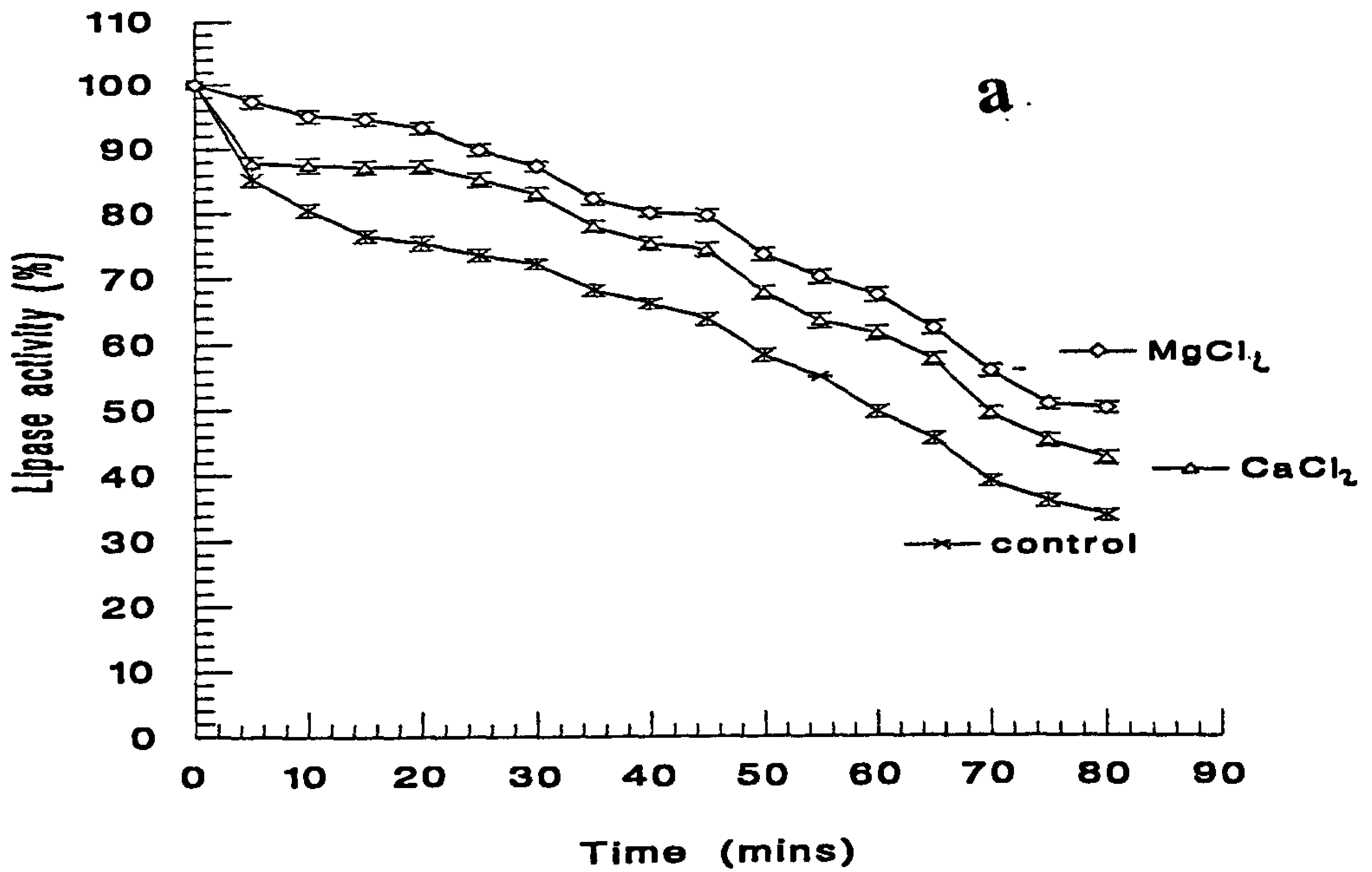
3.9 Effect of metals on lipase activity

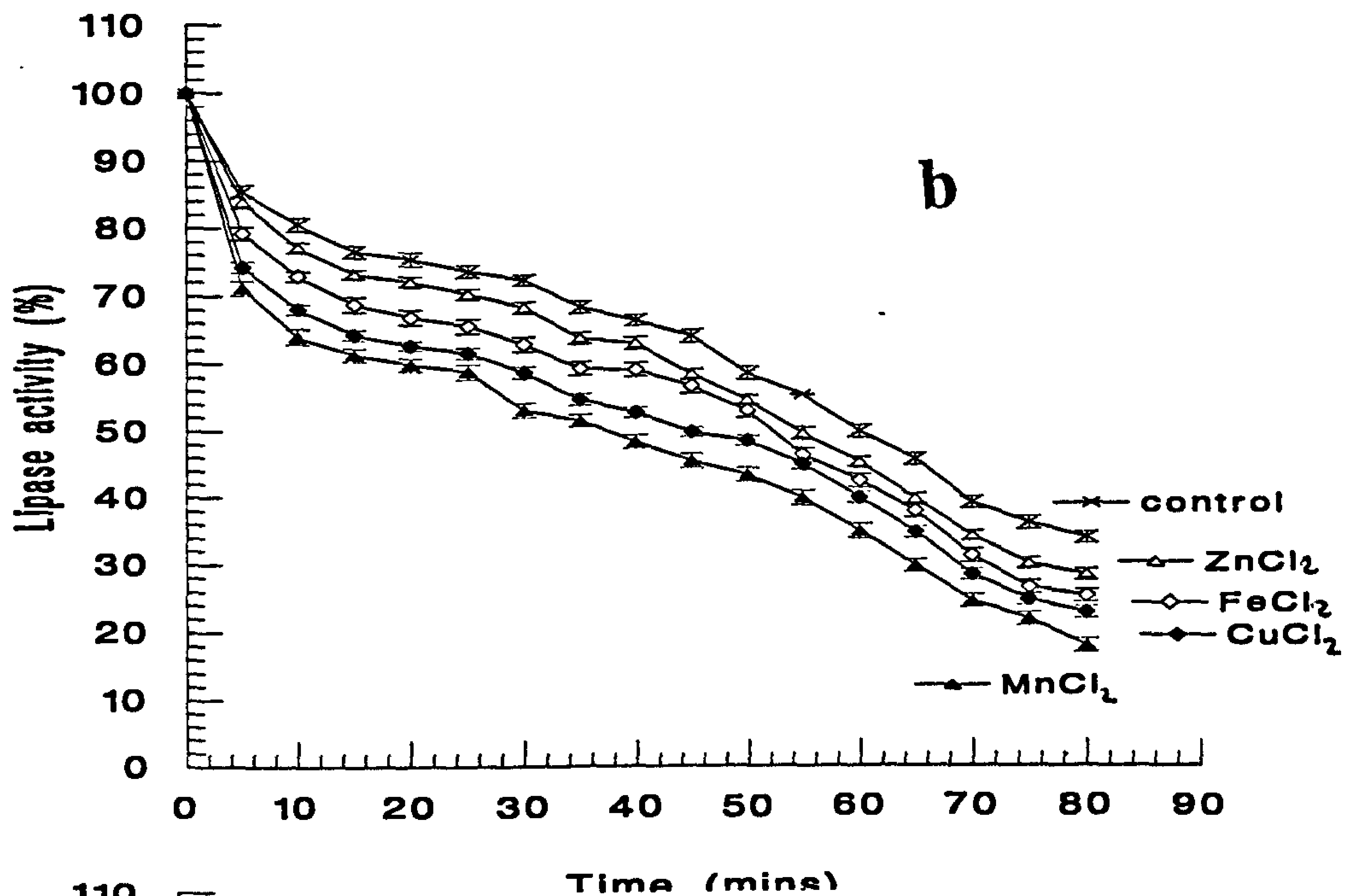
Early studies by Doelman, (1986) revealed that many heavy metals are essential for microbial growth and are required in low concentrations as microelements. Heavy metal-microbe interactions include inhibition of enzymatic activities, binding to cell structures and precipitation of nutrients (Abbas and Edwards, 1990). The presence or absence of heavy metals is also known to affect secondary metabolism, as they are responsible for activation of some of the biosynthetic pathways. Furthermore, many hydrolytic enzymes require divalent cations for activity.

The effects of divalent metals on lipase activity of thermophilic *Streptomyces* was therefore investigated. Figures 3.15, 3.16, 3.17 and 3.18 showed the results obtained for *Streptomyces thermoviolaceus* and *S. thermodiastaticus* cultures grown with olive oil or Tween 80. It was concluded that when cell-free culture supernatants were heated at 70°C and were mixed with divalent metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} lipase activity was inhibited, whereas when Mg^{2+} and Ca^{2+} were used the activity was enhanced. Additionally, it was concluded that the effect of Mg ions over the effect of Ca ions was greater since T_{50} values were higher when the former metal was added prior to the assay for lipase activity (Table 3.6). The presence of EDTA and o-phenanthroline (chelating agents) inhibited lipase activity. When Mg^{2+} and Ca^{2+} divalent ions were added on EDTA treated samples, some restoration of the lost activity was observed (Table 3.7). This showed that when lipases produced by cultures grown in olive oil, vegetable oil and Tween 80 were inhibited by chelating agents, they could be reactivated by addition of Mg^{2+} and Ca^{2+} which suggests that these ions play an important role in lipase activity of the thermophilic *Streptomyces* examined.

Figure 3.13




a
Lipase activity (%)
Time (mins)
MgCl2
CaCl2
control


b
Lipase activity (%)
Time (mins)
control
ZnCl2
FeCl2
CuCl2
MnCl2

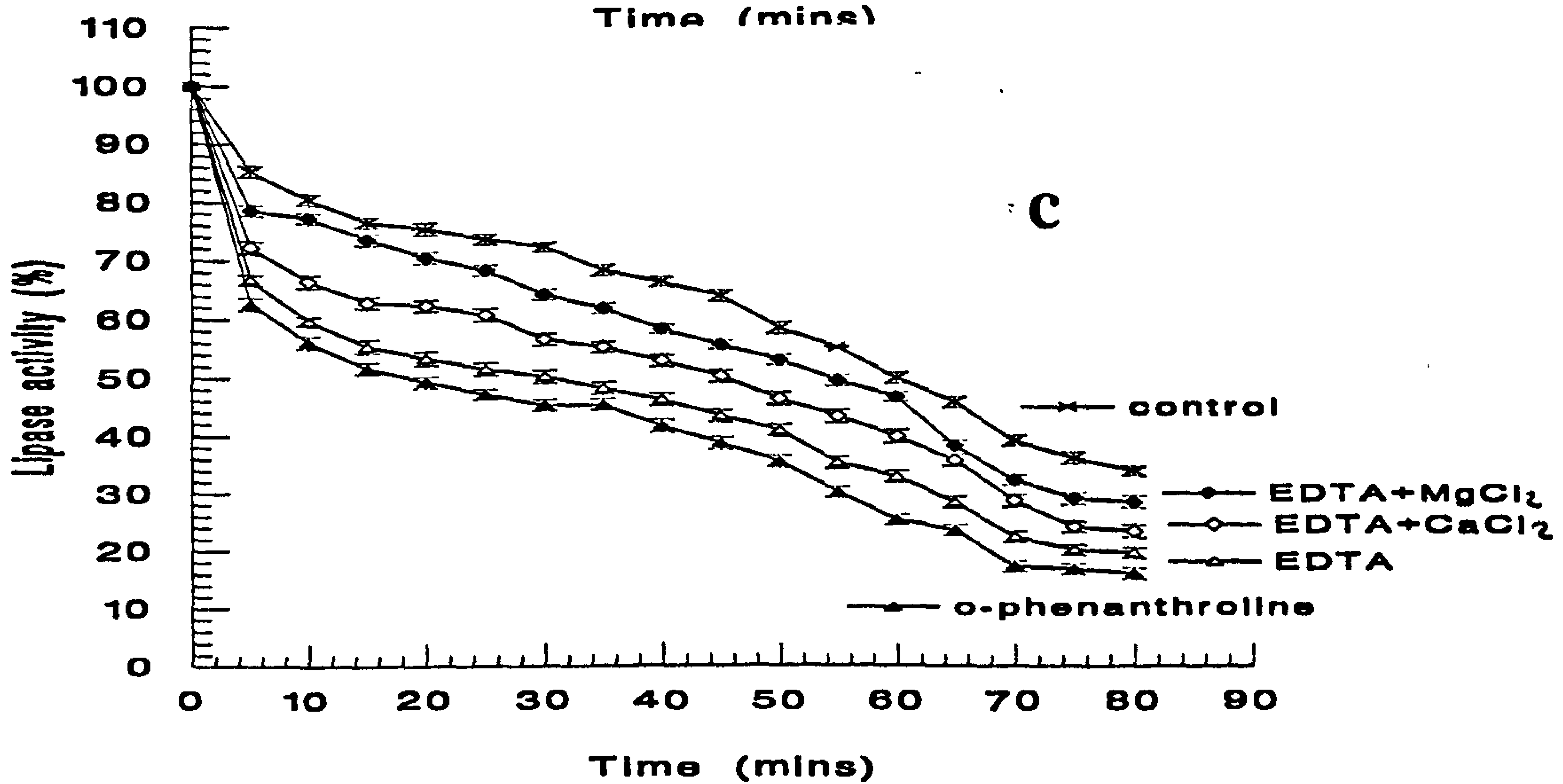
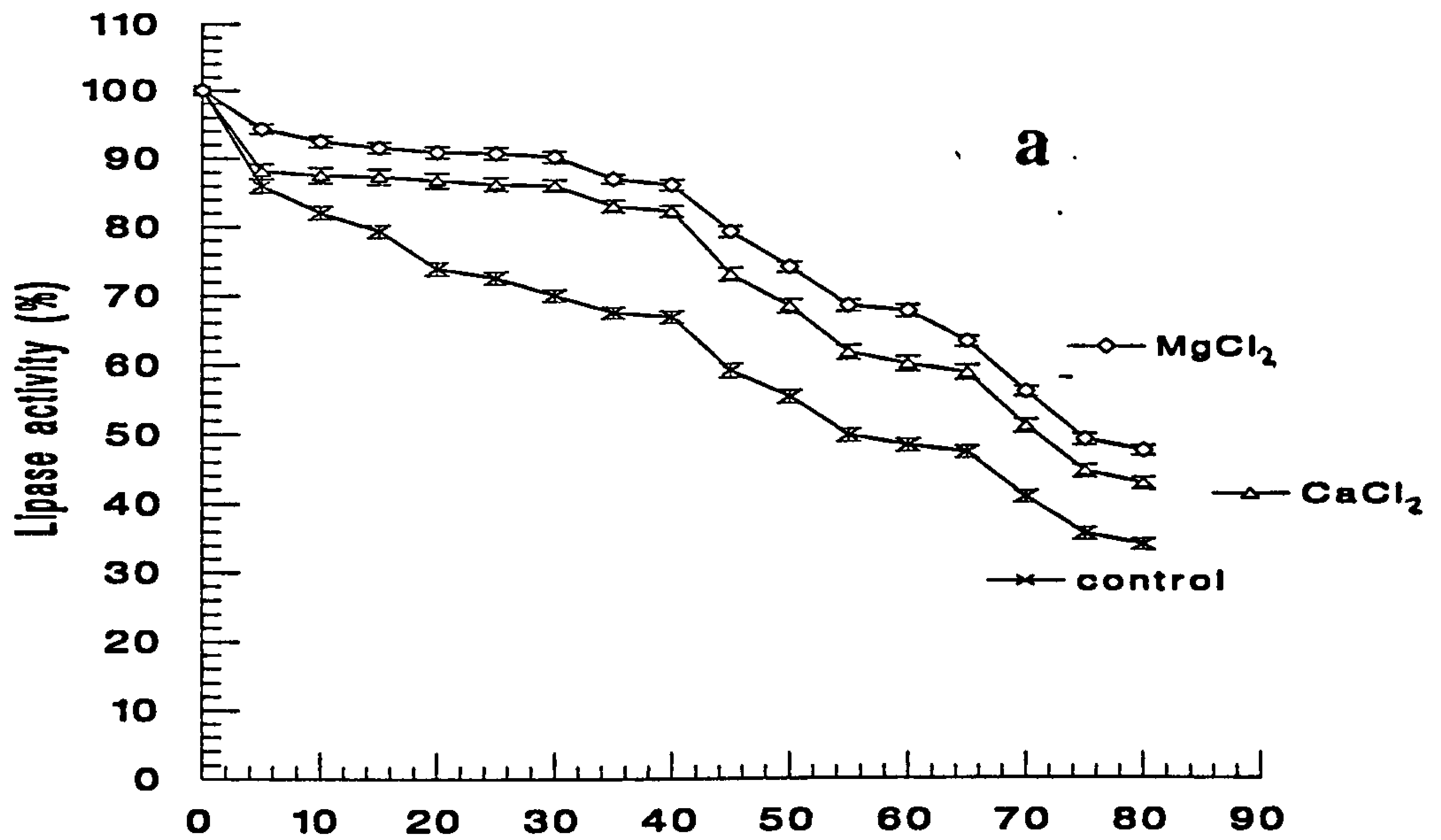
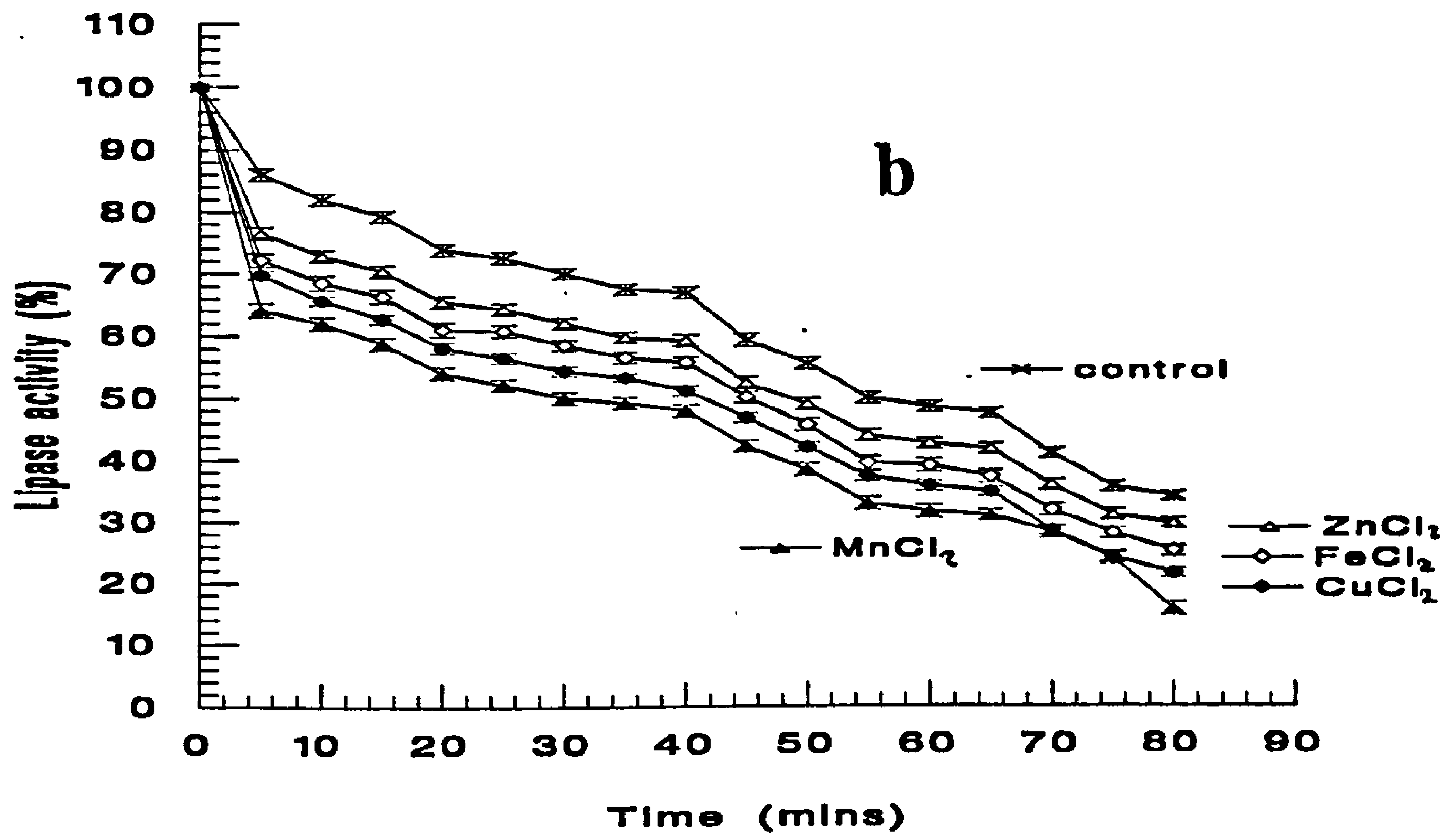

c
Lipase activity (%)
Time (mins)
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline

Figure 3.16 a, b and c

Graph showing the effect of metal ions and inhibitors on the thermostability of lipase activity at 70°C of *Streptomyces thermoviolaceus* from cultures grown at 50°C in the presence of Tween 80. Data are representative of 3 separate replicates. Standard error bars represent the standard errors of the obtained data.


a
Lipase activity (%)
MgCl2
CaCl2
control


b
Lipase activity (%)
control
ZnCl2
FeCl2
CuCl2
MnCl2
Time (mins)

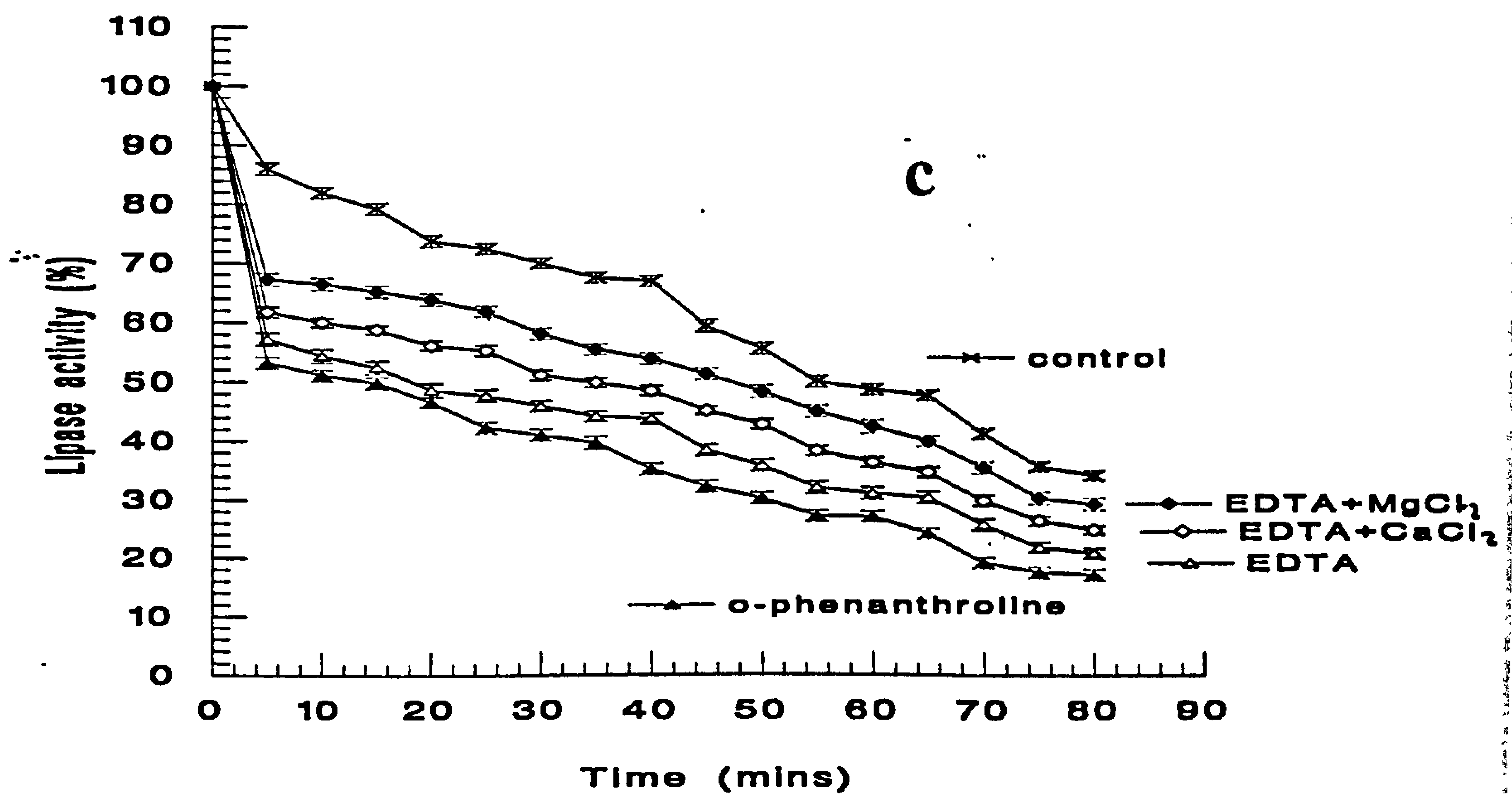
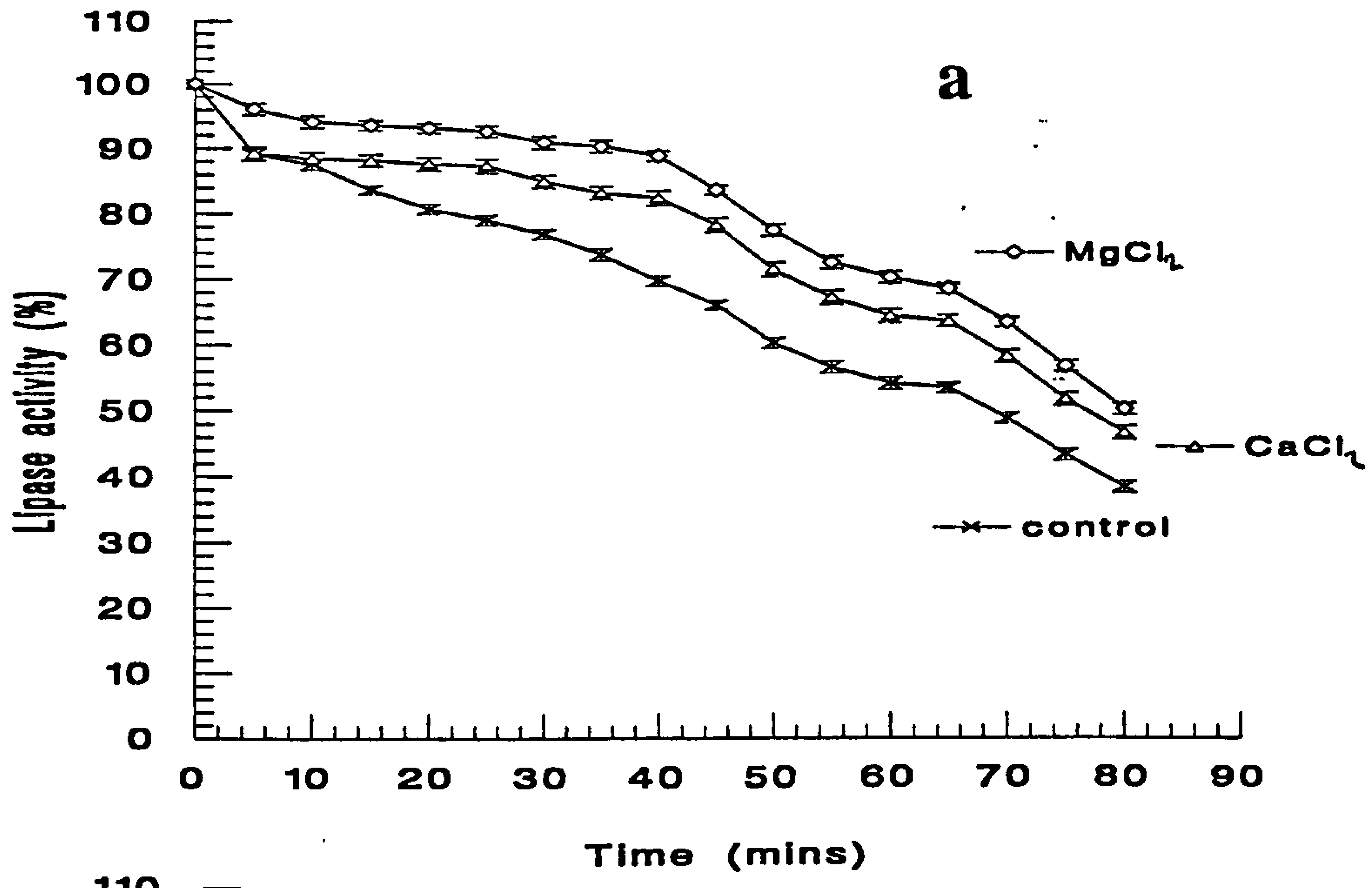
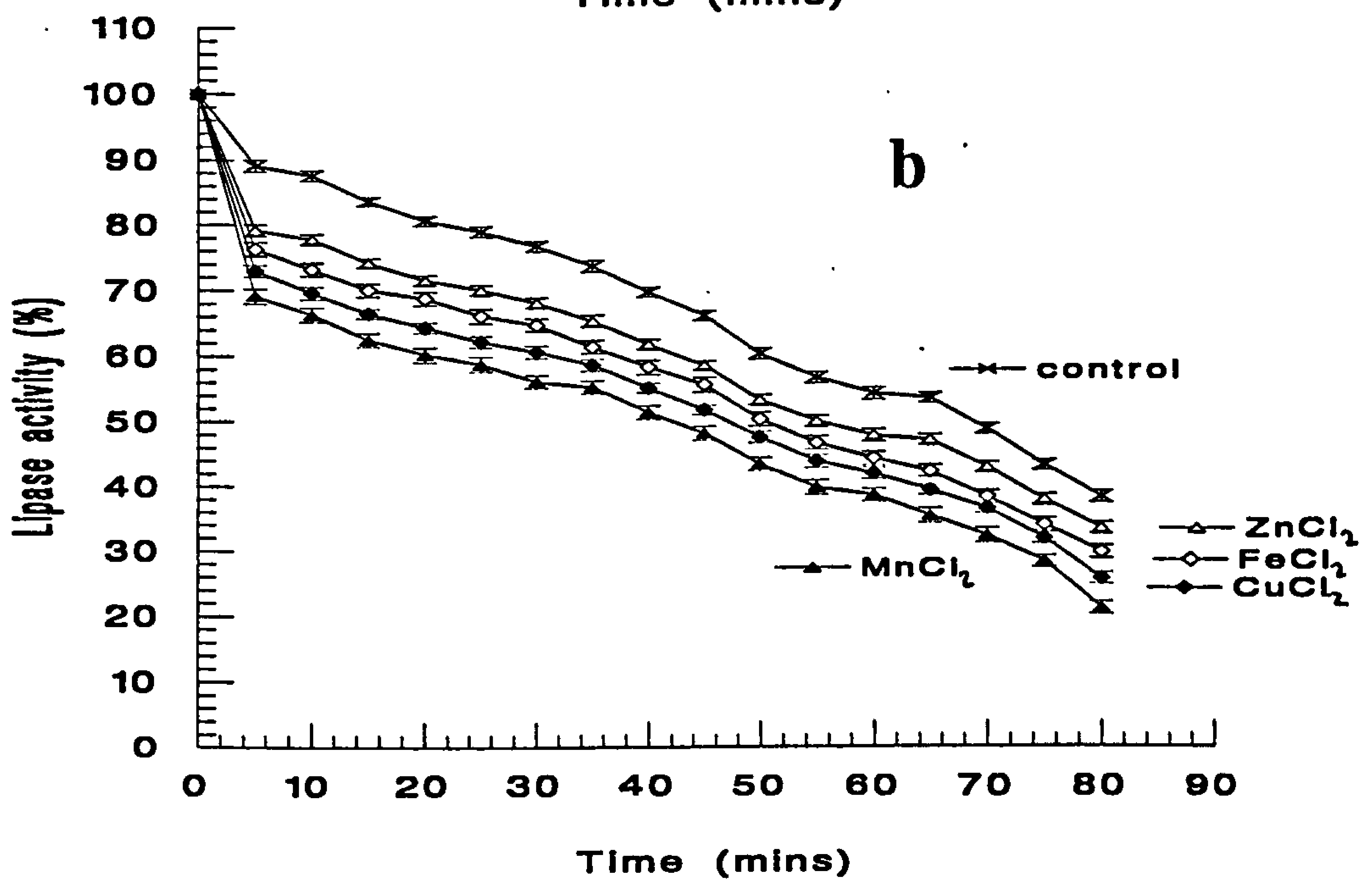

c
Lipase activity (%)
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline
Time (mins)

Figure 3.17 a, b and c

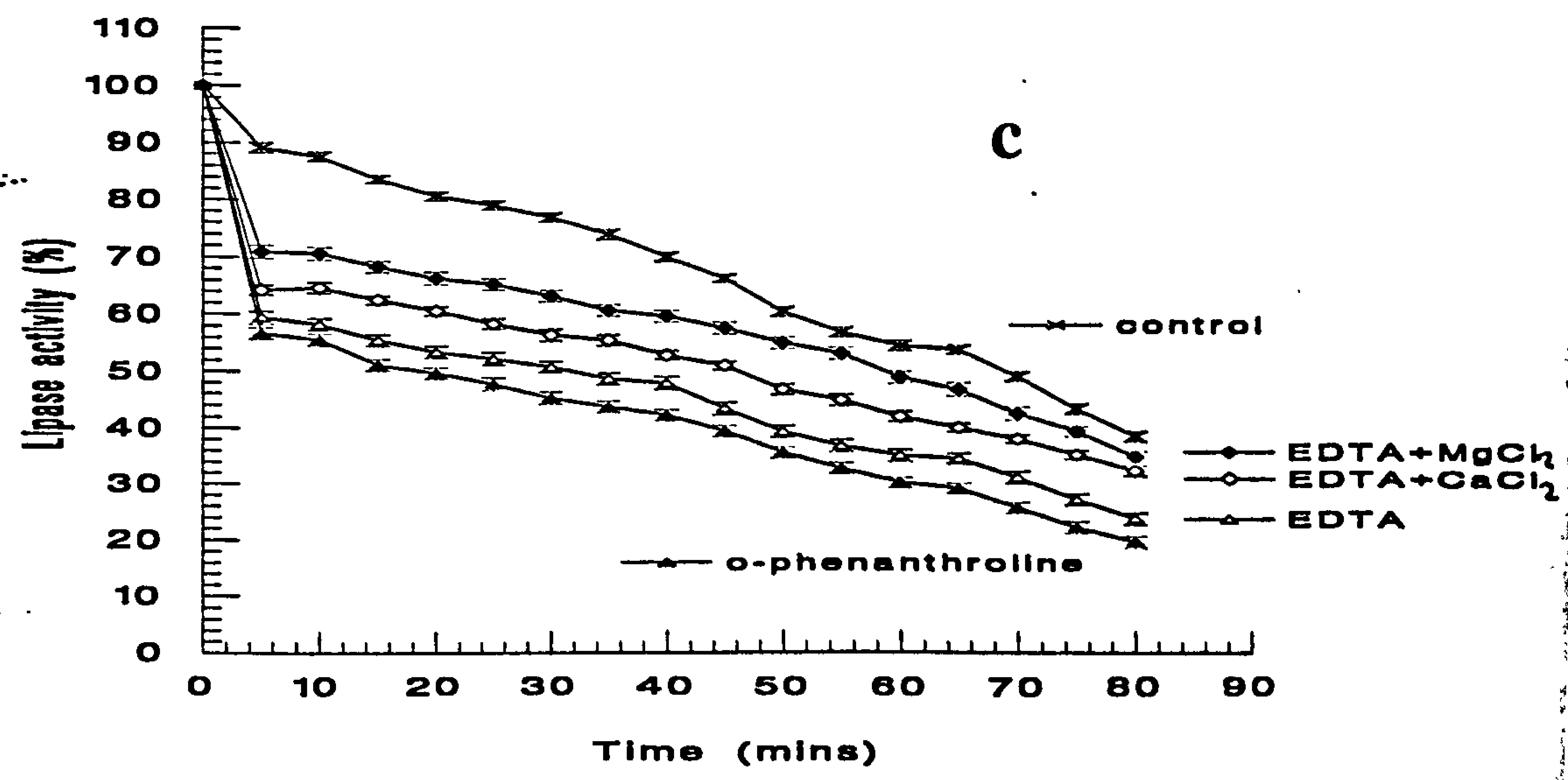
Graph showing the effect of metal ions and inhibitors on the thermostability of lipase activity at 70°C of *Streptomyces thermodiastaticus* from cultures grown at 50°C in the presence of olive oil. Data are representative of 3 separate replicates. Standard error bars represent the standard errors of the obtained data.



a
Lipase activity (%)
Time (mins)
MgCl2
CaCl2
control



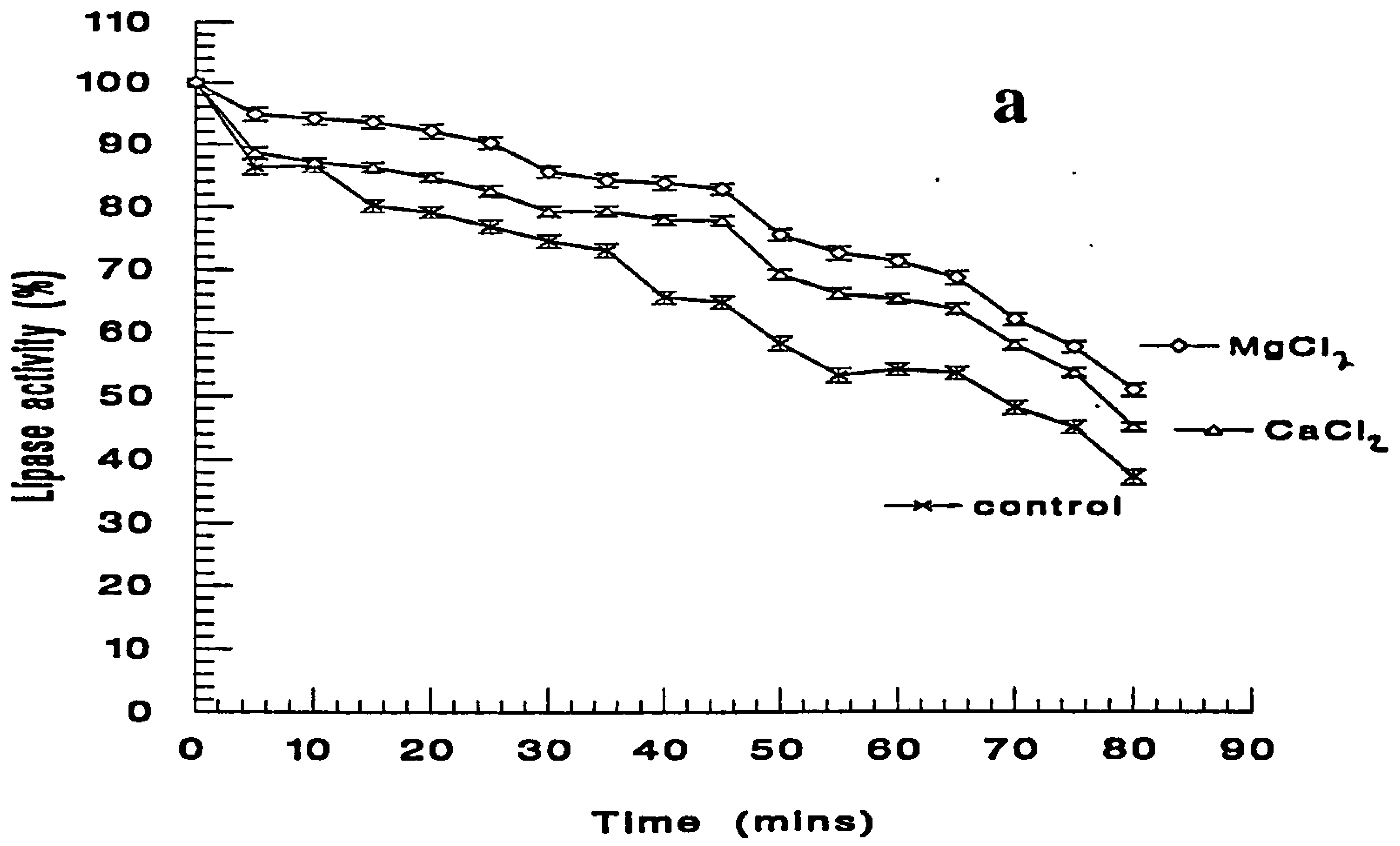
b
Lipase activity (%)
Time (mins)
control
ZnCl2
FeCl2
CuCl2
MnCl2



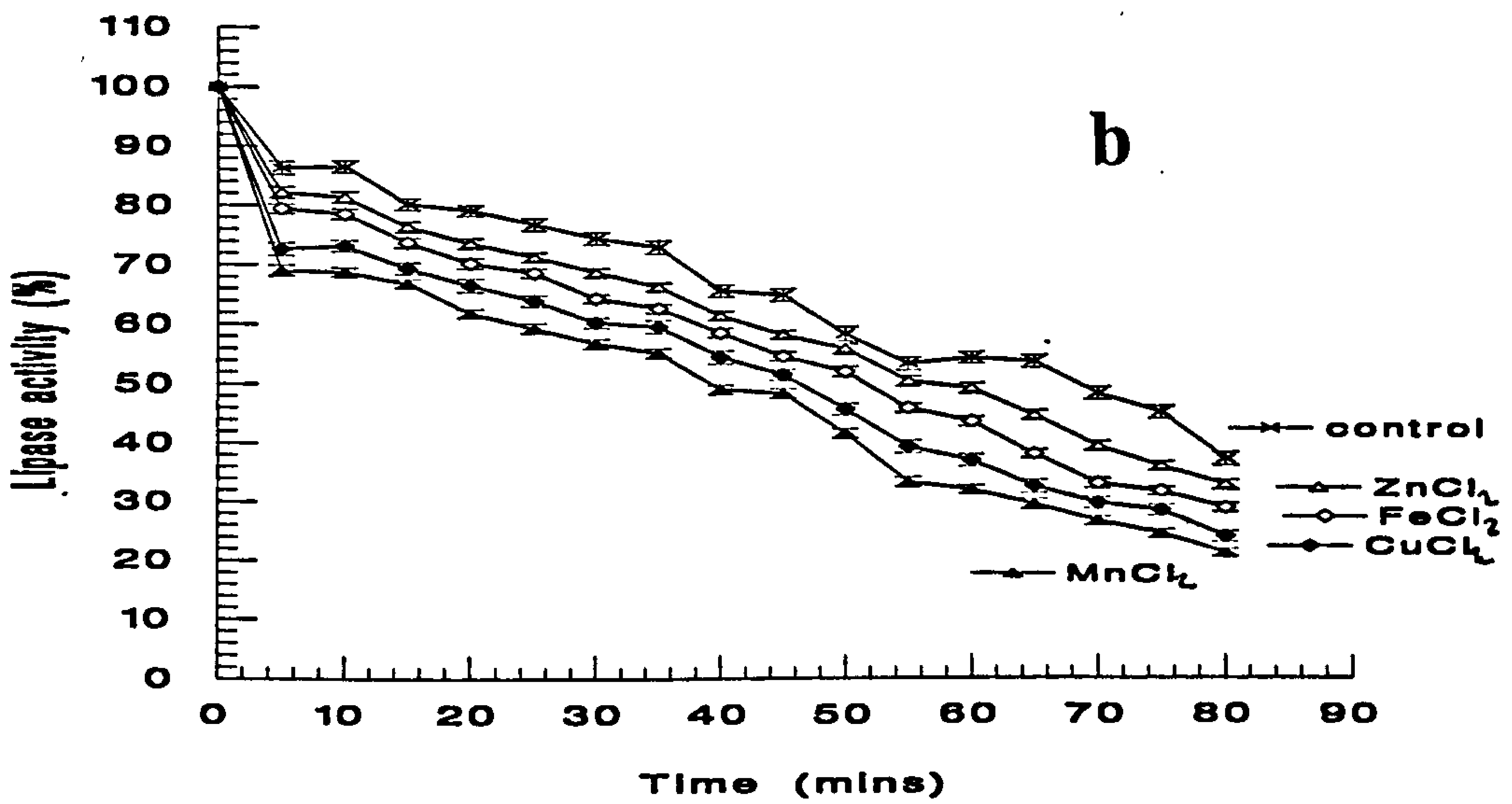
c
Lipase activity (%)
Time (mins)
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline

Figure 3.18 a, b and c

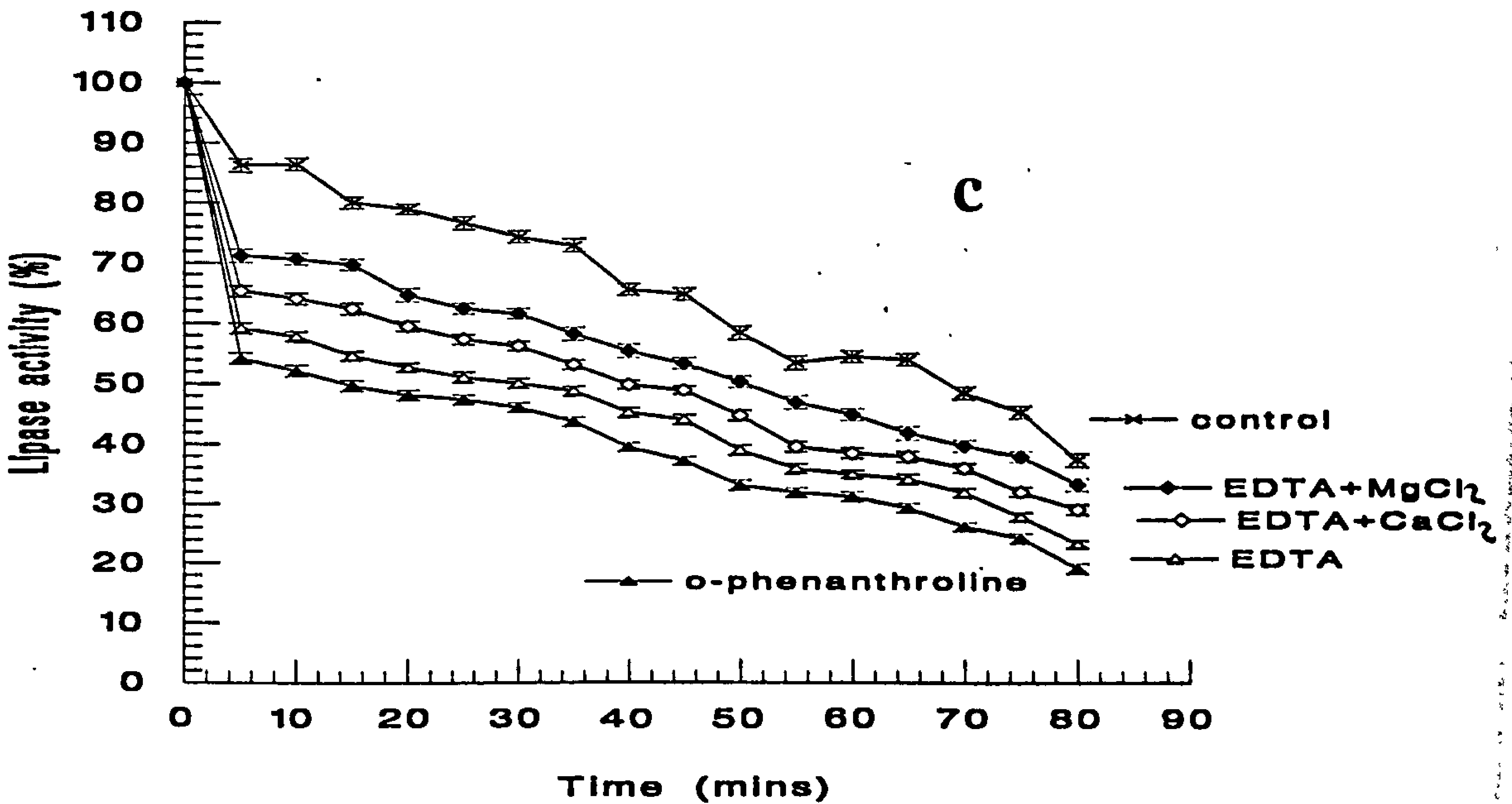
Graph showing the effect of metal ions and inhibitors on the thermostability of lipase activity at 70°C of *Streptomyces thermodiastaticus* from cultures grown at 50°C in the presence of Tween 80. Data are representative of 3 separate replicates. Standard error bars represent the standard errors of the obtained data.



a
Lipase activity (%)
Time (mins)
MgCl2
CaCl2
control



b
Lipase activity (%)
Time (mins)
control
ZnCl2
FeCl2
CuCl2
MnCl2



c
Lipase activity (%)
Time (mins)
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline

Table 3.6 Time taken for the loss of 50% of the total lipase activity (T_{50}) from *S. thermoviolaceus* and *S. thermodiastaticus* grown in the presence of olive oil or Tween 80. The assay temperature was 70°C (mean±SD;n=3).

	S. thermoviolaceus		*S.thermodiastaticus*	
Lipid source	T_{50} at 70°C (mins)		T_{50} at 70°C (mins)	
	MgCl	CaCl	MgCl	CaCl
olive oil	83±0.96	81±0.89	74±0.76	69±0.81
Tween 80	80±0.75	76±0.65	73±0.86	68±0.91

Table 3.7 Effect of metal ions on EDTA treated lipase activity from *Streptomyces thermoviolaceus*, grown in olive oil or Tween 80, for 48hours at 50°C. Results represent 3 separate determinations (mean±SD; n=3).

	Olive oil		**Tween 80**	
Treatment (5mM)	% Activation	% Inhibition	% Activation	% Inhibition
EDTA control	0	0	0	0
$+ZnCl_2$	0	3.6±0.017	0	3.4±0.019
$+FeCl_2$	0	3.9±0.014	0	4.3±0.023
$+CuCl_2$	0	4.7±0.024	0	5.2±0.013
$+MnCl_2$	0	5.5±0.015	0	6.7±0.010
$+MgCl_2$	32±0.027	0	29.4±0.017	0
$+CaCl_2$	27.2±0.024	0	26.8±0.014	0

3.10 Effect of metal ions and inhibitors on intracellular lipase activity

Intracellular lipases from *Streptomyces thermoviolaceus*, grown in olive oil, were treated with different metal ions and lipase inhibitors as shown in Table 3.8. From the results it was concluded that Zn^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} ions have neither inhibitory nor stimulatory effect on lipase activity, while Ca^{2+} and Mg^{2+} ions showed marked stimulatory effects. EDTA and o-phenanthroline showed 69.5 and 58.3% inhibition of the initial activity respectively, suggesting the presence of metallo-lipases.

3.11 Discussion

Koseki, *et al.*, (1989) suggested that lipase activity is closely related to the quality of the formed emulsion. Vorderwülbecke, *et al.*, (1992) used a set of different assays, based on hydrolytic and synthetic activities, to characterise lipases and suggested that lipases should be classified as ester-hydrolyzing enzymes with the capability to form esters. Previous experiments dealing with microbial lipases, used a set of different assays based on hydrolytic and synthetic activities in order to characterise lipases. In this work the spectrophotometric assay with p-nitrophenyl palmitate as the enzyme substrate was used, as it is a time saving, reproducible and easy to handle detection method. Nevertheless, Fay, *et al.*, (1990) suggested that the hydrolysis of p-nitrophenyl palmitate is not always indicative of lipase activity.

Lipases from Gram-positive bacteria have a broad substrate specificity, including the ability to hydrolyse water-soluble substrates. Ishihara, *et al.*, (1989) suggested that automatic feeding of both olive oil as a carbon source and iron, in a fed-batch culture of *P. fluorescens* led to mass production of lipase. A systematic study on regulation of lipase production by *P. aeruginosa* revealed that limitation of carbon sources increased lipase production which was strongly induced by triglycerides and detergents like

Table 3.8 Effect of metal ions and inhibitors on intracellular lipase activity from *Streptomyces thermoviolaceus* grown at 50°C for 48 hours. Olive oil was used as sole carbon source. Data are representative of 4 separate determinations (mean±SD; n=4).

Treatment (5mM)	I/C lipase activity (U/100ml cell-mass/h)	% Activation	% Inhibiton
No addition	450.00	0	0
$ZnCl_2$	450.00	0	0
$FeCl_2$	450.00	0	0
$CuCl_2$	450.00	0	0
$MnCl_2$	450.00	0	0
$CaCl_2$	517.50	15.0±0.017	0
$MgCl_2$	533.70	18.6±0.019	0
EDTA	137.25	0	69.5±0.026
o-phenanthroline	187.65	0	58.3±0.021

Abbreviation

I/C Intracellular

Tweens or Spans, with optimum conditions in Tween 80 grown cultures (Gilbert, *et al.*, 1991). A number of lipids were tested as carbon sources for lipase production. Of all the carbon sources, olive oil was by far the best for both growth and lipase activity. Tween 80, a monooelate ester, acts as an inducer of lipase and the findings that Tween 40 and Tween 60 could not efficiently replaced Tween 80 indicates a degree of specificity with respect to the chemical nature of the inducer. Growth under the lipid sources examined, was satisfactorily reproducible and homogeneous; the cells were largely filamentous, but also comprised small pellets especially in the later stages of growth. After an initial lag phase, the cultures grew exponentially as determined by measurements of dry weight.

Extracellular lipases normally appear in the culture medium when the bacterial cells reach the end of the logarithmic growth phase (Jaeger, *et al.*, 1994). Gowland, *et al.*, (1987) found that lipase production by thermophilic bacterial isolates, occurred predominantly in the stationary phase and suggested that lipase production was the subject of catabolite repression. Hale, *et al.*, (1992) suggested that the appearance of an esterase produced by *Streptomyces scabies* occurred when cells were in the late logarithmic or early stationary phases of growth, suggesting that some factors required for the gene expression may not be available until later stages of growth or that a repressor of expression may be present during active mycelial growth.

Lipase activity and protein secretion were detected during both exponential and stationary phases in the streptomycetes studies and reached peak values when biomass production was at its maximum level. It was also revealed that during stationary phase, constant biomass gave rise to the production of a constant amount of protein and lipase activity which is an indication of an extracellular secretion of protein and lipase. These results coincided with the findings of Hespell and O'Bryan-Shah, (1988) who suggested that the activity from *Butyrivibrio fibrisolvens* strains, increased with cell

growth until the stationary growth phase was reached after which the activity remained fairly stable.

Studies by Janssen, *et al.*, (1994), on a continuous assay of p-nitrophenyl palmitate esterase activity from a thermophilic lipolytic *Bacillus* sp., revealed that since it was difficult to envisage transport of pNP-palmitate into the cell, it was assumed that the esterase was loosely cell-associated which was also observed in the case of lipase production by thermophilic *Streptomyces* species. The amount of lipase activity found in cell-free supernatants was significantly higher than the amount found in cell-mass and it was concluded that the higher extracellular lipase activity was a result of secretion by the mycelial cells. Also, for both extracellular and intracellular lipase activity the amount detected after 48 hours incubation was significantly higher than at 24 hours which was attributed to an increase in biomass.

Extracellular lipases were kept at different temperatures in order to test their stability on storage and it was concluded that cell-free culture supernatants could be stored for long periods at -20°C with minimal loss of activity.

The optimum pH for lipase activity was 8.0, but at pH 2-3 36% of the activity could still be observed but overall the stability was lower at acidic pH.

For all *Streptomyces* species used profound thermostability at 70°C was observed. At 80 and 90°C lower levels of thermotolerance were detected and inhibition of lipase activity was higher. However, because inhibition never reached 100%, the residual activity suggested that more than one lipase activity may possibly be present: a heat labile enzyme activity as well as a highly thermostable one.

Kamel, *et al.*, (1989) suggested that calcium chloride increased the proteolytic activity in *Streptomyces violaceochromogens* and *glaucescens* media and had a marked effect on the permeability of both organisms. Higher concentrations of calcium appeared to increase the secretion of extracellular enzymes. Salas, *et al.*, (1983) showed that there was a high calcium content in spores of *Streptomyces* species and

that for some species it was an essential triggering factor of spore germination (Eaton and Ensign, 1980). Abbas and Edwards, (1990) on studies on the effects of metals on *Streptomyces coelicolor*, suggested that the major regulatory effects of calcium are occurring during the phase of growth-associated processes which are mediated principally by those reactions of primary metabolism. Babcock, *et al.*, (1992) found that expression of an esterase gene from *Streptomyces scabies* is regulated by zinc in both *Streptomyces scabies* and *Streptomyces lividans*. Gilpin, *et al.*, (1995) isolated two phenazines from a *Streptomyces* and found that the compound inhibited some metalloenzymes by chelation of the active site metal ion. Perez, *et al.*, (1995) characterised an intracellular beta-glucosidase from a cellulolytic *Streptomyces* strain and found that the enzyme is inhibited by p-chloromercuribenzoate. Nguyen *et al.*, (1995) found that the activity of glutamine synthetase I (GSI) from *Streptomyces aureofaciens* was significantly stimulated by Co^{2+} but inhibited by other divalent cations. ADP was a strong inhibitor.

More recently, Inyang, *et al.*, (1995) isolated and characterised a D-glucose/xylose isomerase from a new thermophilic strain *Streptomyces* sp. It was found that both the activity and stability of this D-xylose isomerase depend strongly on divalent metal ions. Mg^{2+}, Mn^{2+} and Co^{2+} were found to be of comparable efficiency for the D-xylose isomerase reaction.

Inhibitors were used in an attempt to resolve the type of lipases produced by thermophilic *Streptomyces* species. p-nitrophenyl palmitate activity in culture supernatant fluids was inhibited to a great extent in the presence of metallo lipase inhibitors EDTA and o-phenanthroline and to a lesser extent by PMSF, a serine lipase inhibitor. This suggested the presence of two types of enzymes, a metallo-lipase and a serine-lipase.

Extracellular lipase activity proved to be inhibited to divalent metal ions such as Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} whereas it was activated by Mg^{2+} and Ca^{2+} ions with the

former having a greater effect. Inhibition by EDTA and o-phenanthroline was partially restored by adding Mg^{2+} and Ca^{2+} ions. This indicates a dependence of extracellular lipase from thermophilic *Streptomyces* to these divalent metal ions. In the case of intracellular lipases divalent metal ions proved to have neither inhibition nor stimulatory effect whereas Ca^{2+} and Mg^{2+} ions proved to have stimulatory effects.

Chapter 4

Laboratory scale fermentation experiments

4.1 Introduction

Batch cultures may be considered as a closed system containing a limited amount of medium, in which the inoculated culture passes through a number of phases. Batch culture systems are used in the majority of industrial fermentation processes and are particularly suited to fermentations where the bulk of product formation occurs after the exponential growth phase. One disadvantage is that efficient product formation only occurs during a fraction of each fermentation cycle. The main function of a fermenter is to provide a controlled environment which allows for efficient growth of cells and product formation. As a result, a series of experiments was performed to investigate lipase production and activity in *Streptomyces* under the controlled environment provided by laboratory-scale fermentations.

Previous fermentation studies on thermophilic *Streptomyces* were focused on *Streptomyces thermoviolaceus* since it makes a useful model for studying secondary metabolism by producing a coloured and easily identified antibiotic, granaticin (Maehr, *et al.*, 1979; Snipes, *et al.*, 1979; James and Edwards, 1989). Preliminary flask cultures experiments, reported in chapter 3, revealed that *Streptomyces thermoviolaceus* and *S. thermodiastaticus* grew rapidly in a simple salts medium with an appropriate lipid carbon source. The best biomass yields and maximum lipase activity were also produced by these two organisms, especially when olive oil was used as the sole carbon source. As a result, fermentation experiments were focused on these two organisms.

A temperature shift experiment, involving batch cultures in flasks, was performed in order to investigate the properties of *Streptomyces* lipase with respect to growth temperatures. The results of this experiment were further examined in subsequent fermentation experiments.

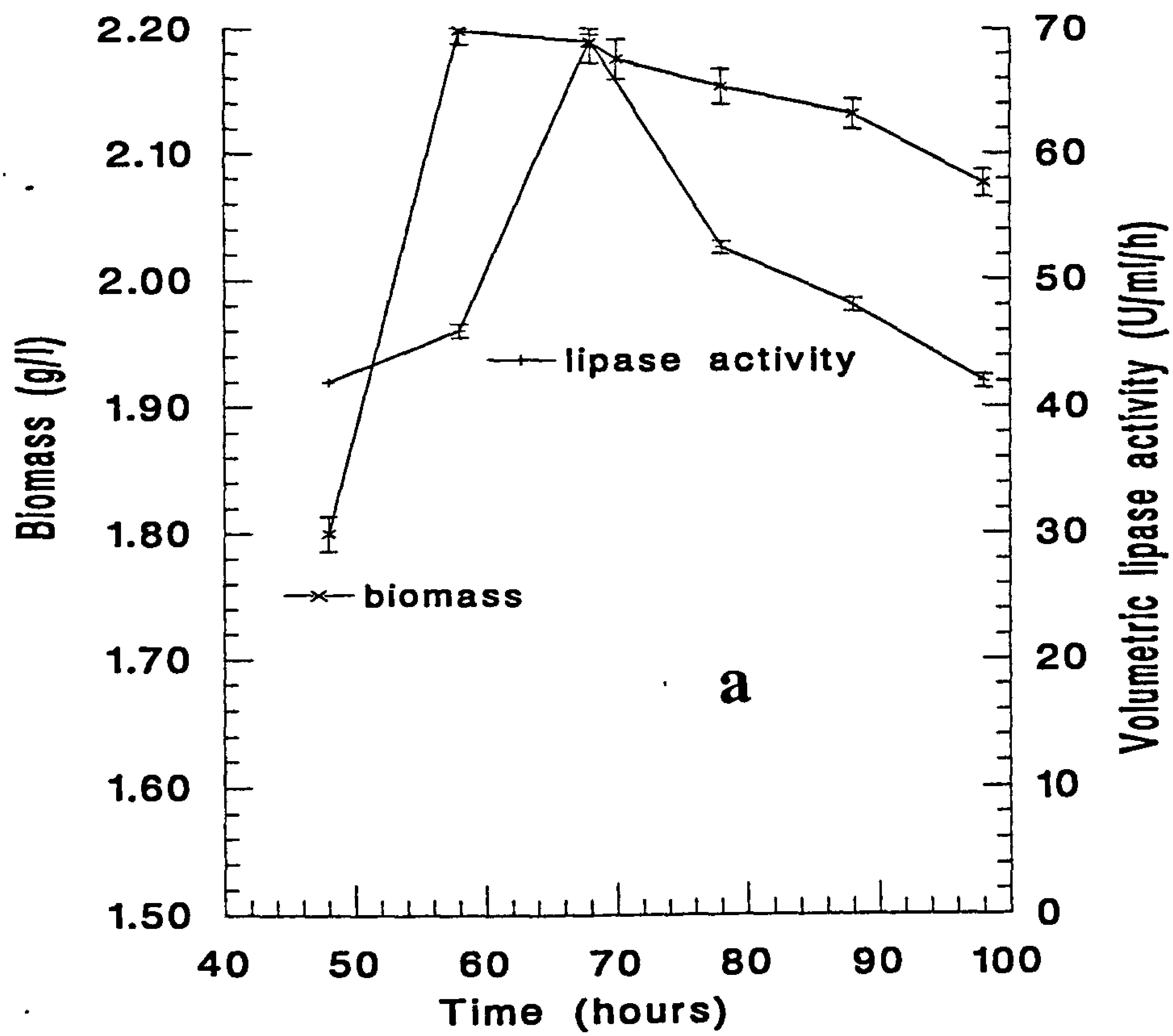
Laboratory scale fermentation experiments were carried out using an LH500 series fermentation unit. Temperature was maintained at 30 or 50°C by a thermistor linked to a temperature control unit. The pH was controlled to 8.0 by using a pH probe connected to a pH control unit.

4.2 Flask cultures temperature shift experiment

Hollick, (1982) suggested that enzymes produced by microorganisms reflect the temperature of growth. In order to examine the properties of *Streptomyces* lipase with respect to growth temperature, a temperature shift experiment was performed. Olive oil was used as the carbon source and *Streptomyces thermoviolaceus* was grown at 30°C for 48 hours in flask cultures. After 48 hours, half of the flasks were shifted to 50°C while the other half were kept at 30°C. Samples were collected from both temperatures every 10 hours up to 100 hours. Cell-free culture supernatants were assayed for extracellular protein and lipase activity. Figure 4.1 shows the results obtained when cultures were kept at 30°C. It was observed that biomass production was maximum after 58 hours. Lipase activity was highest at 68 hours of incubation. In cultures shifted to 50°C after 48 hours incubation at 30°C, biomass production was maximum after 50 hours whilst lipase activity peaked at 58 hours, reflecting the higher metabolic rate at the higher temperature (Figure 4.2). It was observed that at the end of growth, lipase activity decayed when growth took place at 30°C. In cultures shifted to 50°C, lipase activity was more stable up to 100 hours, proving the thermostability of the lipase activity at the higher temperature. The extracellular protein profile showed that protein concentration increased up to 70 hours and then remained constant when growth took place at 30°C. Similar findings were observed when the culture was shifted to 50°C. In order to test the effect of temperature on batch culture fermentations, 30°C was used as the low temperature and 50°C as the high temperature for growth.

Figure 4.1

Graph showing a) the production of biomass and secretion of volumetric lipase activity and b) the secretion of volumetric extracellular protein by *Streptomyces thermoviolaceus* grown in the presence of olive oil at 30°C after 48h initial incubation at the same temperature. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


2.20
2.10
2.00
1.90
1.80
1.70
1.60
1.50
Biomass (g/l)
70
60
50
40
30
20
10
0
Volumetric lipase activity (U/ml/h)
lipase activity
biomass
a
40
50
60
70
80
90
100
Time (hours)

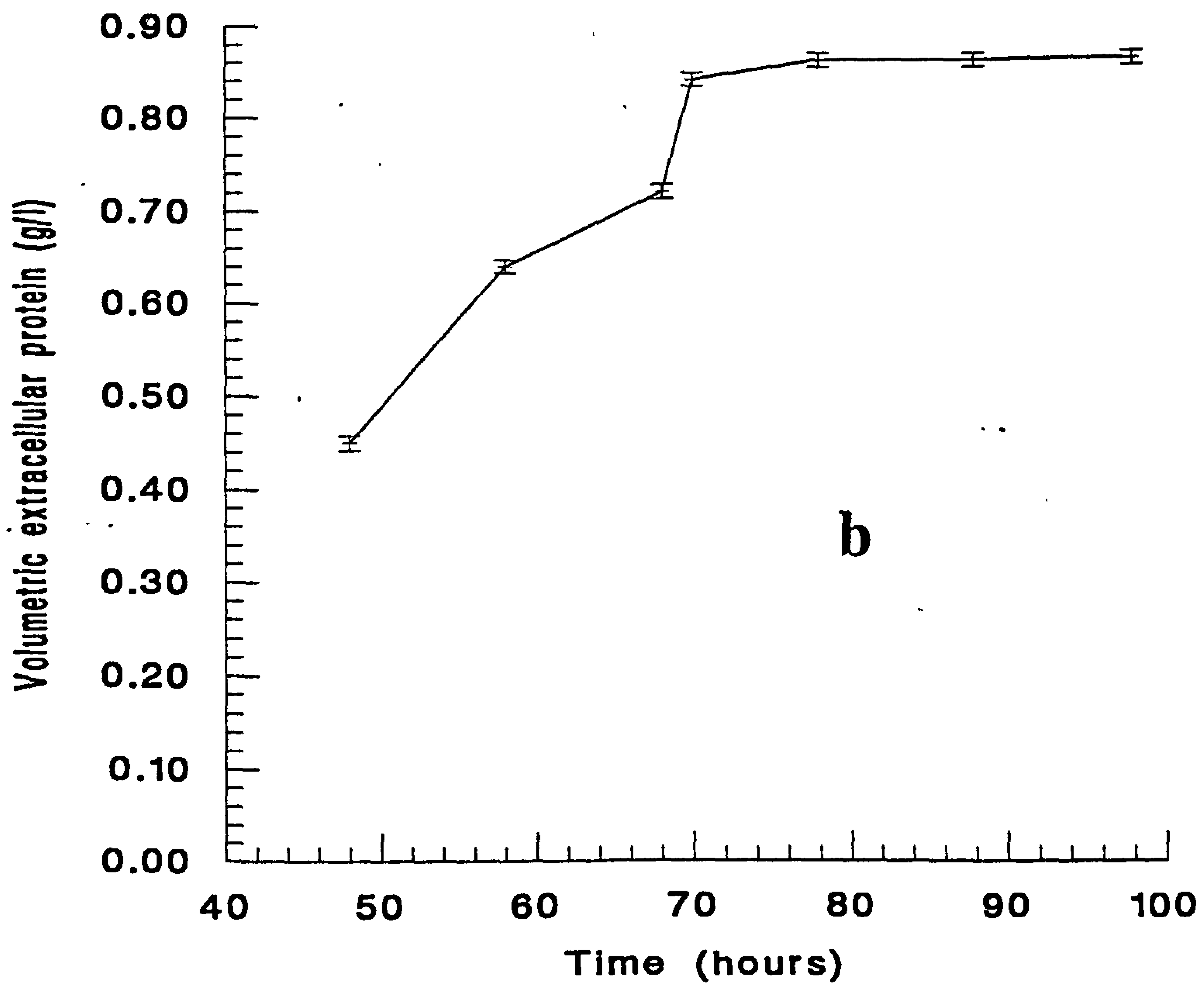
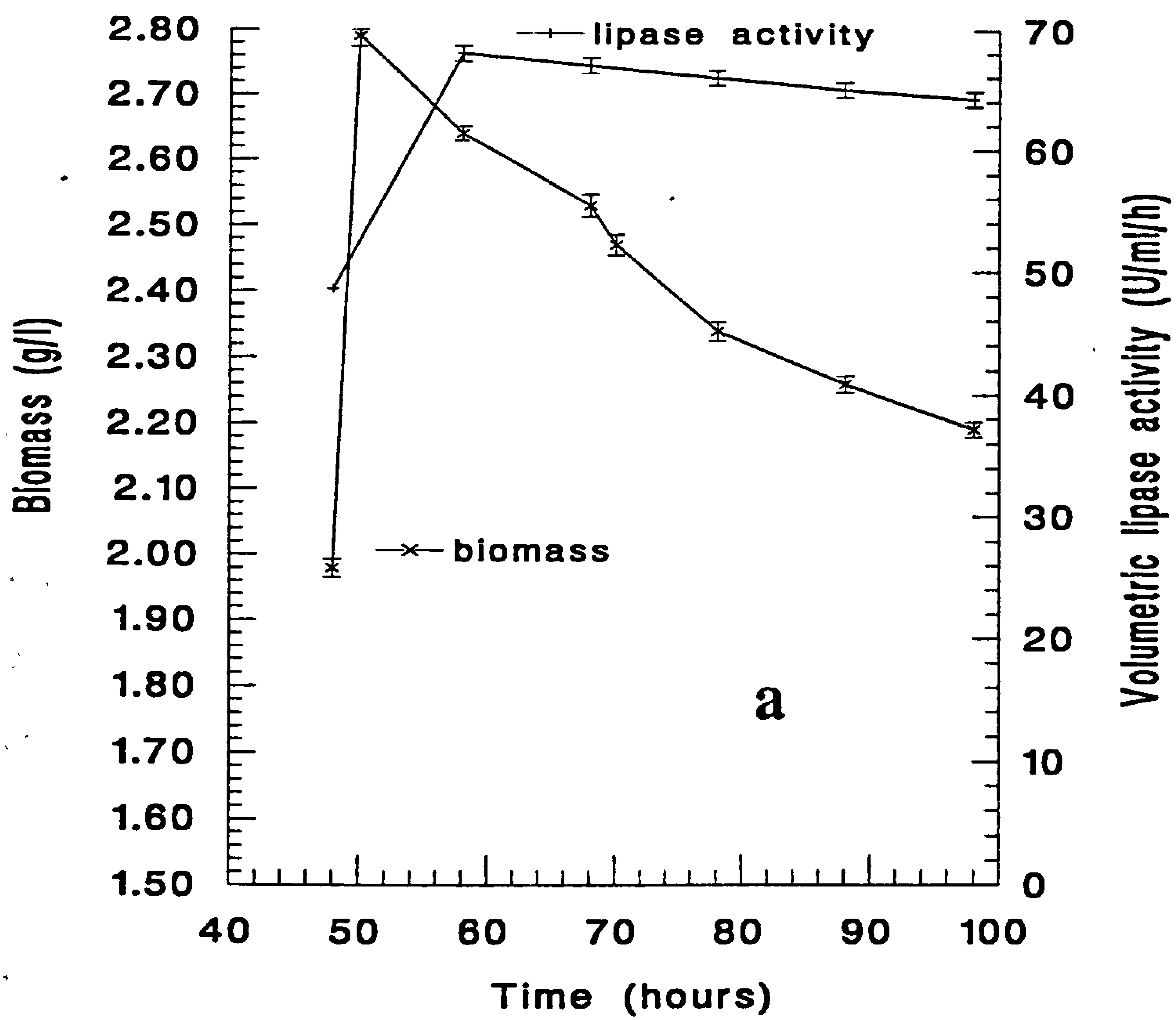
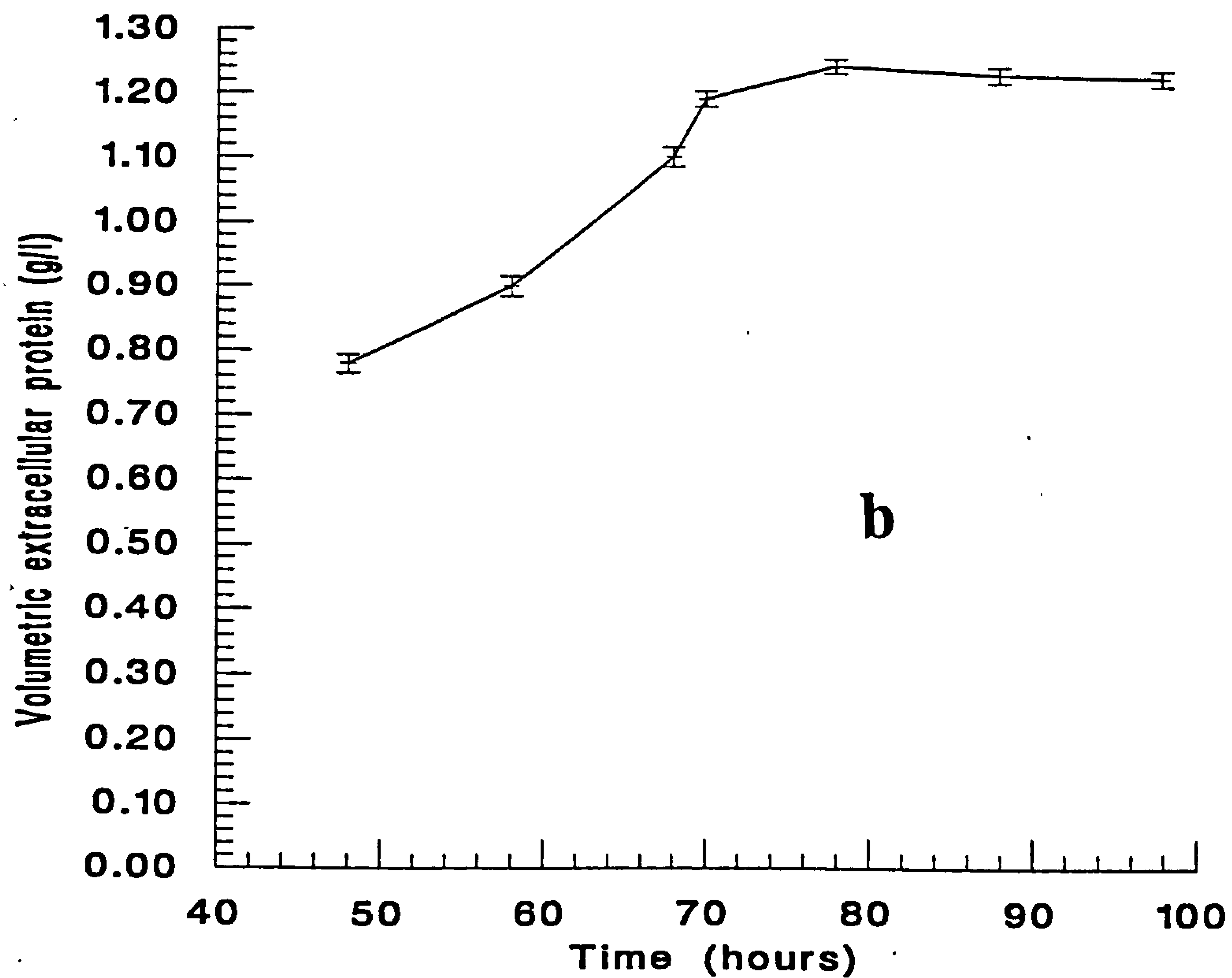

0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Volumetric extracellular protein (g/l)
b
40
50
60
70
80
90
100
Time (hours)

Figure 4.2

Graph showing a) the production of biomass and secretion of volumetric lipase activity and b) the secretion of volumetric extracellular protein by *Streptomyces thermoviolaceus* grown in the presence of olive oil at 50°C after 48h initial incubation at 30°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



lipase activity
biomass
Biomass (g/l)
Volumetric lipase activity (U/ml/h)
Time (hours)
a



Volumetric extracellular protein (g/l)
Time (hours)
b

4.2.1 Thermostability of lipases during flask cultures temperature shift experiment

In order to test if thermophilic *Streptomyces* produce significant thermostable lipases when shifted to a higher temperature, the thermostability of lipase from flask cultures of *Streptomyces thermoviolaceus* was tested. Figure 4.3a showed that when growth took place at 30°C, in the presence of olive oil as the sole carbon source, the time taken for the loss of 50% of the total lipase activity (T_{50}) was decreased significantly as the culture grew older: 31 mins at 48h, 26 mins at 60h and 16 mins at 72h. Figure 4.3b showed that when the cultures were shifted to 50°C, the thermostability of lipase 12, 24 and 36 hours after the shift, remained the same at approximately 68 mins. T_{50} values were not decreased significantly as the culture grew older. This suggested that the organism produced a thermostable lipase when growth was shifted to a higher temperature, whilst at 30°C, a proportion of the lipase activity could be ascribed to a more thermolabile enzyme not detectable in the organism at 50°C.

4.3 Thermostability of lipases from flask cultures grown at 30 and 50°C

To examine the effects of culture temperature on lipase thermostability, cell-free culture supernatant from *Streptomyces thermoviolaceus*, grown at 30 or 50°C in olive oil, was heated at 70°C and assayed for lipase activity with time. Results from Figure 4.4 confirmed that lipase produced in 30°C grown cultures was more thermolabile since T_{50} was approximately 30 mins whereas, in 50°C grown cultures the T_{50} value was approximately 67 mins. This implied either that growth temperature played an important role in determining the thermotolerance of lipase activity produced, or that different enzymes were induced at different growth temperatures.

a

Lipase activity (%)

48h incubation

72h incubation

60h incubation

Time (mins)

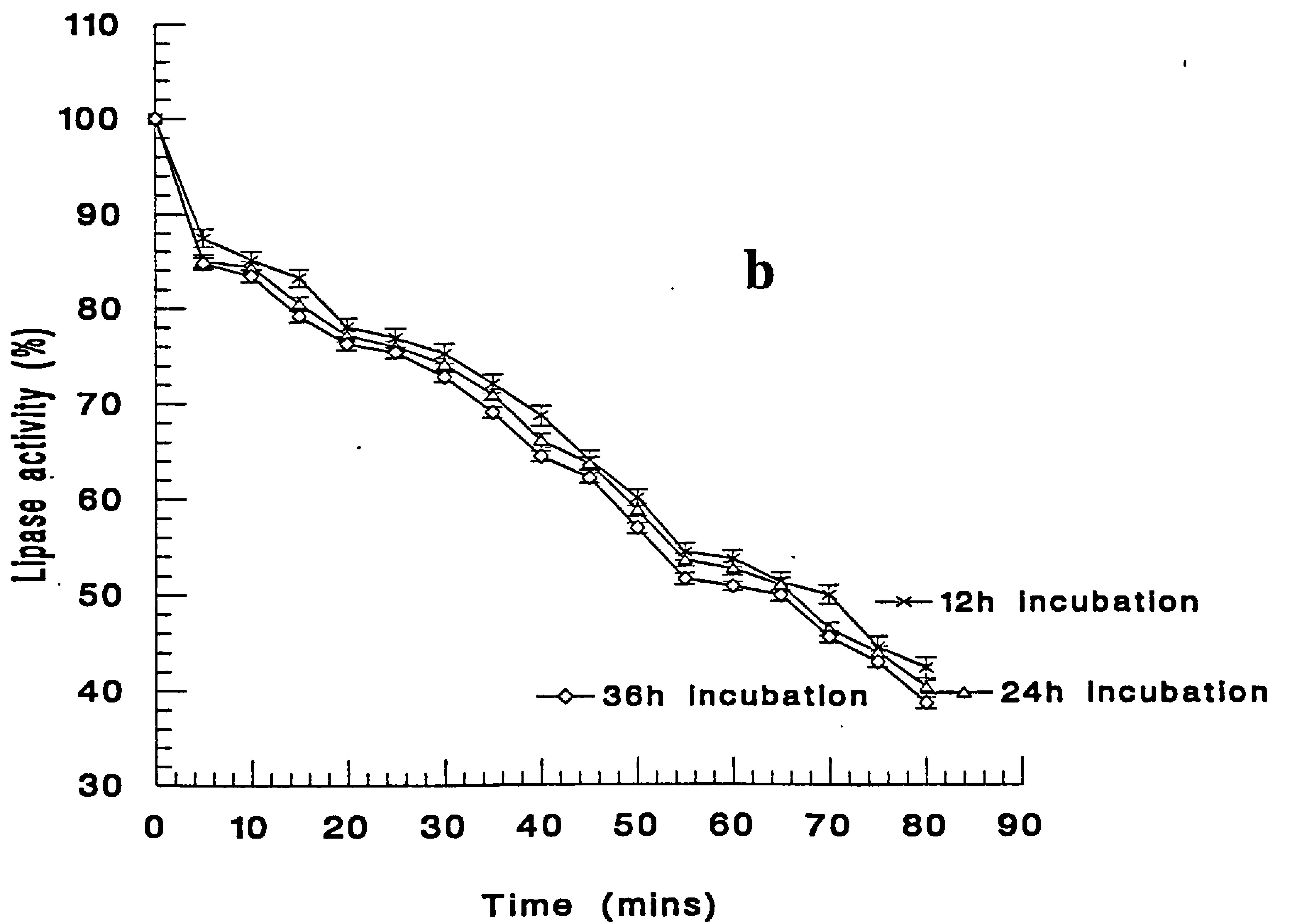
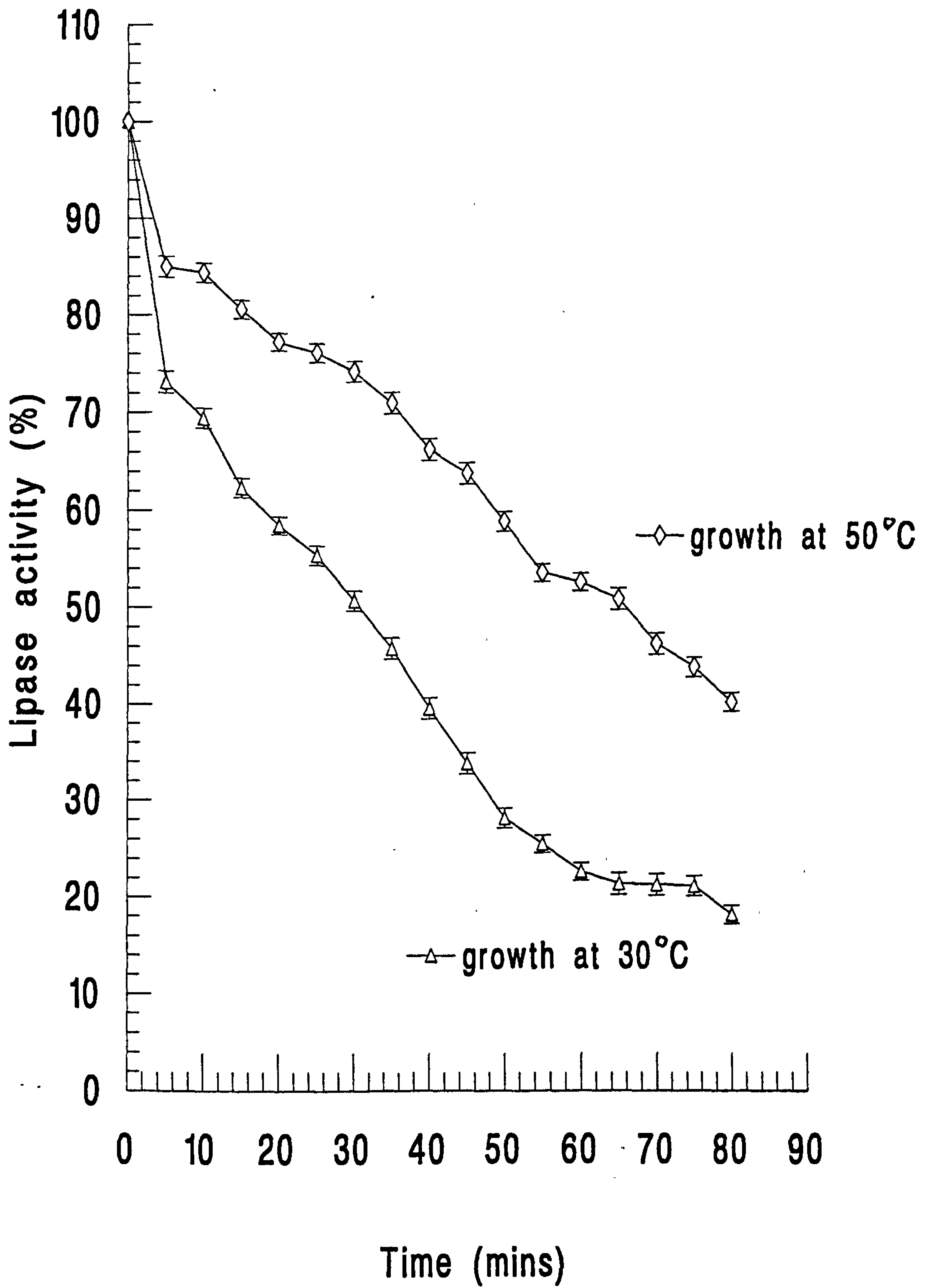


Figure 4.4

Effect of culture temperature on lipase activity thermostability at 70°C of *Streptomyces thermoviolaceus* grown in the presence of olive oil at 30 or 50°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



Lipase activity (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Time (mins)
growth at 50°C
growth at 30°C

4.4 Effect of pH on growth, lipase activity and extracellular protein

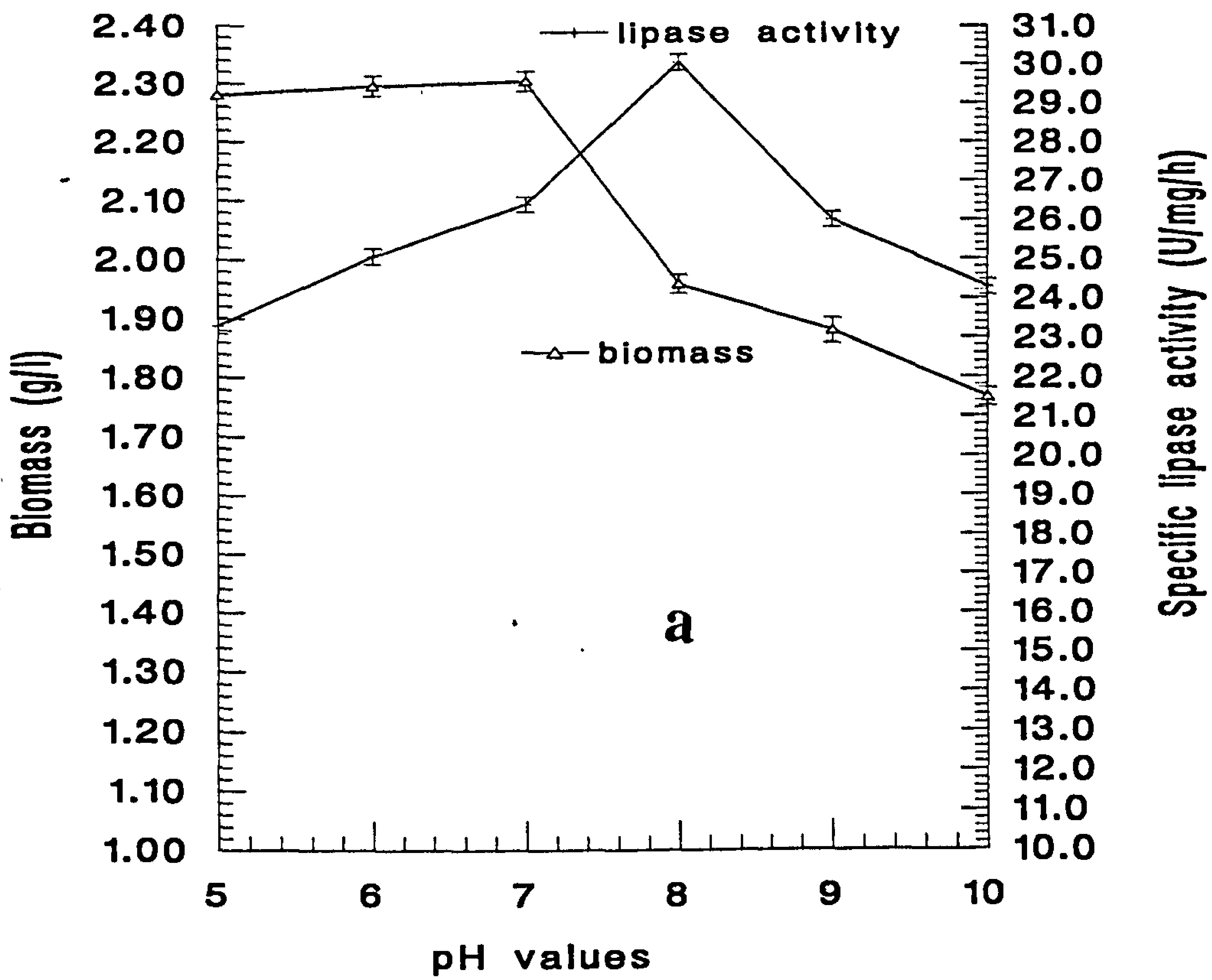
In order to examine the effect of pH on growth and lipase production, *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown in a fermenter controlled environment, at pH values ranging from 5 to 10. In each case, the pH was kept at the required value by using a pH probe connected to a pH control unit. Lipase activity and extracellular protein were expressed as specific values obtained by dividing the amount of lipase activity and extracellular protein produced at a particular time interval, by the amount of biomass produced at the same time. Both organisms were grown in batch culture fermentation at 50°C, using olive oil as the carbon source. Results from *Streptomyces thermoviolaceus* are represented in Figure 4.5. Biomass (peak value) was approximately 2.302 g/l between pH 5.0 and 7.0 and thereafter fell with increasing pH to around 1.776 g/l at pH 10.0. Lipase activity reached its peak value, 30.04 U/mg/h, at pH 8.0 and it was lower between pH 5.0-7.0 where biomass was at its maximum. These results coincided with those in flask culture experiments during which the optimum pH for lipase activity was again 8.0. As a result pH 8.0 was chosen for subsequent batch culture fermentations. Extracellular protein levels exhibited a similar profile to the one for biomass production. Protein values rose to a maximum at pH 6.0 to 7.0 and then fell to around 0.175 at pH 10.0. A similar pattern of results was observed when *Streptomyces thermodiastaticus* was used as the test organism as shown in Figure 4.6.

4.5 Effect of carbon source on growth and production of lipase and extracellular protein

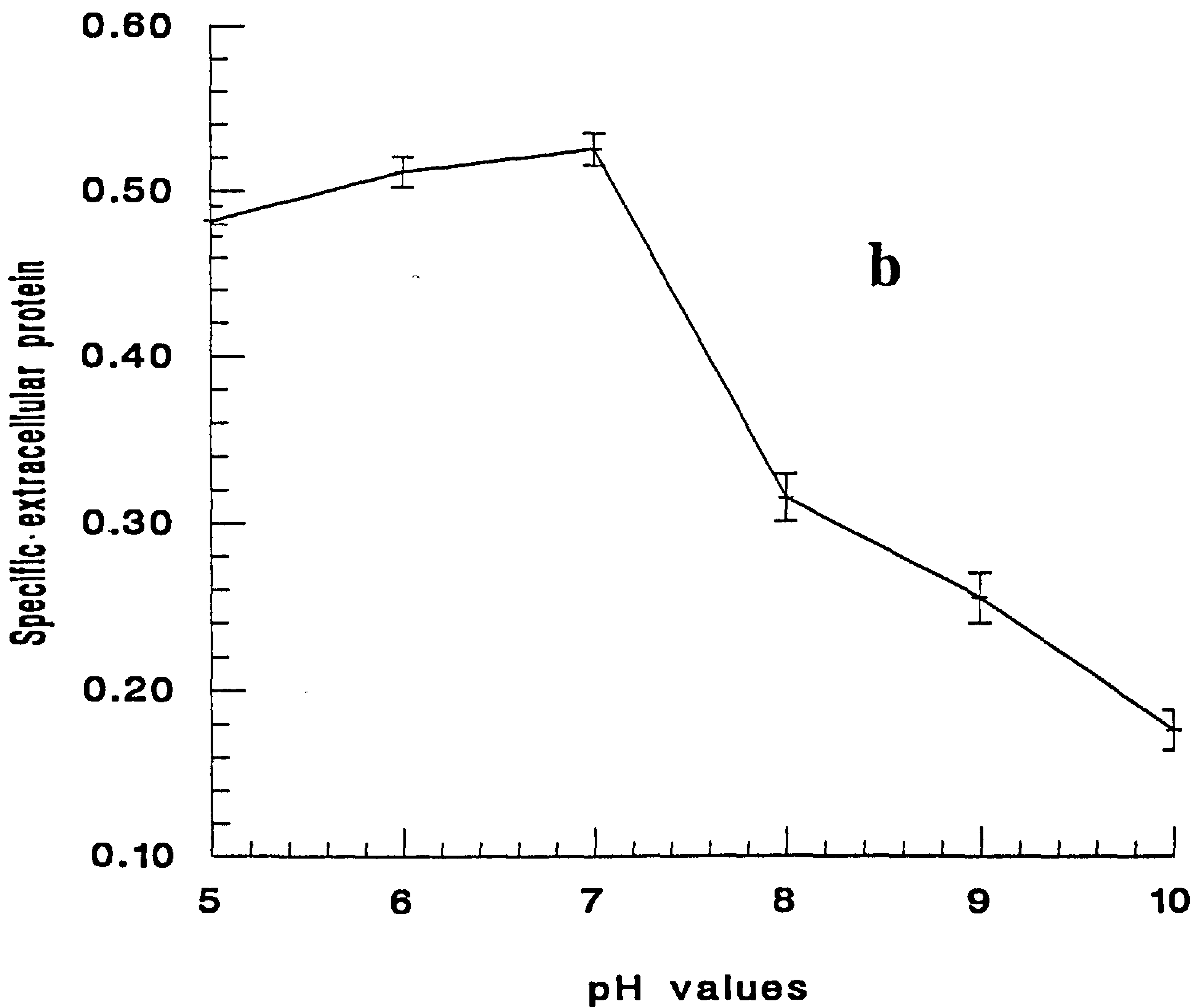
Tables 4.1 and 4.2 show the summary of a number of experiments in which *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown using different

Figure 4.5

Effect of pH on a) biomass production and secretion of lipase activity and b) secretion of extracellular protein by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C. For every parameter examined, its maximum value in the fermentation was taken as the reference point. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



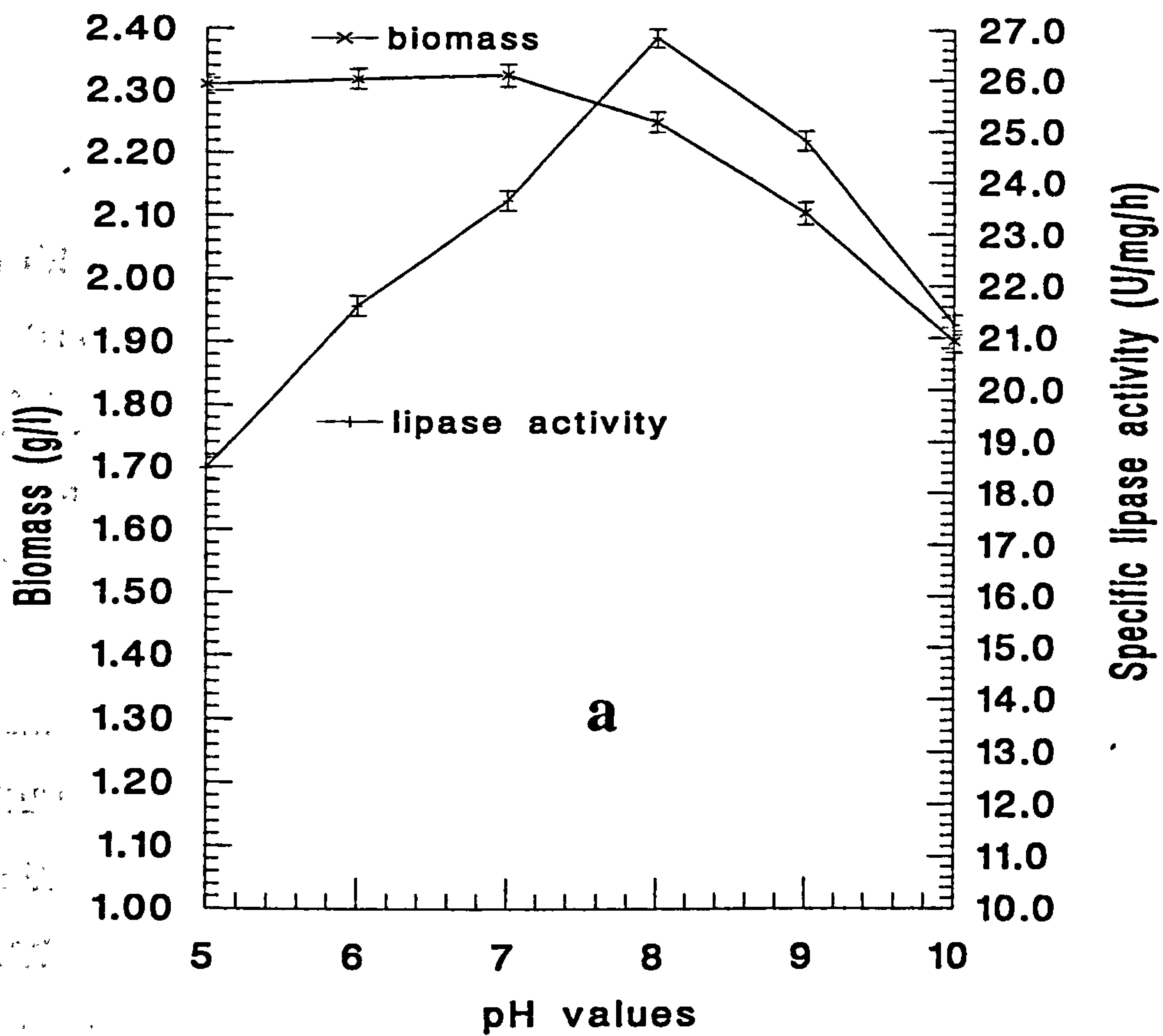
lipase activity
biomass
a
Biomass (g/l)
Specific lipase activity (U/mg/h)
pH values
2.40
2.30
2.20
2.10
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
31.0
30.0
29.0
28.0
27.0
26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
5
6
7
8
9
10



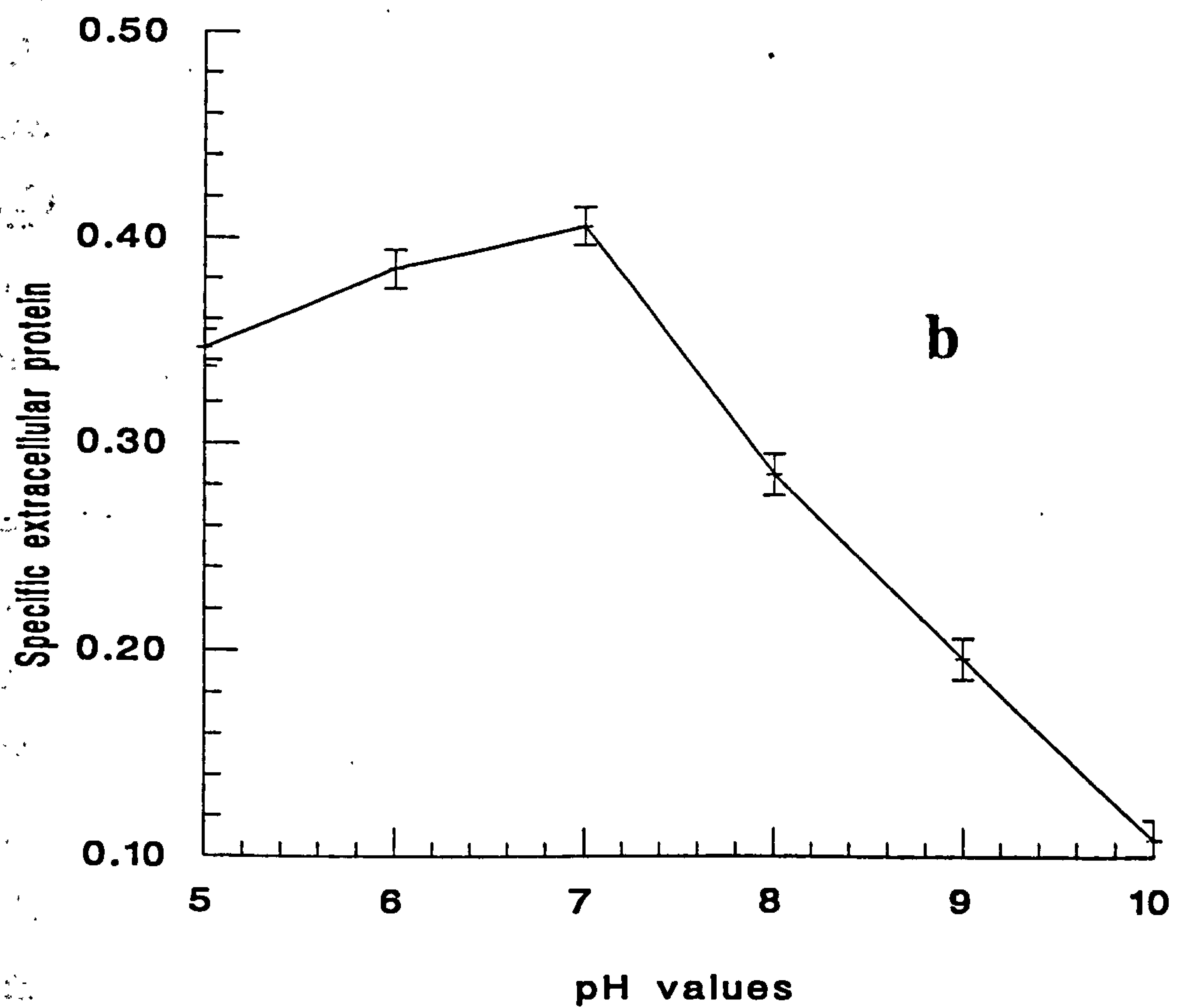
b
Specific extracellular protein
pH values
0.60
0.50
0.40
0.30
0.20
0.10
5
6
7
8
9
10

Figure 4.6

Effect of pH on a) biomass production and secretion of lipase activity and b) secretion of extracellular protein by *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 50°C. For every parameter examined, its maximum value in the fermentation was taken as the reference point. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



biomass
lipase activity
Biomass (g/l)
Specific lipase activity (U/mg/h)
a
2.40
2.30
2.20
2.10
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
27.0
26.0
25.0
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
5
6
7
8
9
10
pH values



Specific extracellular protein
b
0.50
0.40
0.30
0.20
0.10
5
6
7
8
9
10
pH values

Table 4.1 Effect of carbon source on growth and production of lipase and extracellular protein by *Streptomyces thermoviolaceus* grown in a fermenter at 50°C.

carbon source	maximum biomass (g/l)	biomass at inflexion of growth (g/l)	maximum volumetric lipase activity (U/ml/h)	maximum volumetric extracellular protein (g/l)	z1 (1/h)	z2 (1/h)
olive oil	2.302	1.58	68.50	1.20	0.333	0.077
Tween 80	2.150	1.52	65.58	0.98	0.318	0.067
vegetable oil	2.053	1.51	62.53	0.83	0.291	0.058
Tween 40	1.936	1.49	55.44	0.65	0.278	0.052
Tween 60	1.879	1.49	51.17	0.57	0.262	0.047

Table 4.2 Effect of carbon source on growth and production of lipase and extracellular protein by *Streptomyces thermodiastaticus* grown in a fermenter at 50°C.

carbon source	maximum biomass (g/l)	biomass at inflexion of growth (g/l)	maximum volumetric lipase activity (U/ml/h)	maximum volumetric extracellular protein (g/l)	z1 (1/h)	z2 (1/h)
olive oil	2.325	1.40	62.13	0.94	0.166	0.066
Tween 80	2.201	1.36	59.28	0.86	0.155	0.057
vegetable oil	2.140	1.33	58.30	0.72	0.143	0.048
Tween 40	1.918	1.32	48.25	0.61	0.131	0.042
Tween 60	1.836	1.33	43.50	0.49	0.127	0.039

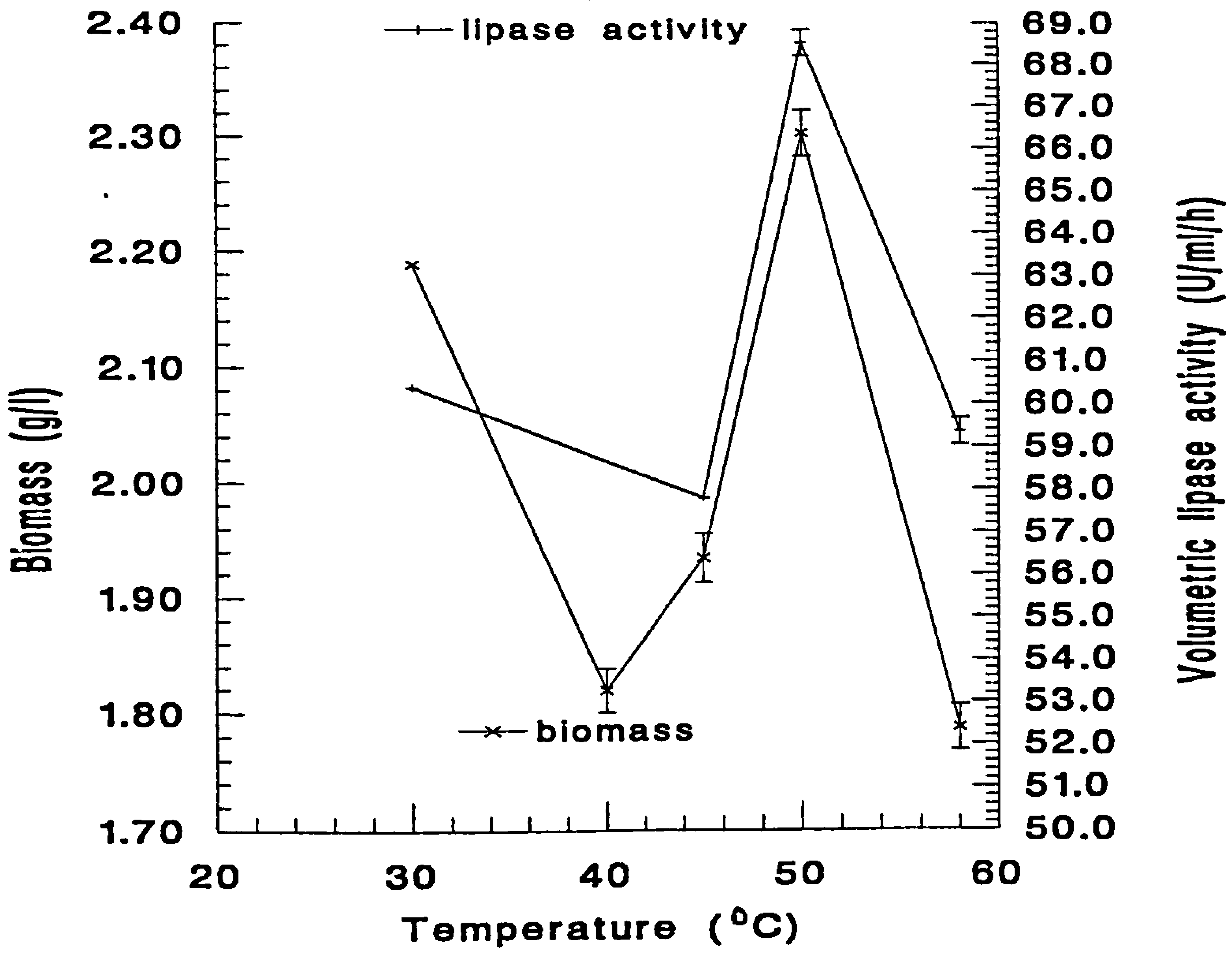
carbon sources under fermenter-controlled conditions at 50°C. Maximum biomass, biomass at inflexion of growth, volumetric lipase activity and extracellular protein were determined for each carbon source. In each case, cultures exhibited biphasic growth and the growth rates of the first and the second slower phase of growth were calculated and denoted as z1 and z2 respectively. Similar results were observed by James and Edwards, (1991) on the effect of carbon source on secondary metabolism in *Streptomyces thermoviolaceus*. Most rapid growth occurred on olive oil and Tween 80. The growth rates of the second growth phases for Tween 40 and 60 were the slowest. The point of inflexion for the change in growth rate occurred at a similar biomass concentration for the carbon sources used. It became clear that olive oil was the best carbon source for both lipase activity and extracellular protein since maximum values for all parameters were observed in the presence of olive oil, and all subsequent experiments were based on this carbon source. A direct relationship between z2 and lipase activity or extracellular protein was observed for every carbon source and the faster the growth rate the more lipase activity or extracellular protein was produced.

4.6 Effect of temperature on growth, lipase activity and extracellular protein

Fermentations were set up at different temperatures with olive oil as the carbon source. The chosen temperatures were 30. 45, 50 and 58°C. Figure 4.7 summarises the effect of temperature for *Streptomyces thermoviolaceus*. The doubling time when the organism was grown at 30°C was approximately 11.2 hours. By increasing the incubation temperature, it fell to 3 hours at 50°C. Above this temperature, it rose again to 9.4 hours at 58°C, indicating that cellular metabolism became thermolabile. Yield of biomass decreased up to 40°C and then increased steadily reaching the peak value at 50°C. After this temperature, it decreased steadily reaching the lowest value at 58°C.

Figure 4.7

Effect of temperature on a) biomass production and secretion of volumetric lipase activity and b) secretion of volumetric extracellular protein and doubling time by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30, 45, 50 and 58°C. For every parameter examined, its maximum value in the fermentation was taken as the reference point. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


a
lipase activity
biomass
Biomass (g/l)
Volumetric lipase activity (U/ml/h)
Temperature (°C)
2.40
2.30
2.20
2.10
2.00
1.90
1.80
1.70
69.0
68.0
67.0
66.0
65.0
64.0
63.0
62.0
61.0
60.0
59.0
58.0
57.0
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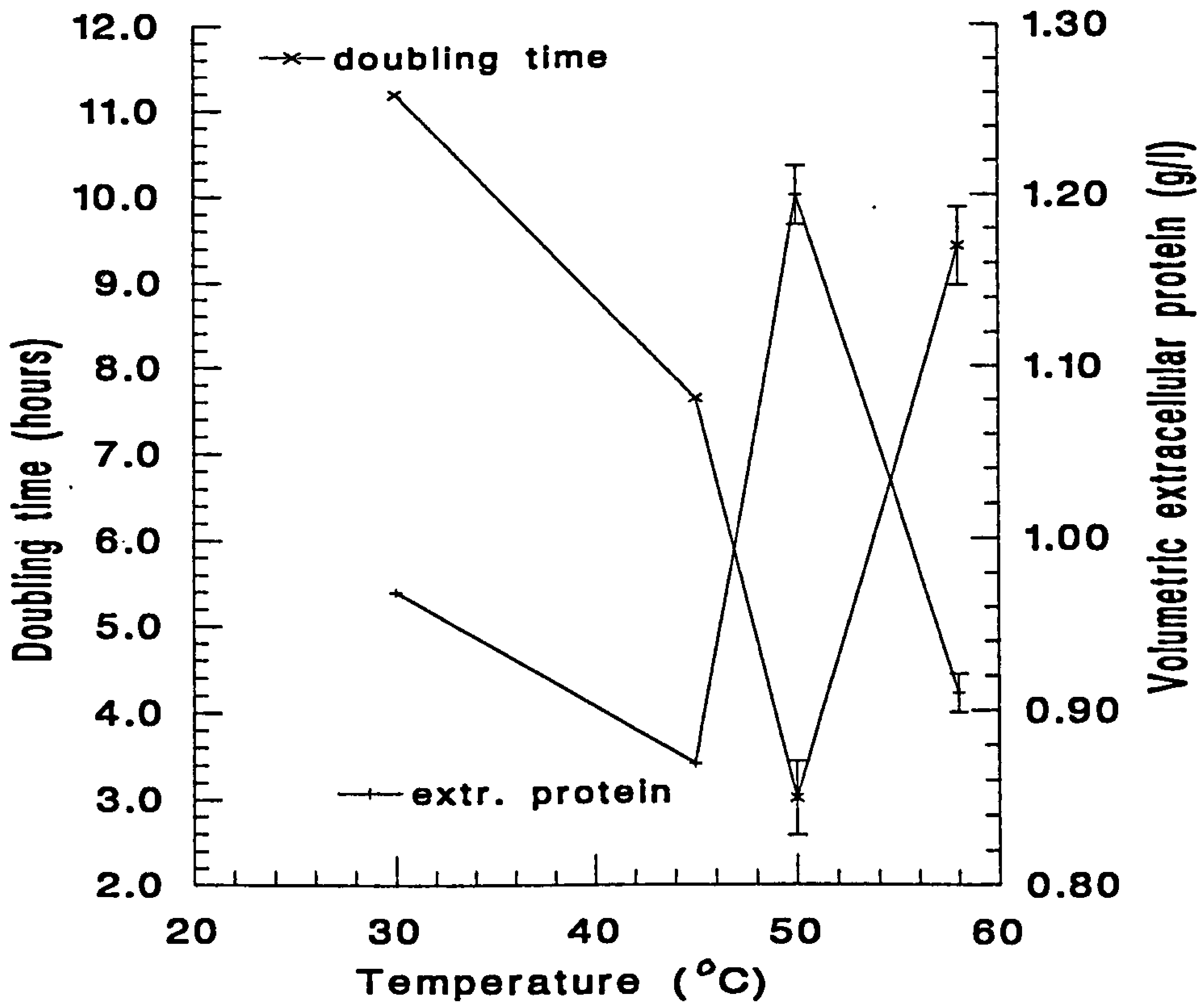
55.0
54.0
53.0
52.0
51.0
50.0
20
30
40
50
60


b
doubling time
extr. protein
Doubling time (hours)
Volumetric extracellular protein (g/l)
Temperature (°C)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.30
1.20
1.10
1.00
0.90
0.80
20
30
40
50
60

Volumetric lipase activity decreased up to 45°C above which it rose again reaching the maximum value at 50°C after which it decreased gradually. Volumetric extracellular protein production showed a similar pattern reaching its maximum value at 47°C. These results verified the ones obtained from flask cultures, in that the secretion of volumetric lipase activity and extracellular protein is proportional to biomass production. From the results it was observed that the temperatures of 30 and 50°C were proved to be the most efficient for the production of lipase activity and extracellular protein and so subsequent fermentation experiments were focused on these two temperatures.

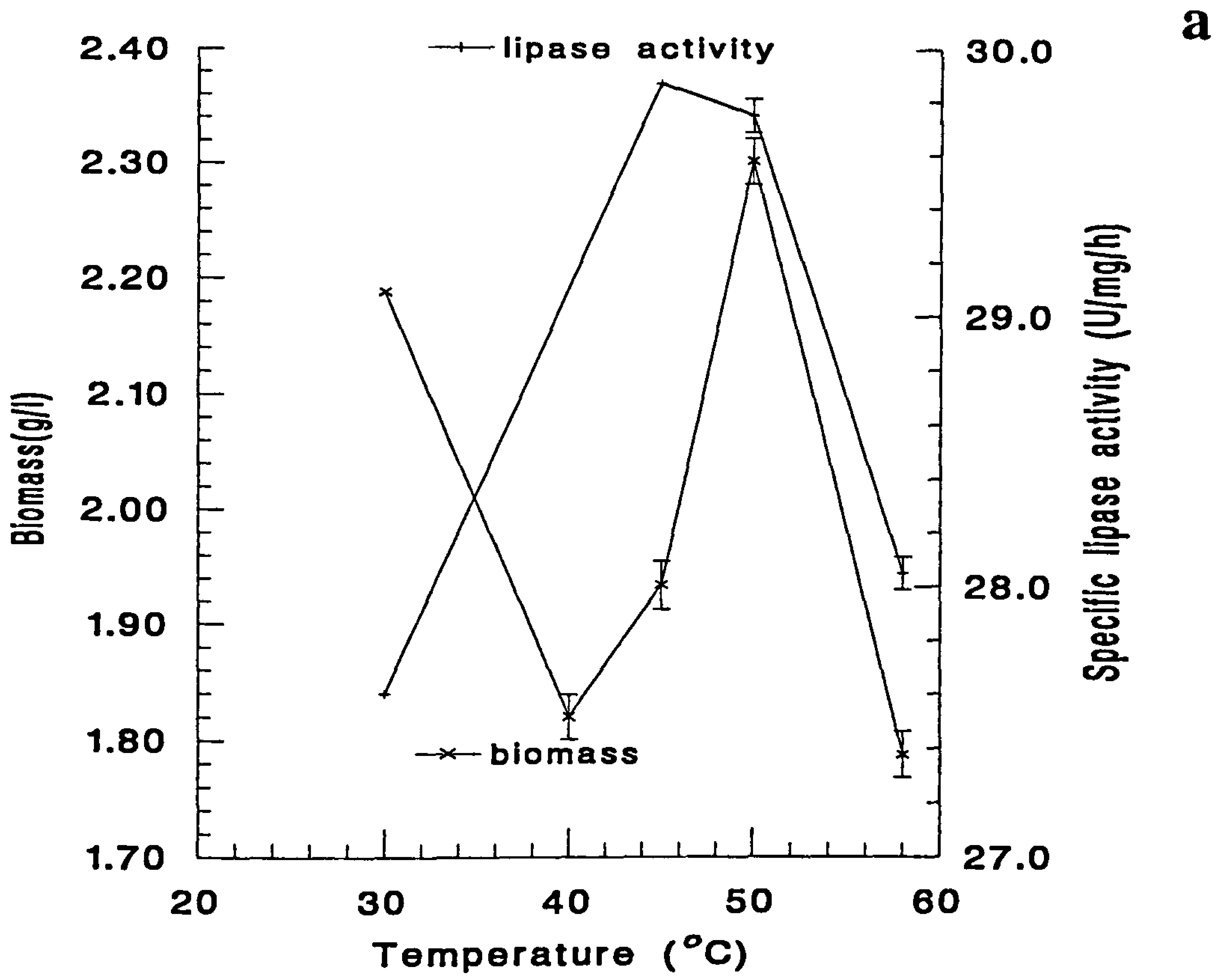
Figure 4.8 summarises the effect of temperature on the secretion of specific lipase activity and extracellular protein of *Streptomyces thermoviolaceus*. In contrast to the volumetric production, specific lipase activity increased constantly from 30 to 45°C, temperature at which it reached its maximum value. Above this temperature it decreased gradually. Specific extracellular protein in agreement with the volumetric results, reached its maximum point at 47°C. By contrast, an increase between 30 and 45°C was observed. If specific values were to be taken into account then the optimum temperature for the secretion of lipase activity would be 45°C.

4.7 Growth at low and high growth temperatures

Batch fermentations representative of low (30°C) and high (50°C) growth temperatures were studied for *Streptomyces thermoviolaceus* and *S. thermodiastaticus*. Results for *S. thermoviolaceus* are shown in Figures 4.9 and 4.10. At 30°C, biomass reached a maximum value of 2.258 g/l after 52 hours with a doubling time of around 11.2 hours up to 30 hours. Thereafter growth slowed down. The cessation of the more rapid growth phase was accompanied by an increase in the volumetric lipase activity produced, which reached a maximum value of 55.25 U/ml/h after 48 hours. After this

Figure 4.8

Effect of temperature on a) biomass production and secretion of specific lipase activity and b) secretion of specific extracellular protein and doubling time by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30, 45, 50 and 58°C. For every parameter examined, its maximum value in the fermentation was taken as the reference point. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


a
lipase activity
biomass
Biomass(g/l)
Specific lipase activity (U/mg/h)
Temperature (°C)
2.40
2.30
2.20
2.10
2.00
1.90
1.80
1.70
30.0
29.0
28.0
27.0
20
30
40
50
60

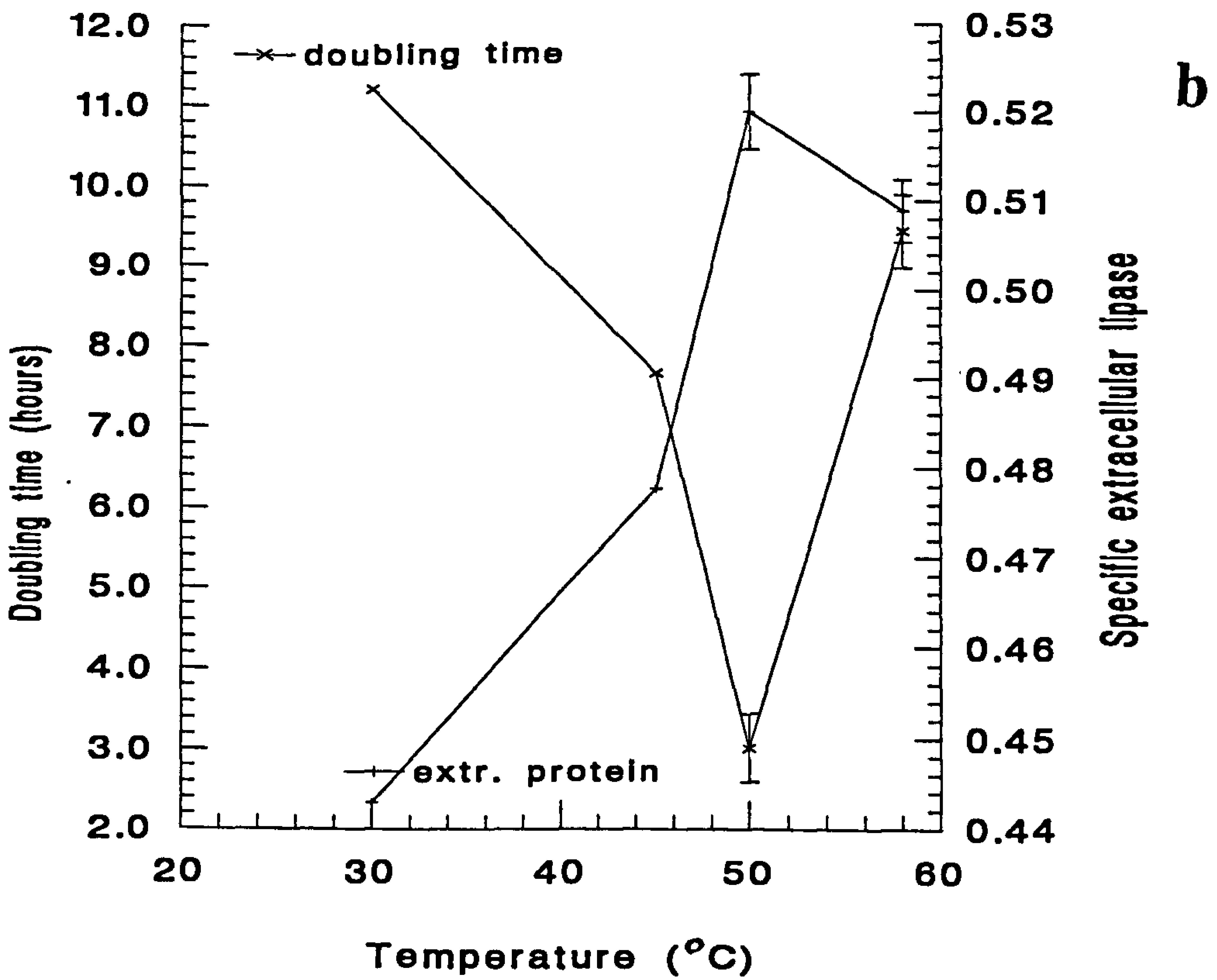
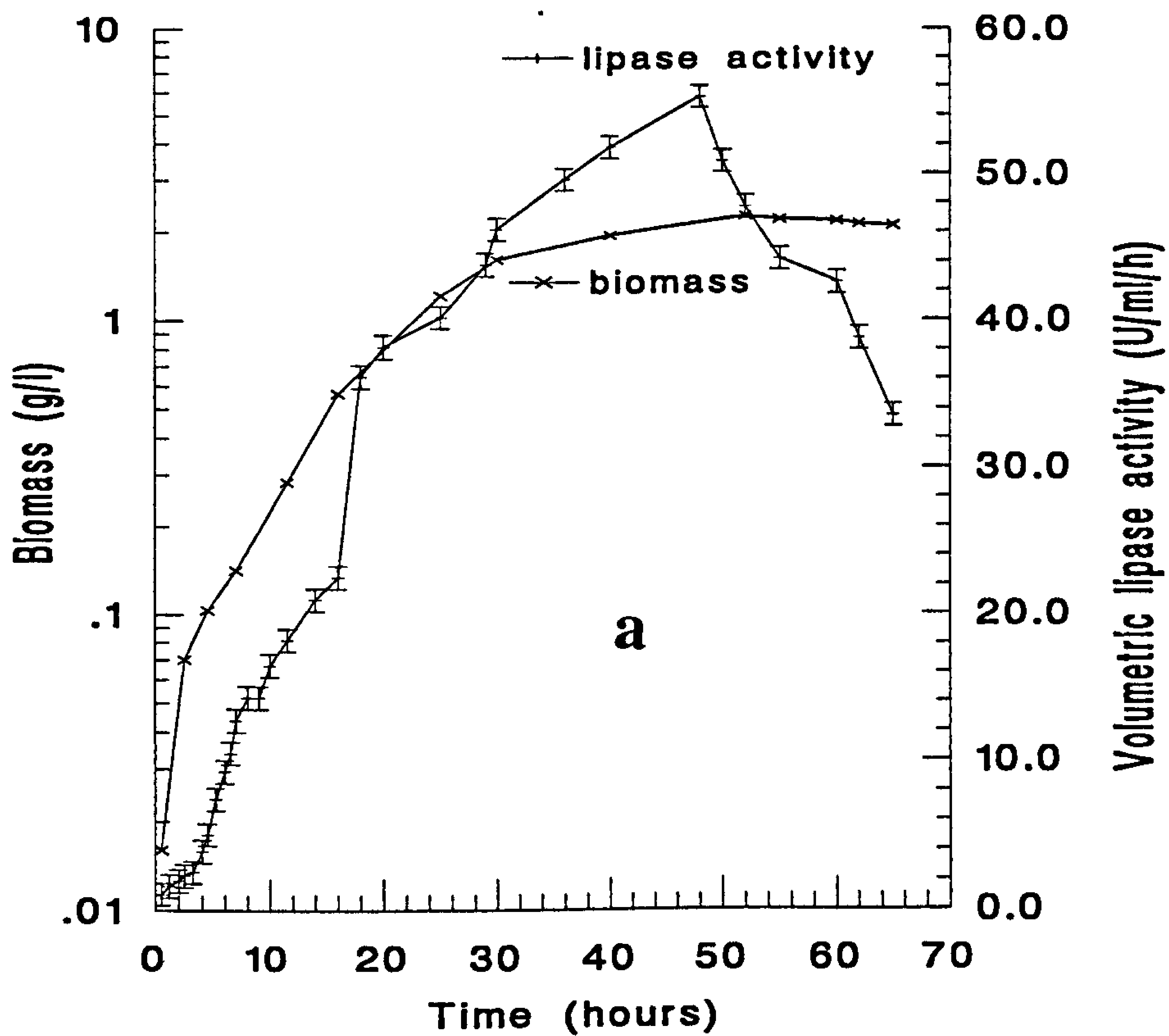
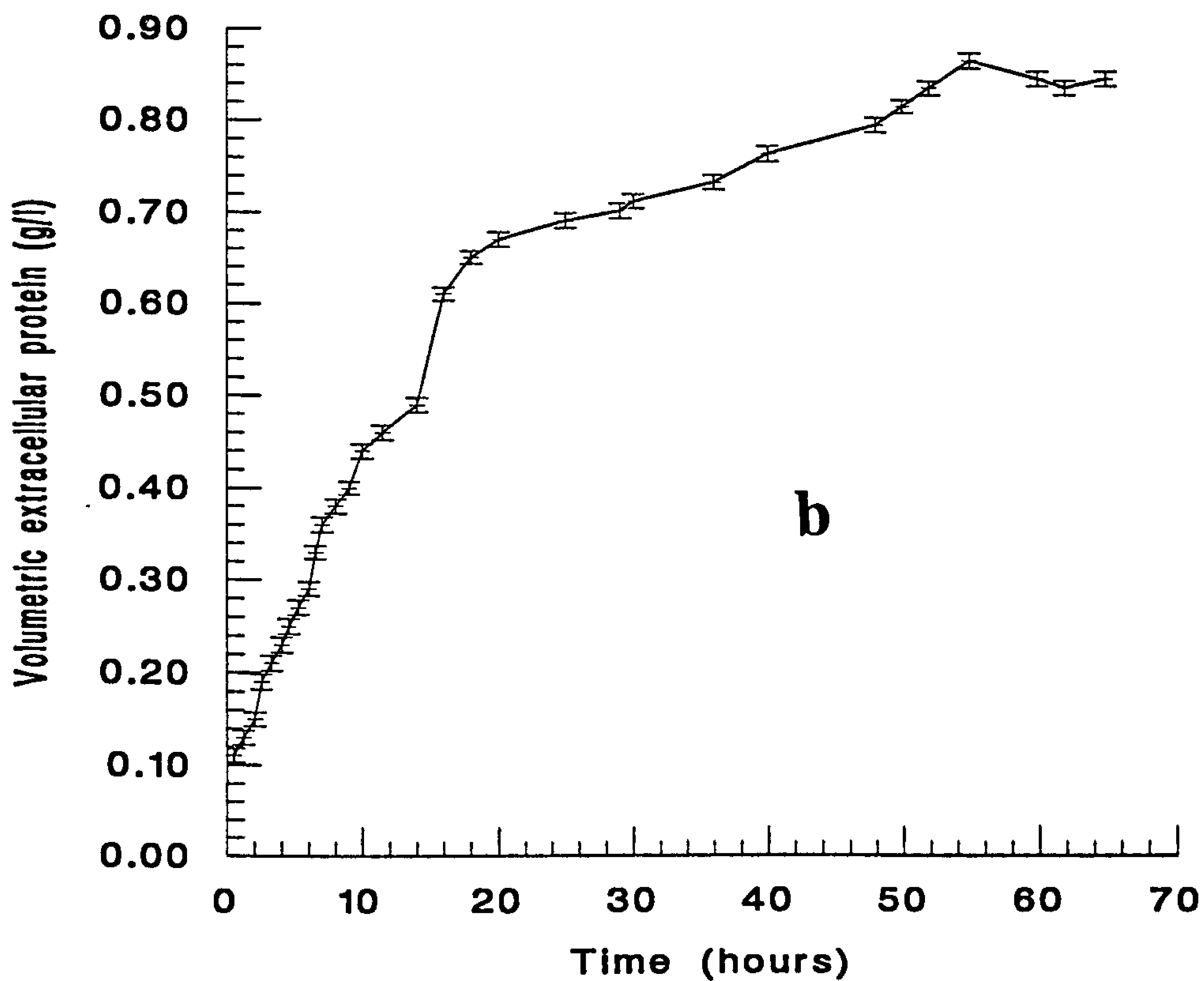

b
doubling time
extr. protein
Doubling time (hours)
Specific extracellular lipase
Temperature (°C)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
0.53
0.52
0.51
0.50
0.49
0.48
0.47
0.46
0.45
0.44
20
30
40
50
60

Figure 4.9

Graph showing a) production of biomass and secretion of volumetric lipase activity and b) secretion of volumetric extracellular protein by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



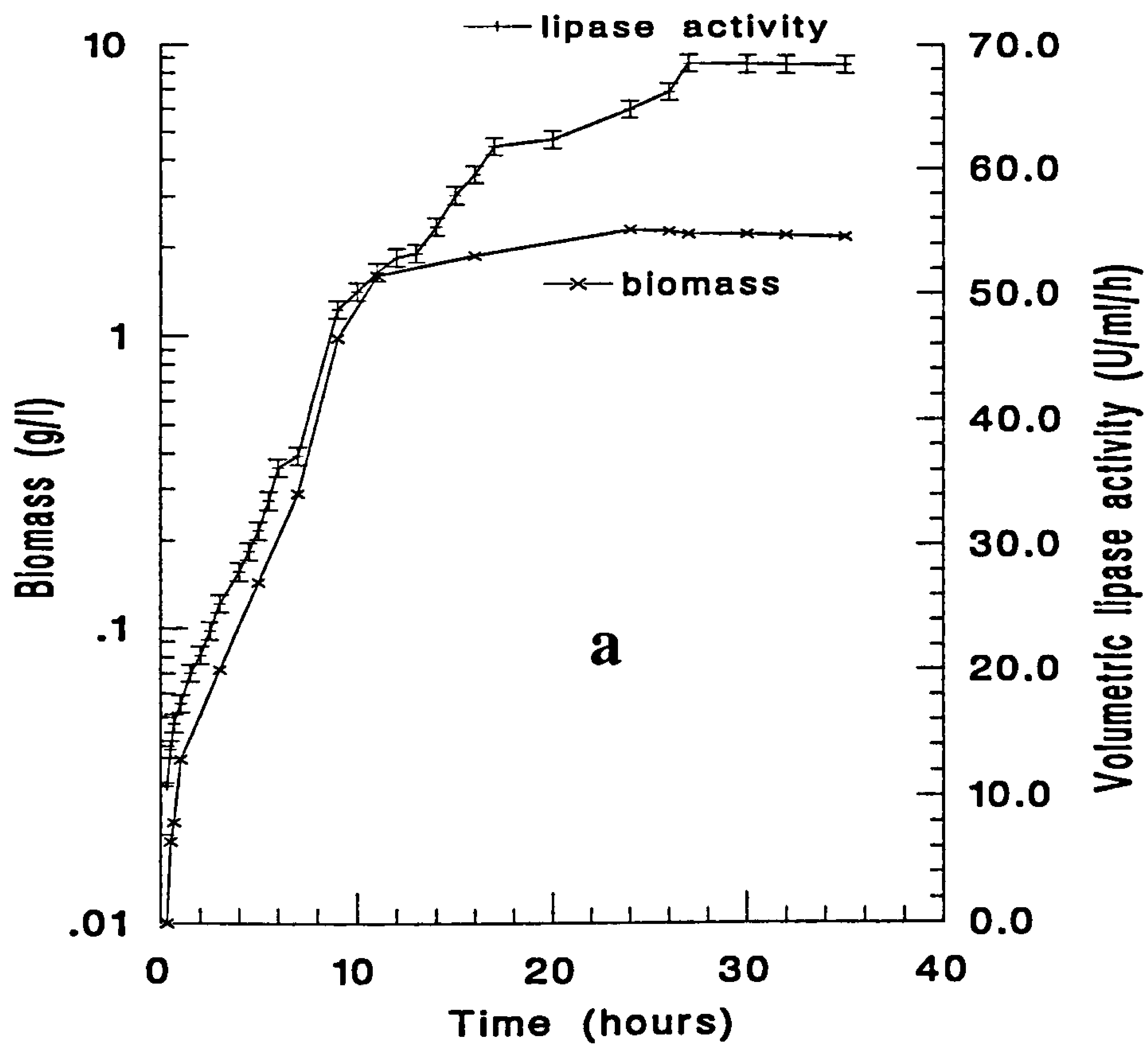
lipase activity
biomass
a
Biomass (g/l)
Volumetric lipase activity (U/ml/h)
Time (hours)
10
1
.1
.01
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
10
20
30
40
50
60
70



b
Volumetric extracellular protein (g/l)
Time (hours)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70

Figure 4.10

Graph showing a) production of biomass and secretion of volumetric lipase activity and b) secretion of volumetric extracellular protein by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


lipase activity
biomass
Biomass (g/l)
Volumetric lipase activity (U/ml/h)
Time (hours)
a
10
1
.1
.01
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
10
20
30
40

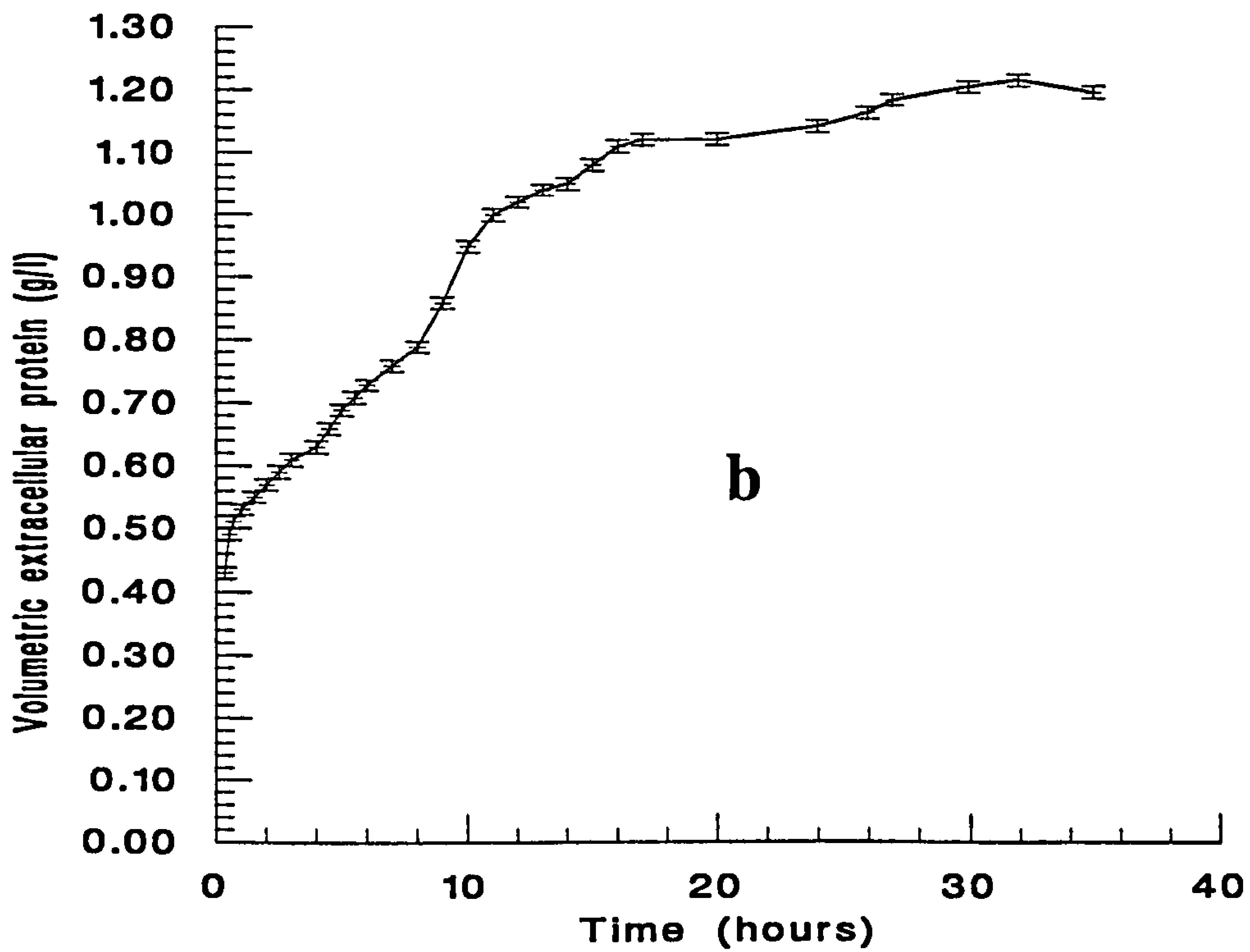
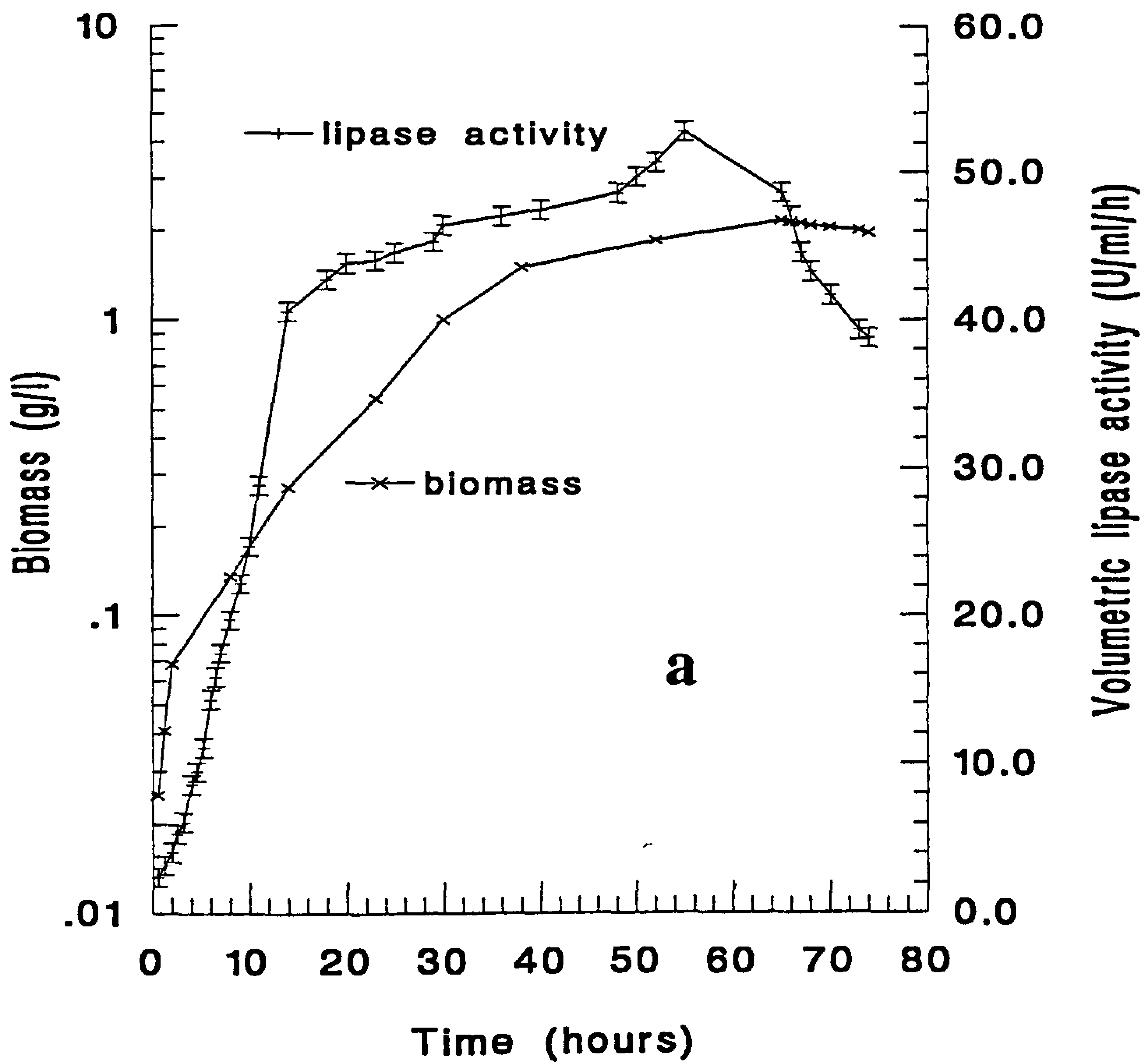
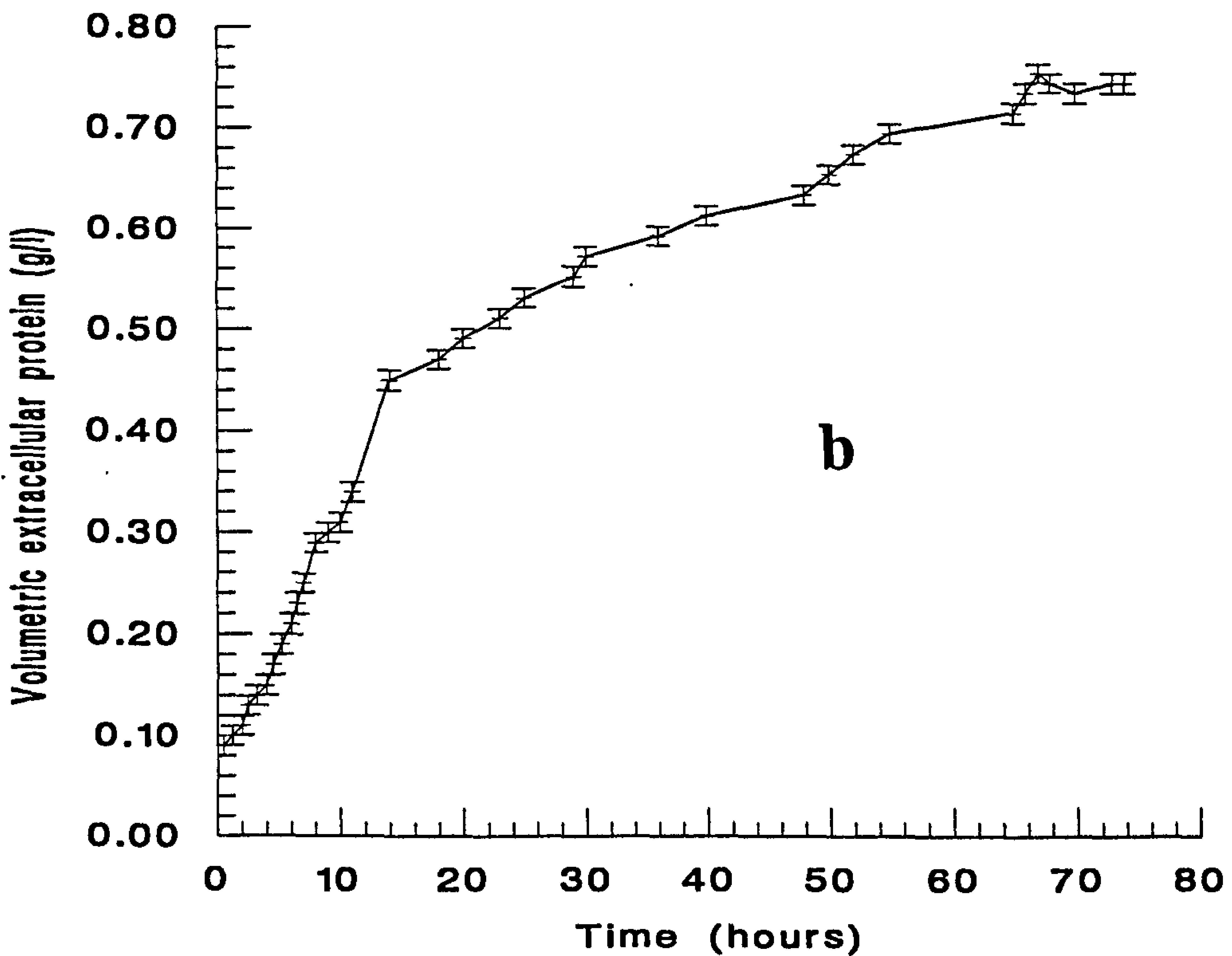

Volumetric extracellular protein (g/l)
Time (hours)
b
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40

Figure 4.11

Graph showing a) production of biomass and secretion of volumetric lipase activity and b) secretion of volumetric extracellular protein by *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 30°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



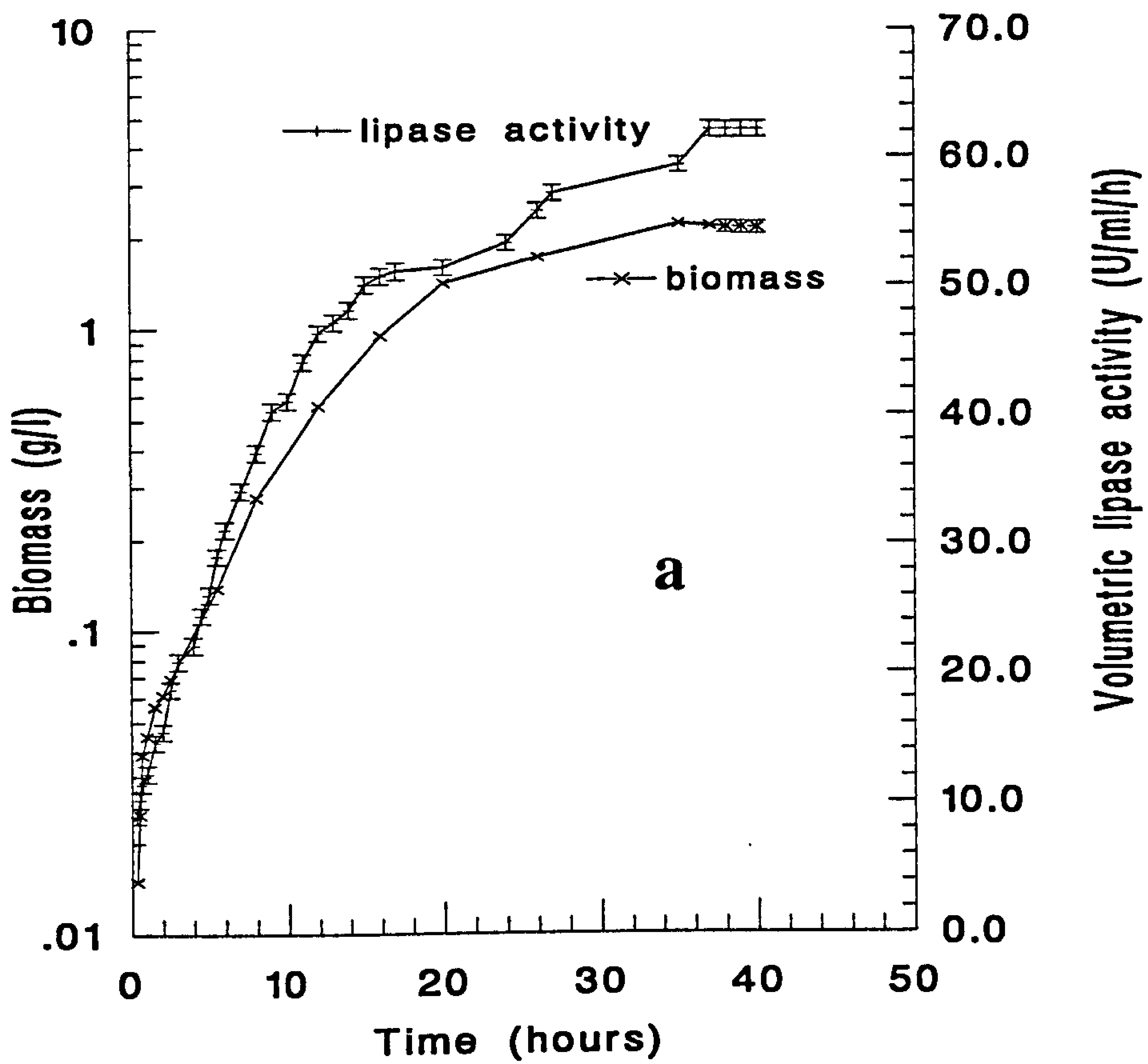
lipase activity
biomass
a
Biomass (g/l)
Volumetric lipase activity (U/ml/h)
Time (hours)
10
1
.1
.01
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
10
20
30
40
50
60
70
80



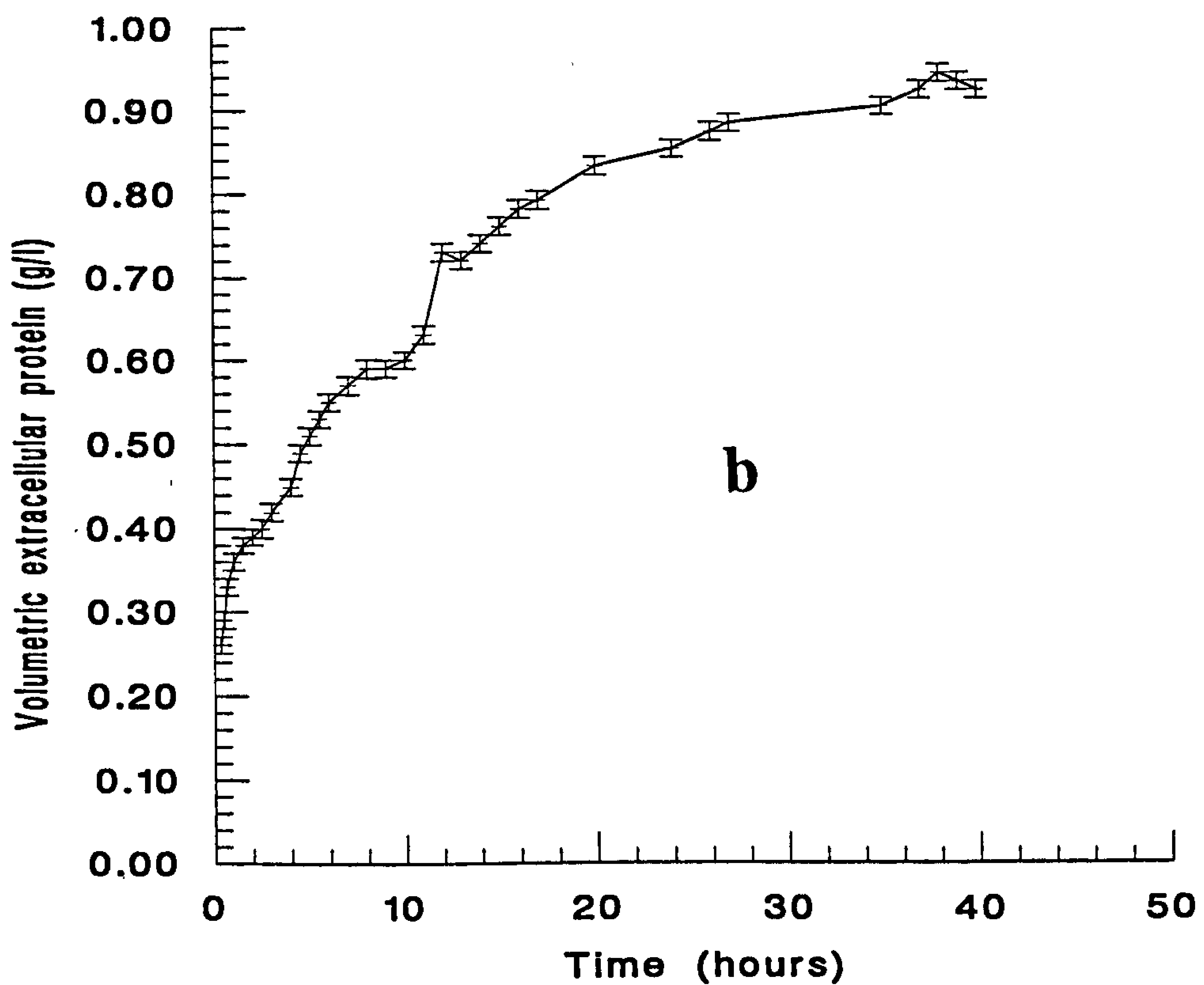
b
Volumetric extracellular protein (g/l)
Time (hours)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80

Figure 4.12

Graph showing a) production of biomass and secretion of volumetric lipase activity and b) secretion of volumetric extracellular protein by *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 50°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



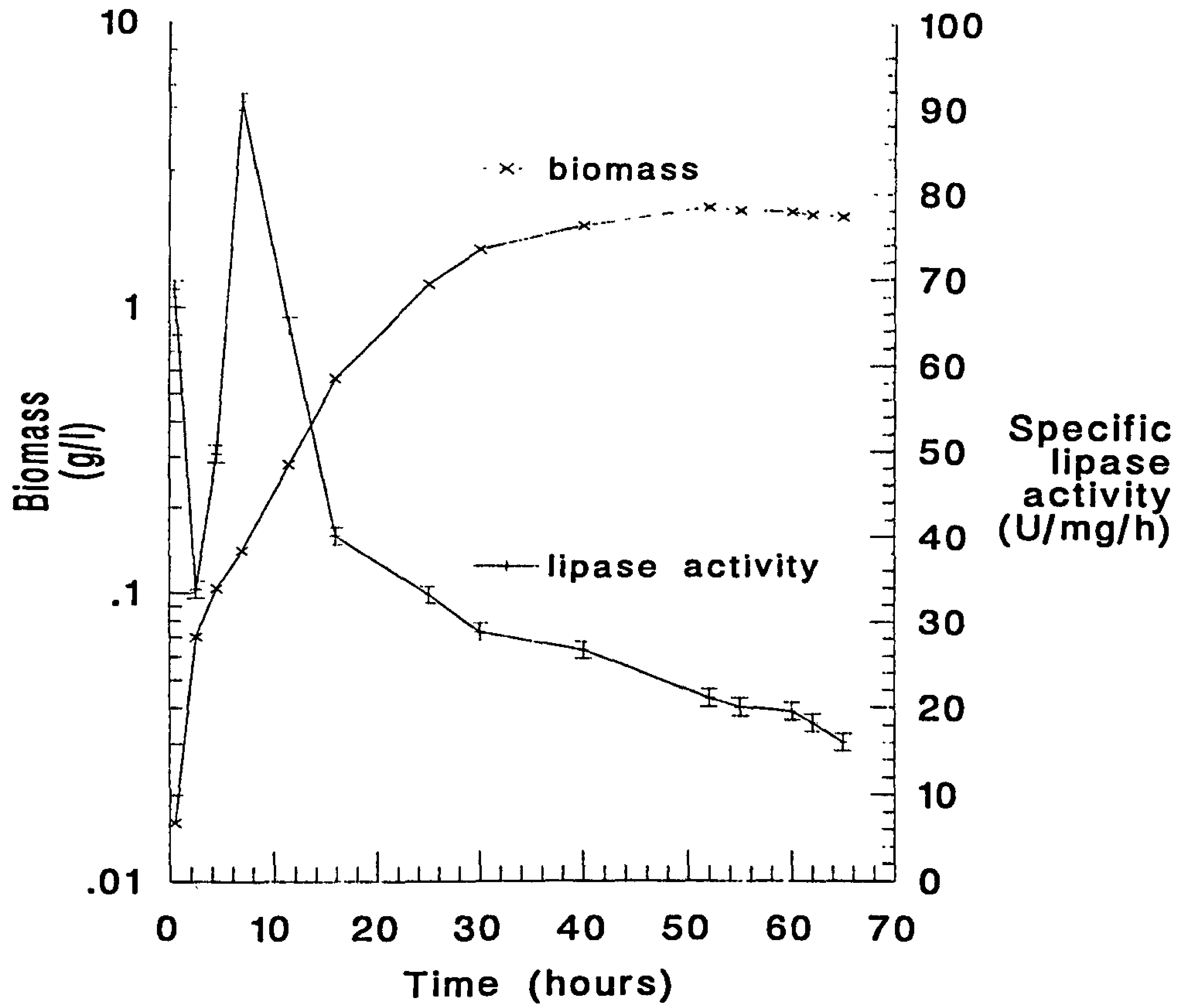
10
1
.1
.01
Biomass (g/l)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Volumetric lipase activity (U/ml/h)
lipase activity
biomass
a
0
10
20
30
40
50
Time (hours)



1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Volumetric extracellular protein (g/l)
b
0
10
20
30
40
50
Time (hours)

Figure 4.13

Graph showing a) production of biomass and secretion of specific lipase activity and b) secretion of specific extracellular protein by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


a
Biomass (g/l)
10
1
.1
.01
biomass
lipase activity
Specific lipase activity (U/mg/h)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (hours)

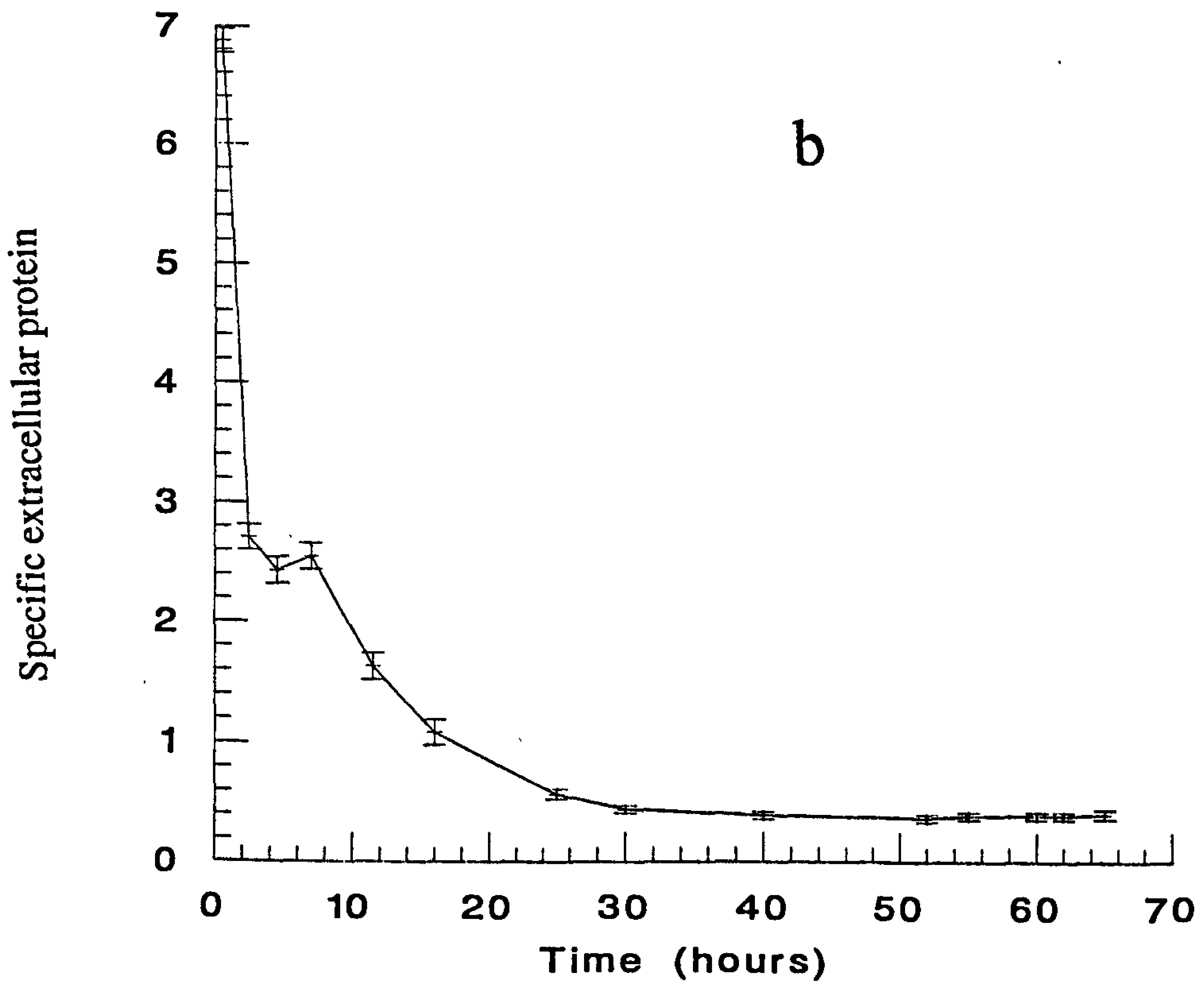
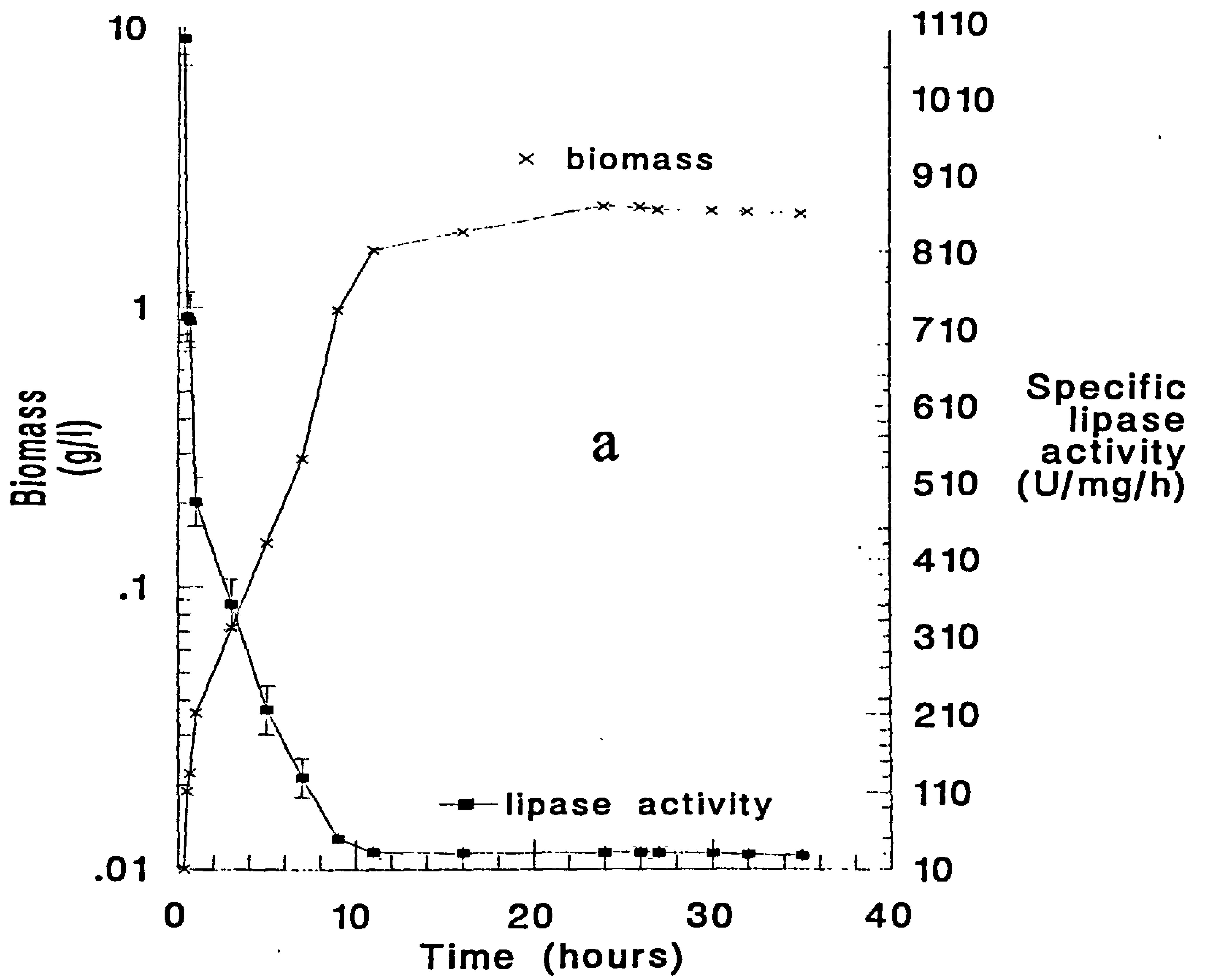
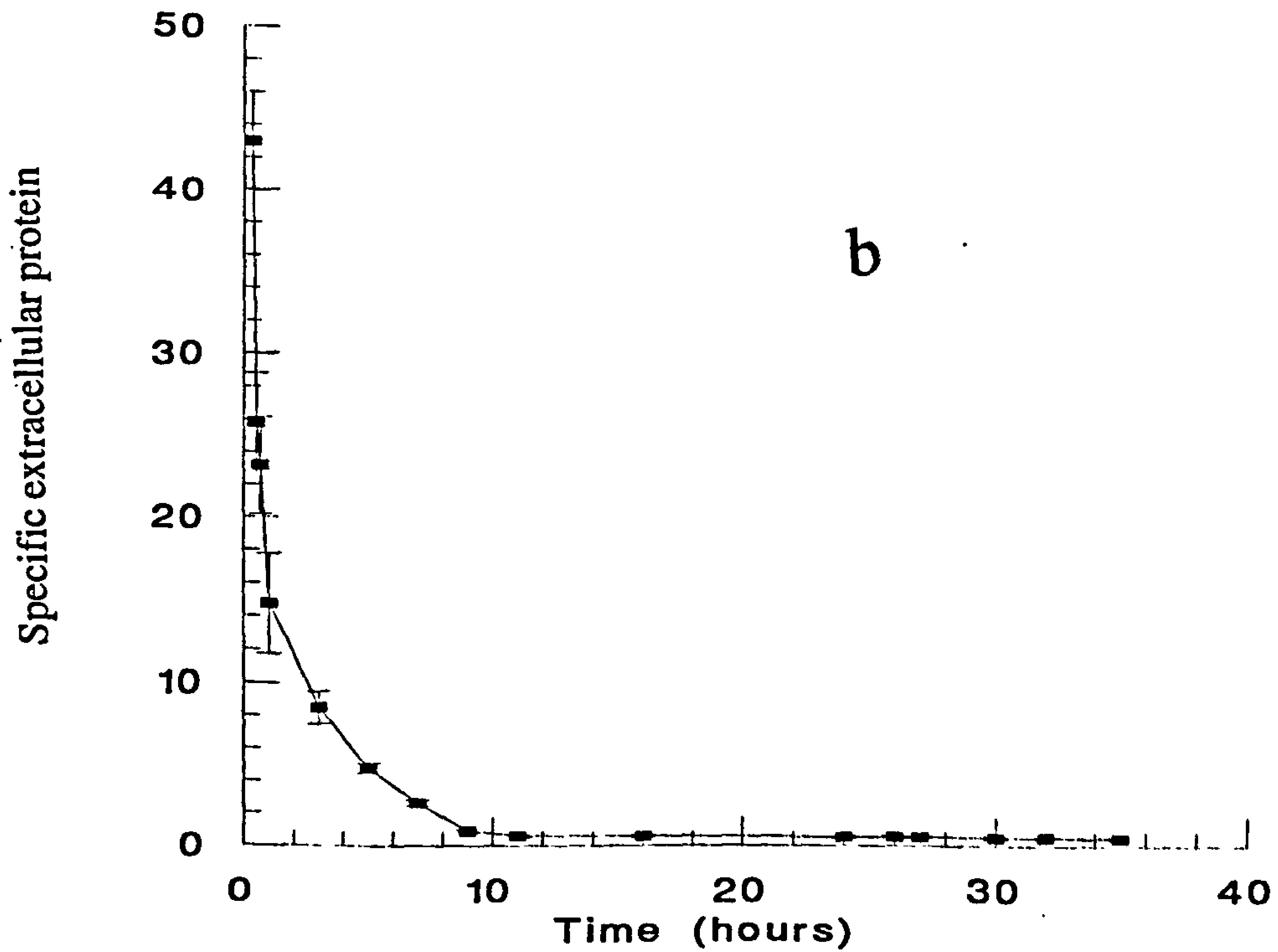

b
Specific extracellular protein
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
Time (hours)

Figure 4.14

Graph showing a) production of biomass and secretion of specific lipase activity and b) secretion of specific extracellular protein by *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



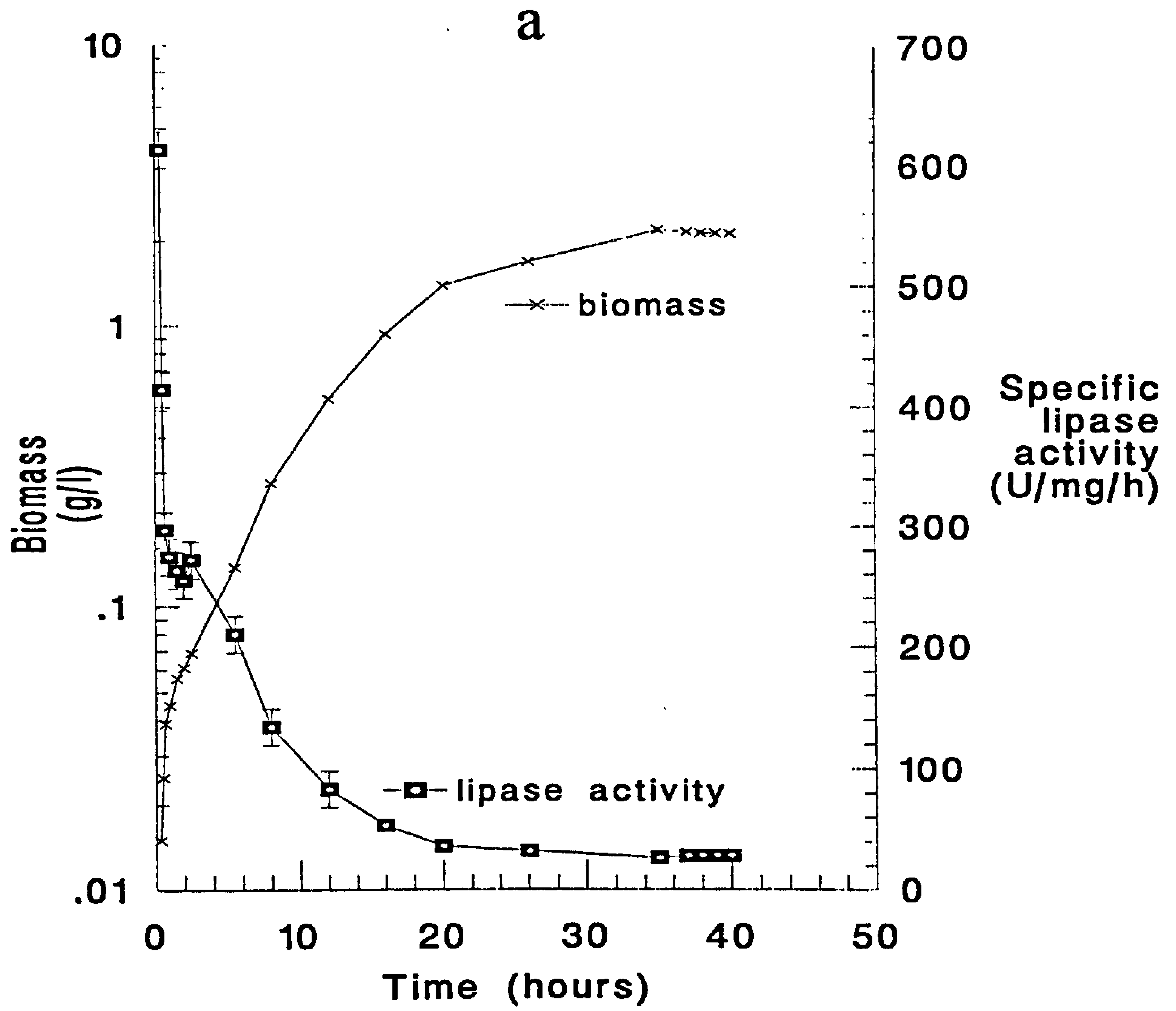
10
1
.1
.01
Biomass (g/l)
1110
1010
910
810
710
610
510
410
310
210
110
10
Specific lipase activity (U/mg/h)
× biomass
a
lipase activity
0
10
20
30
40
Time (hours)



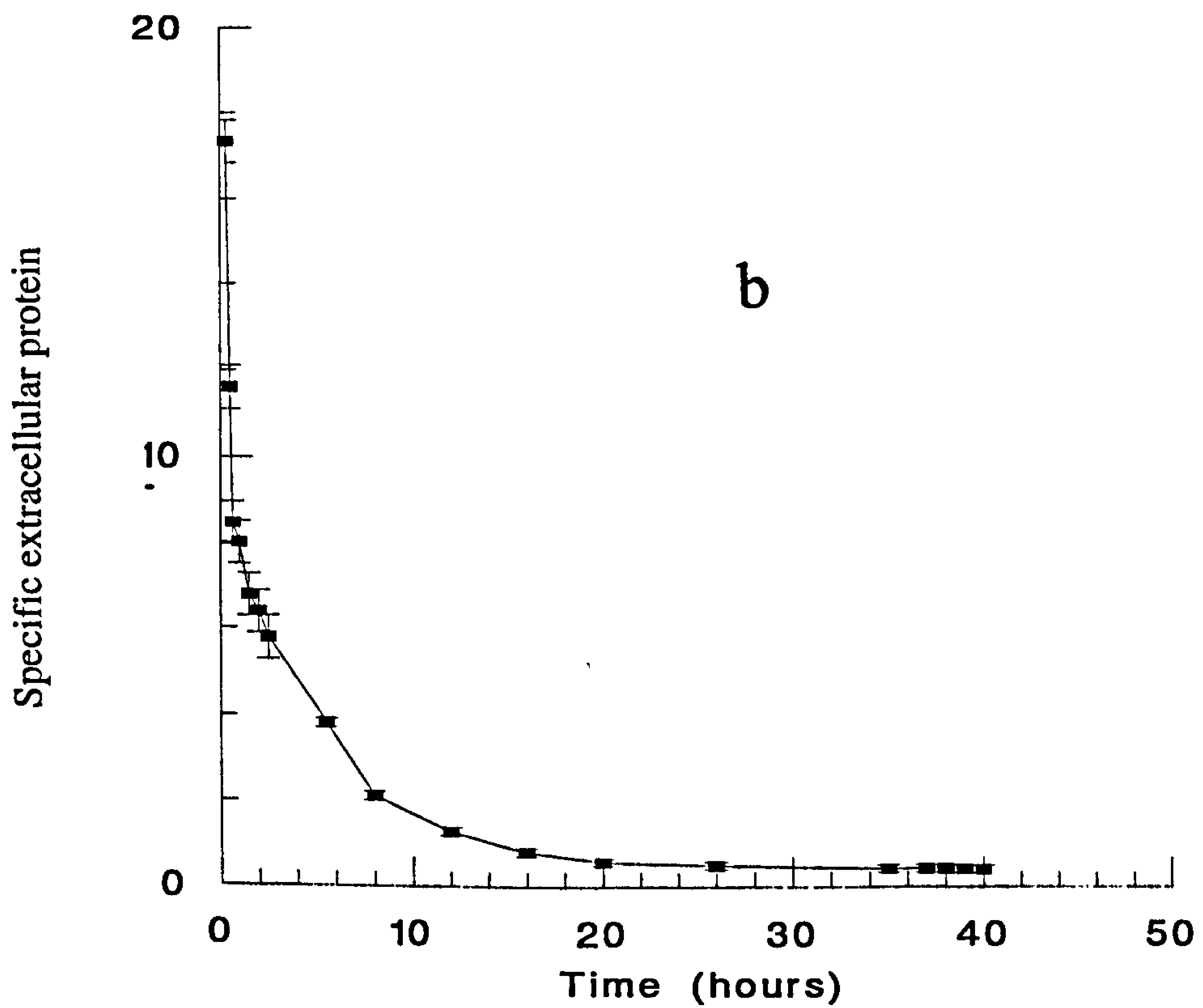
50
40
30
20
10
0
Specific extracellular protein
b
0
10
20
30
40
Time (hours)

Figure 4.16

Graph showing a) production of biomass and secretion of specific lipase activity and b) secretion of specific extracellular protein by *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 50°C. Data represent 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



a
10
1
.1
.01
Biomass (g/l)
700
600
500
400
300
200
100
0
Specific lipase activity (U/mg/h)
biomass
lipase activity
0
10
20
30
40
50
Time (hours)



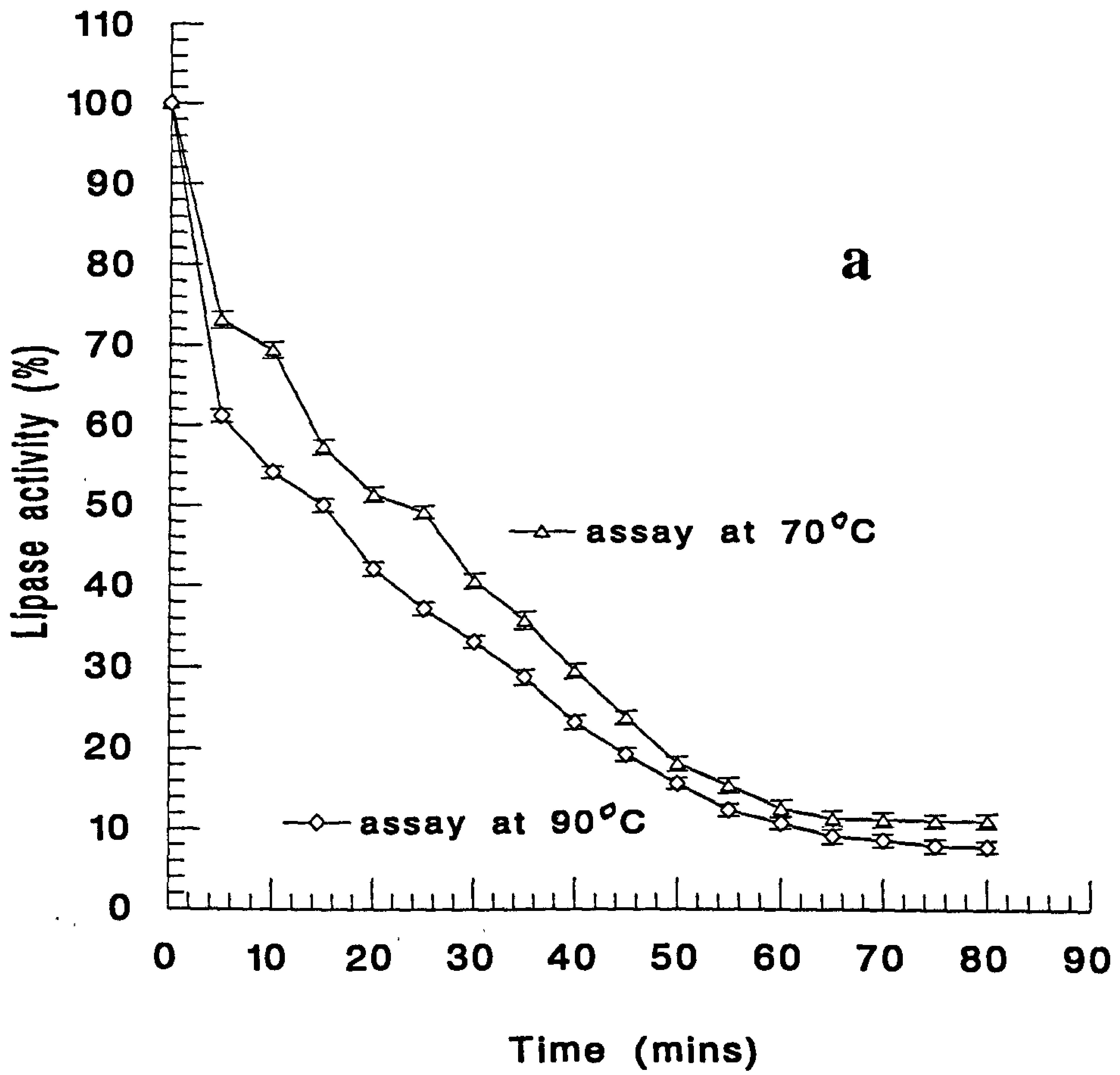
b
20
10
0
Specific extracellular protein
0
10
20
30
40
50
Time (hours)

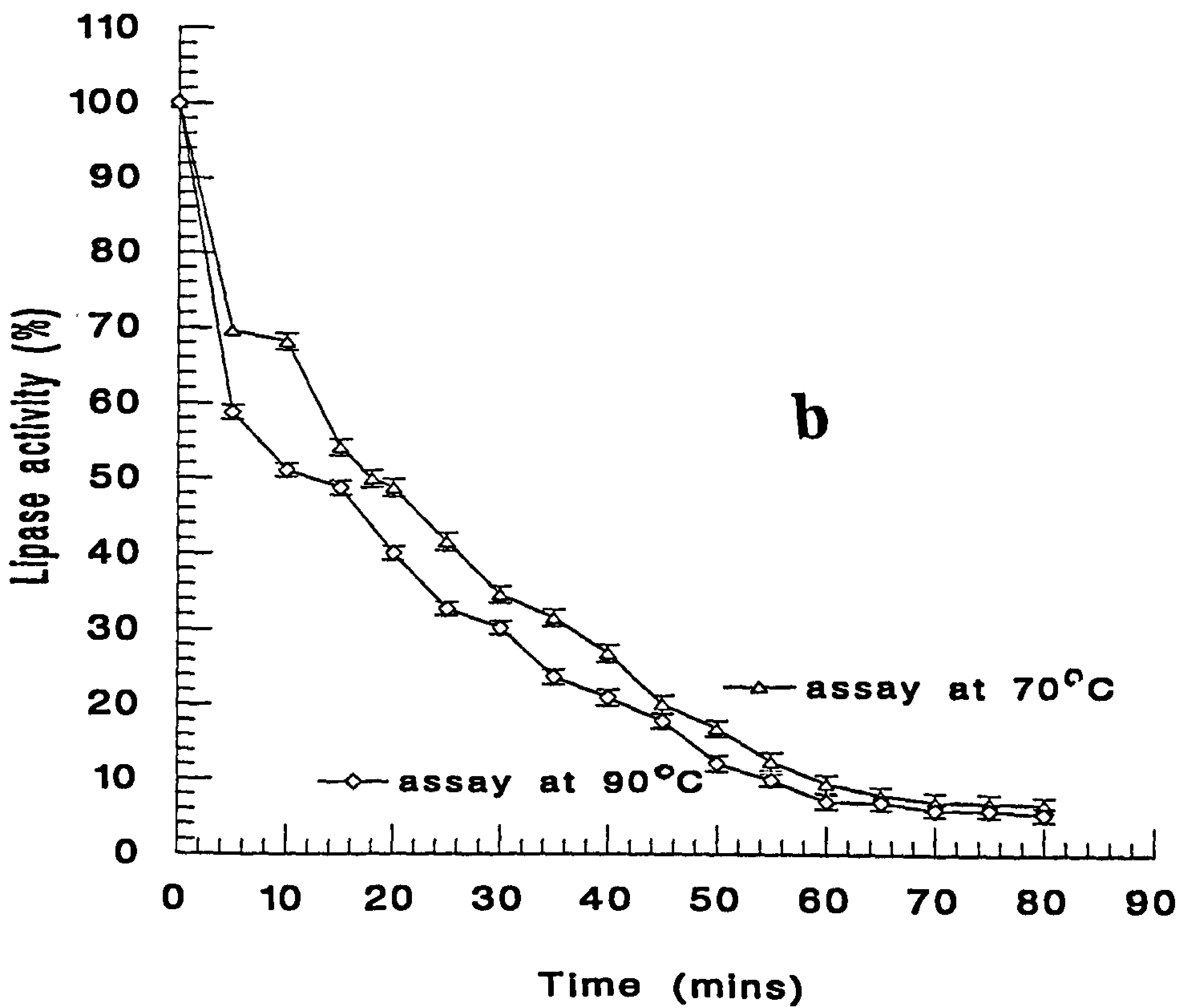
Figure 4.17a

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30°C and heated at 70 or 90°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.

Figure 4.17b

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 30°C and heated at 70 or 90°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


a
Lipase activity (%)
Time (mins)
assay at 70°C
assay at 90°C
0
10
20
30
40
50
60
70
80
90
100
110
0
10
20
30
40
50
60
70
80
90


b
Lipase activity (%)
Time (mins)
assay at 70°C
assay at 90°C
0
10
20
30
40
50
60
70
80
90
100
110
0
10
20
30
40
50
60
70
80
90

30°C. It was concluded that the lipase produced at 30°C was thermolabile since T_{50} values were very low. For *Streptomyces thermoviolaceus* T_{50} was 16 mins when the assay took place at 90°C and 23 mins at 70°C. The corresponding values for *Streptomyces thermodiastaticus* were 12 and 18 mins respectively. These results confirmed flask culture experiments findings in that lipase activity is more thermostable at 70 than at 90°C. When growth took place at 50°C, T_{50} values were much slower as shown in Figures 4.18a and 4.18b. For *Streptomyces thermoviolaceus* the respective values when the assay took place at 70 and 90°C where 64 and 40 mins, whereas for *Streptomyces thermodiastaticus* they were 59 and 36 mins respectively. Table 4.3 shows a summary of the T_{50} values obtained for both organisms at 30 and 50°C growth temperatures.

In order to monitor the thermostability of lipase activity throughout growth, culture supernatants were obtained at regular time intervals during the fermentation and assayed for lipase activity. From Figures 4.19 and 4.20 it was concluded that for both growth temperatures, % inhibition of the activity showed a proportional relationship with time and it was concluded that thermostability of lipase activity at 90°C assay over 70°C assay was decreased as the cultures grew older.

4.9 Effect of lipase inhibitors and metals on lipase activity assayed at 70°C

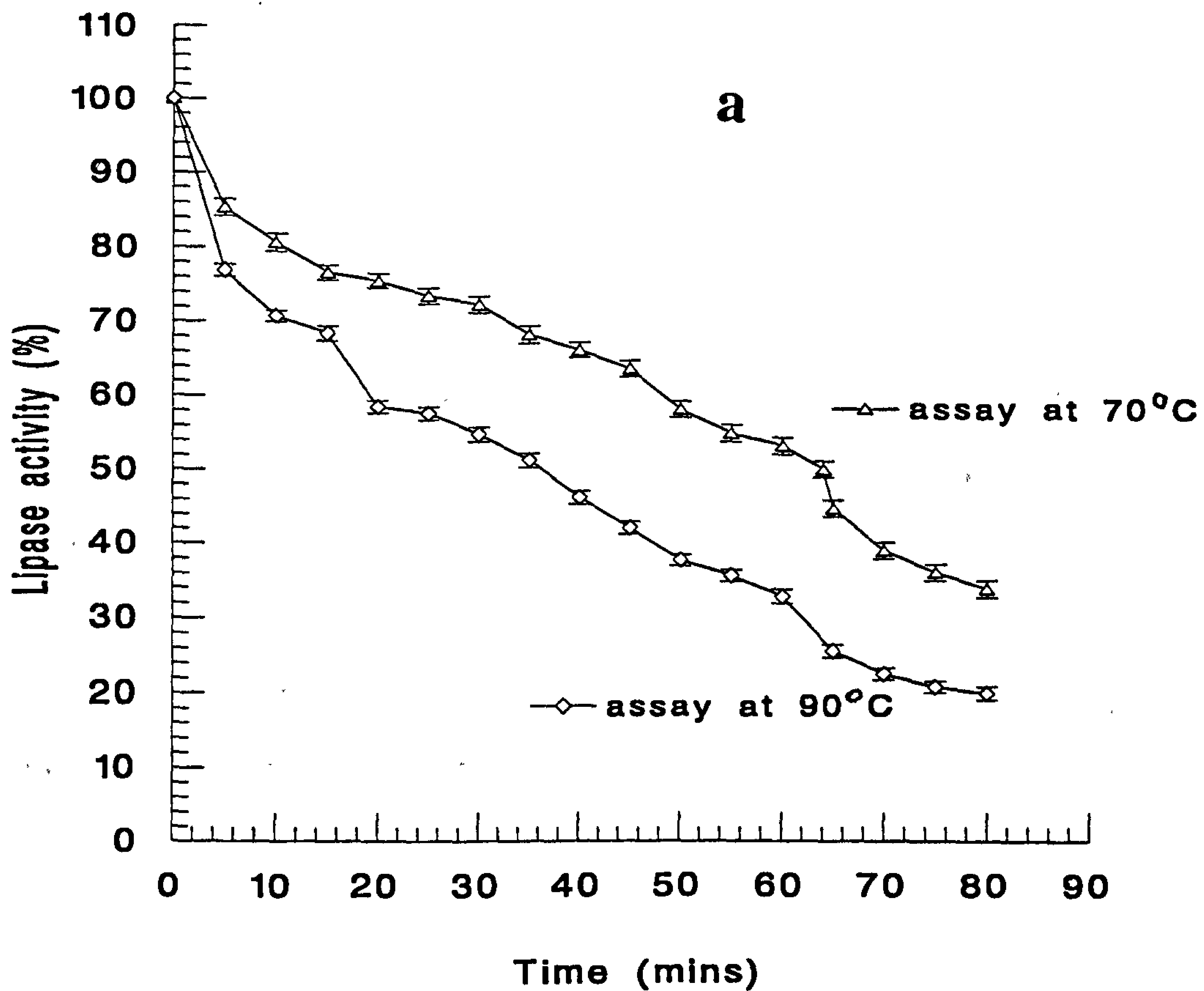
Batch culture cell-free supernatants from *Streptomyces thermoviolaceus* and S. *thermodiastaticus*, grown at 50°C, were assayed for lipase activity after the samples were treated with different metals and inhibitors. As in the case with flask culture experiments, EDTA, o-phenanthroline and PMSF showed a considerable degree of inhibition after a period of 80 mins. $CaCl_2$ and $MgCl_2$ salts enhanced the lipase activity and by adding these metals to EDTA treated samples some of the lost activity was restored. A summary of the results is shown in Figures 4.21 and 4.22. It was suggested

Figure 4.18a

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C and heated at 70 or 90°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.

Figure 4.18b

Effect of different temperatures on the thermostability of lipase activity of *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 50°C and heated at 70 or 90°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


a
Lipase activity (%)
Time (mins)
assay at 70°C
assay at 90°C
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90

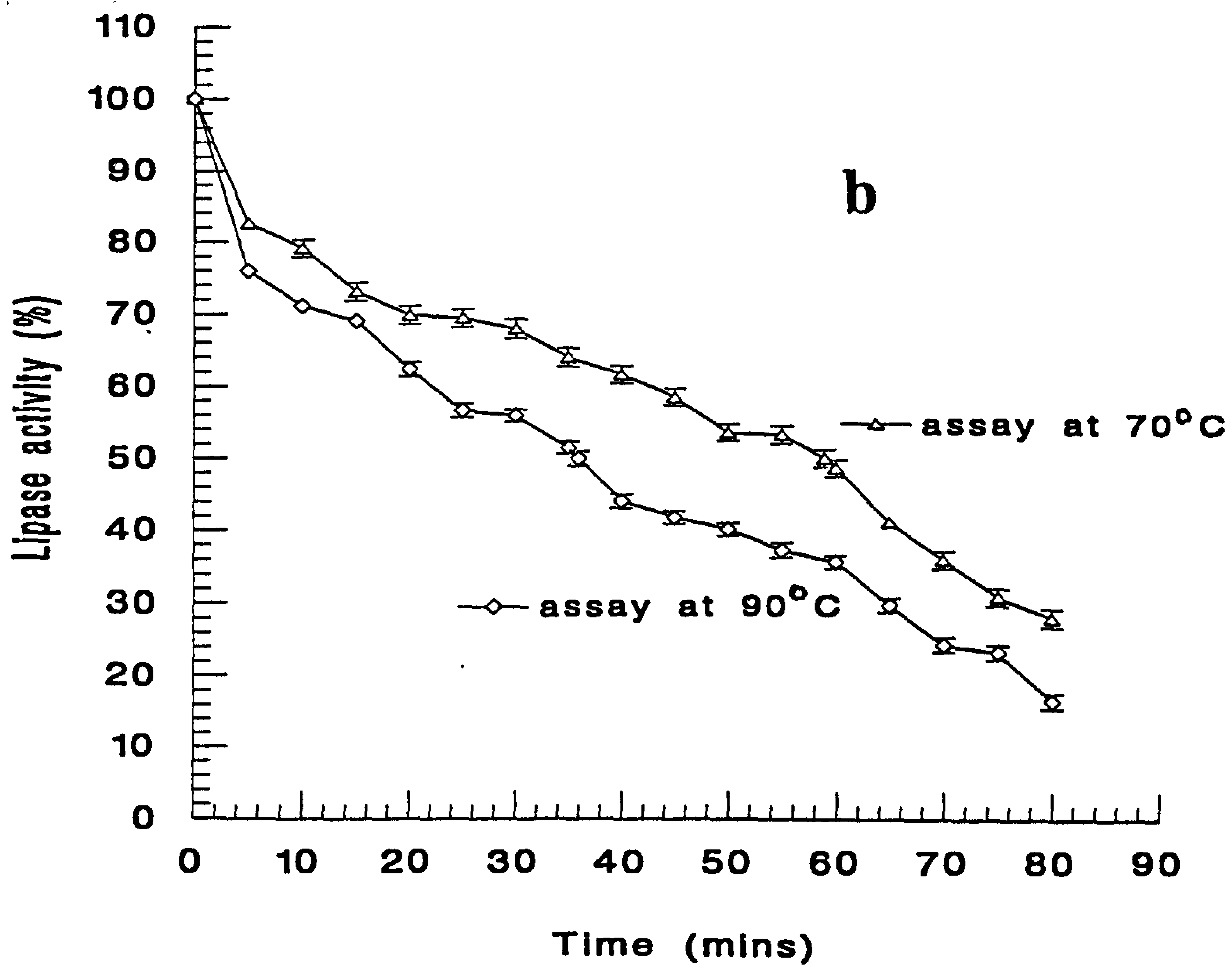
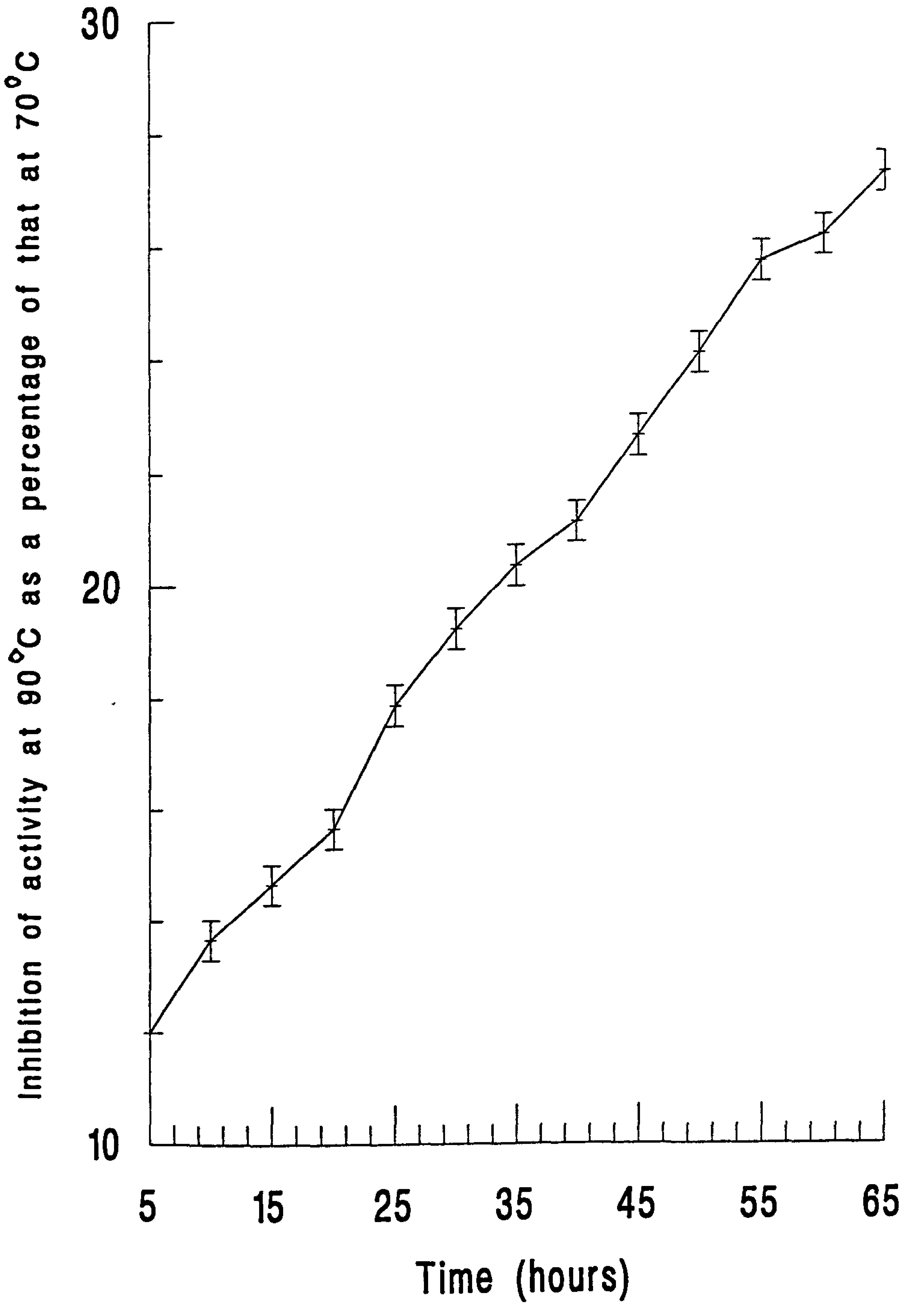

b
Lipase activity (%)
Time (mins)
assay at 70°C
assay at 90°C
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90

Table 4.3 T_{50} values for lipase activity assayed at 70 or 90°C (for cultures grown at 30 or 50°C). Data are representative of 3 separate determinations (mean±SD; n=3).

	Growth at 30°C, T_{50} (mins)		Growth at 50°C, T_{50} (mins)	
Organisms	**Assay at 70°C**	**Assay at 90°C**	**Assay at 70°C**	**Assay at 90°C**
Streptomyces thermoviolaceus	23±0.36	16±0.33	64±0.41	40±0.38
Streptomyces thermodiastaticus	18±0.31	12±0.29	59±0.43	36±0.35

Figure 4.19

Graph showing the inhibition of lipase activity at 90°C as a percentage of that at 70°C of *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 30°C. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



Inhibition of activity at 90°C as a percentage of that at 70°C
30
20
10
5
15
25
35
45
55
65
Time (hours)

Figure 4.20

Graph showing the inhibition of lipase activity at 90°C as a percentage of that at 70°C of *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.

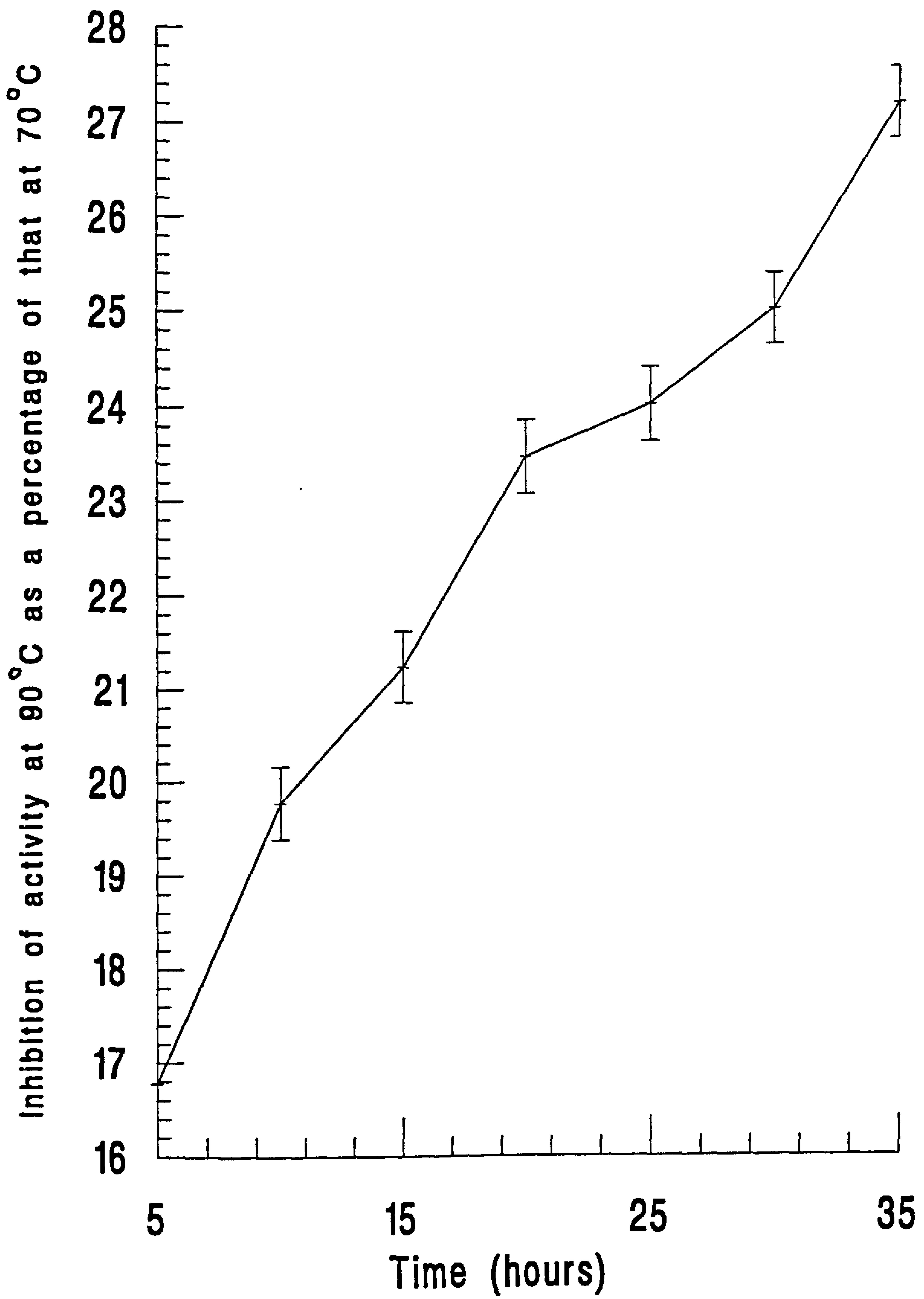
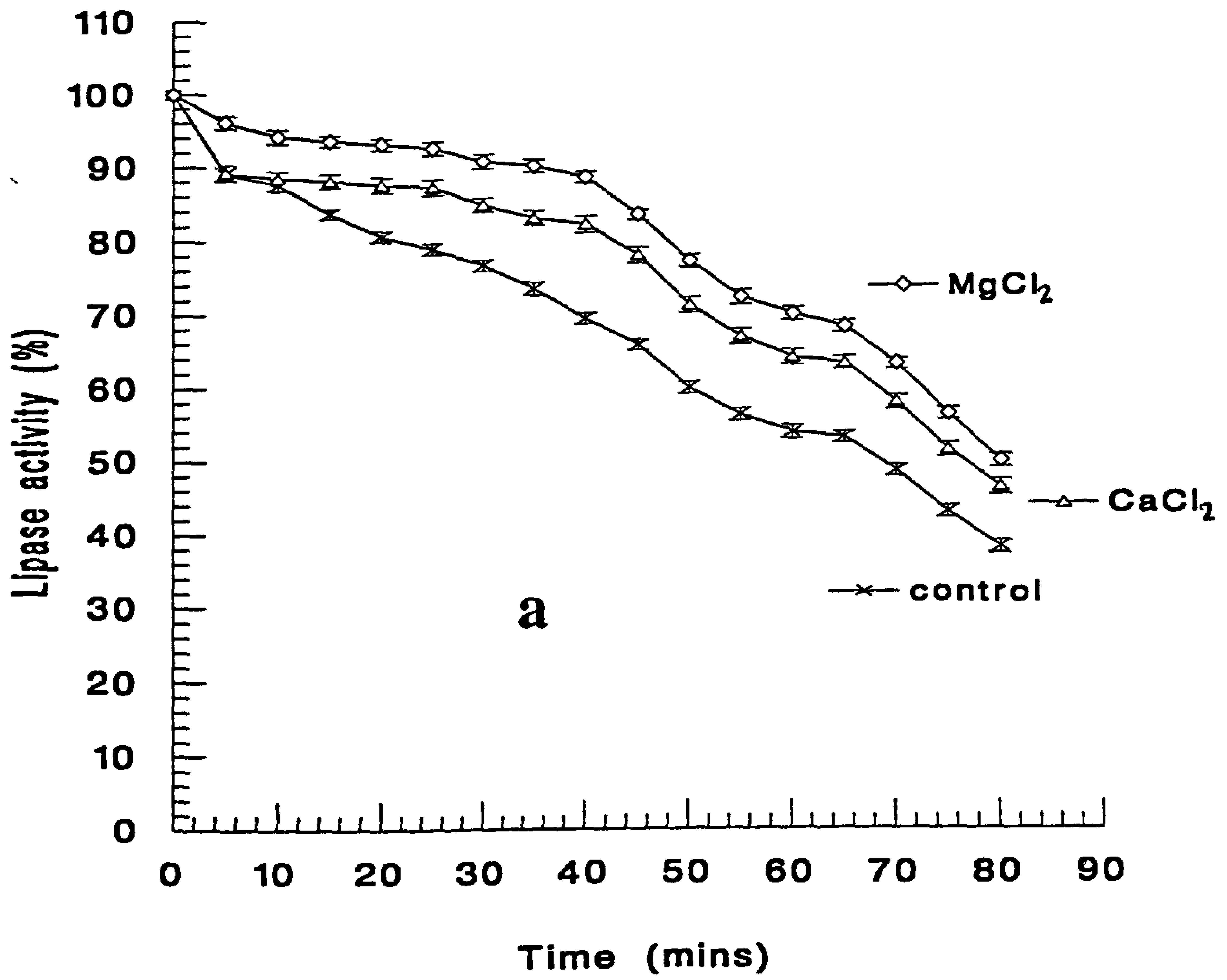
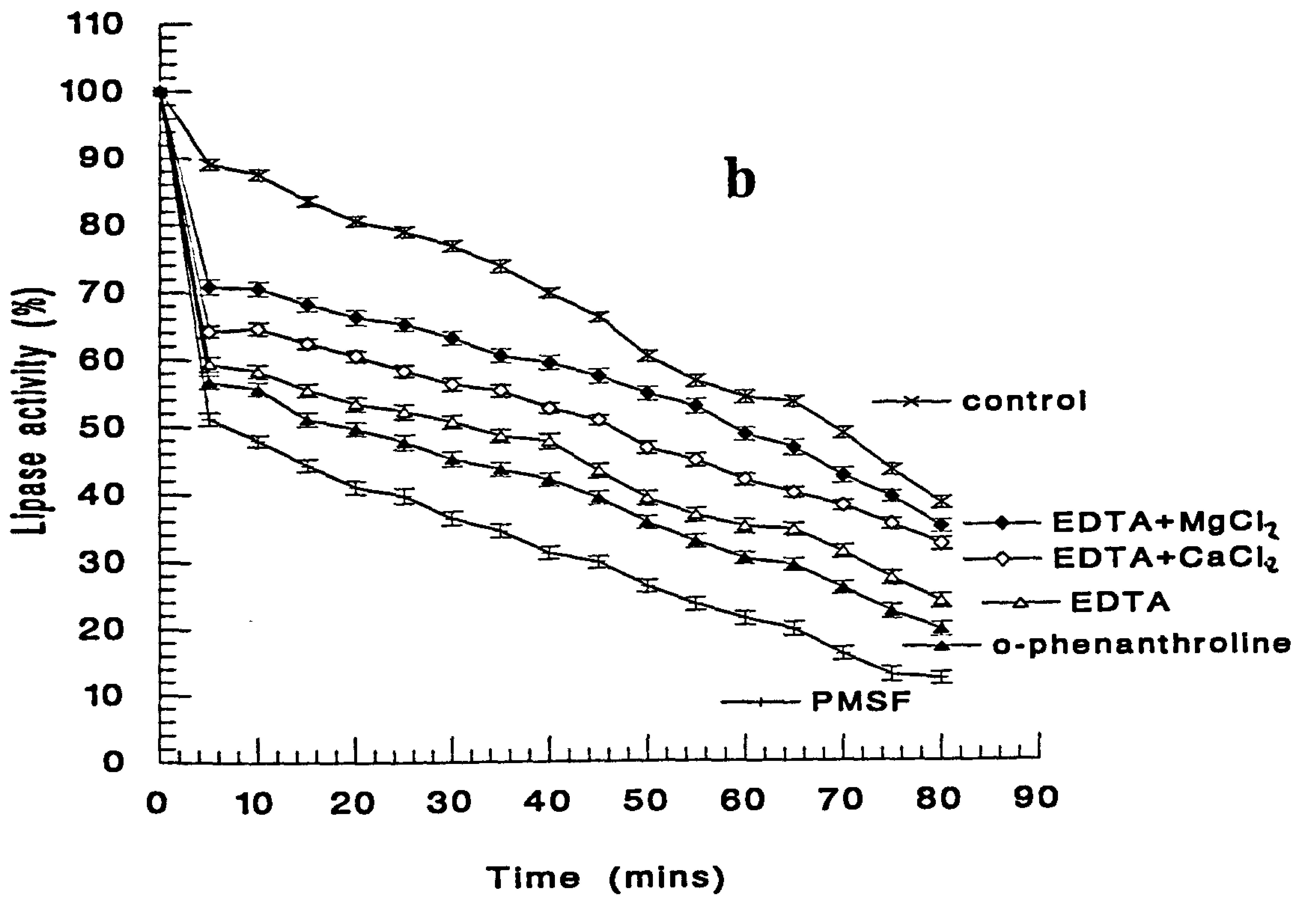

Inhibition of activity at 90°C as a percentage of that at 70°C
28
27
26
25
24
23
22
21
20
19
18
17
16
5
15
25
35
Time (hours)

Figure 4.21

Graph showing the effect of metal ions and inhibitors on the thermostability of lipase activity at 70°C of *Streptomyces thermoviolaceus* from batch culture fermentations grown in the presence of olive oil at 50°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.



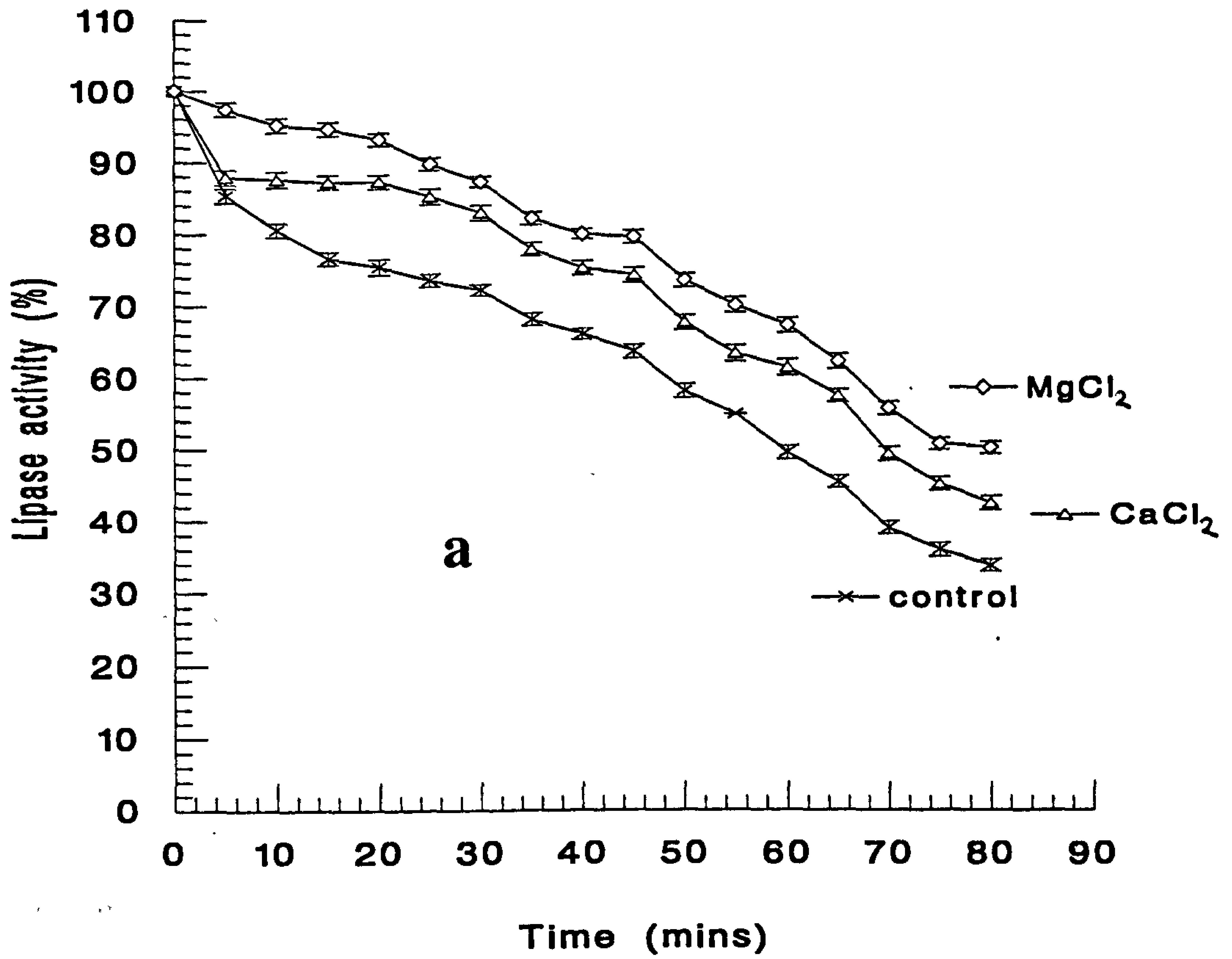
110
100
90
80
70
60
50
40
30
20
10
0
Lipase activity (%)
0
10
20
30
40
50
60
70
80
90
Time (mins)
MgCl2
CaCl2
control
a

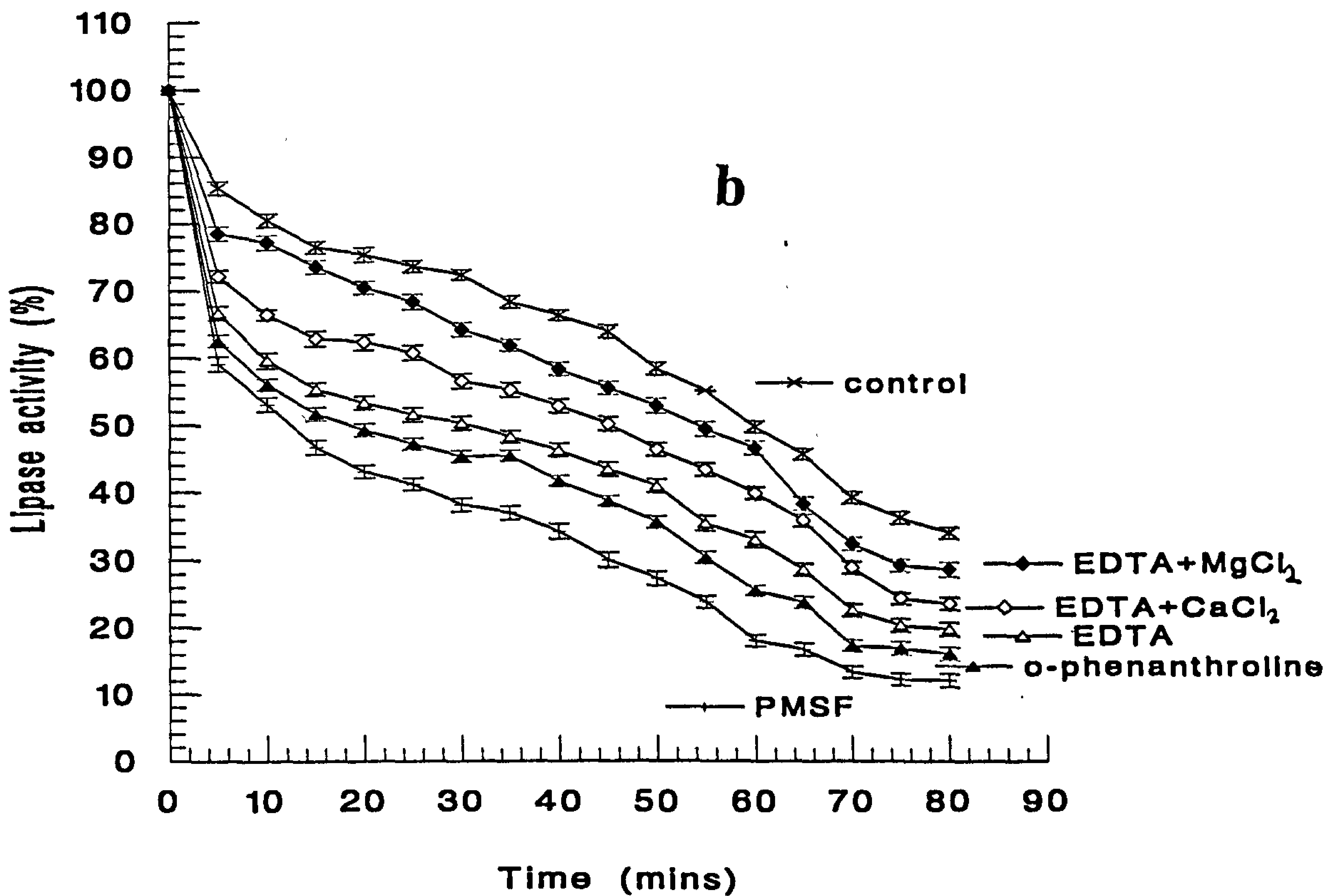


110
100
90
80
70
60
50
40
30
20
10
0
Lipase activity (%)
0
10
20
30
40
50
60
70
80
90
Time (mins)
b
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline
PMSF

Figure 4.22

Graph showing the effect of metal ions and inhibitors on the thermostability of lipase activity at 70°C of *Streptomyces thermodiastaticus* from batch culture fermentations grown in the presence of olive oil at 50°C. The maximum value of lipase activity in the fermentation was taken as the reference point. Data are representative of 3 separate determinations. Standard error bars represent the standard errors of the obtained data.


110
100
90
80
70
60
50
40
30
20
10
0
Lipase activity (%)
0
10
20
30
40
50
60
70
80
90
Time (mins)
a
MgCl2
CaCl2
control


110
100
90
80
70
60
50
40
30
20
10
0
Lipase activity (%)
0
10
20
30
40
50
60
70
80
90
Time (mins)
b
control
EDTA+MgCl2
EDTA+CaCl2
EDTA
o-phenanthroline
PMSF

that the lipase produced has a specific binding site for these two metals which when present make the molecule more compact and hence the thermostability is increased.

4.10 Discussion

Flask culture temperature shift experiments revealed that lipase activity produced its peak value earlier at 50°C than 30°C, which reflected the higher metabolic rate at the higher temperature. Extracellular protein concentration was unaffected by the change in temperature. Lipase produced in 30°C grown cultures was more thermolabile than that in 50°C grown cultures, indicating that growth temperature played an important role in lipase activity thermotolerance, or in expression and synthesis of different heat tolerant enzymes.

Streptomyces thermoviolaceus and *S. thermodiastaticus* were grown at 50°C with different carbon sources in a fermenter. The results suggested that irrespective of the carbon source employed, growth was biphasic. The first growth phase is rapid. This may be due to excess oxygen which gradually becomes a limiting factor until a critical point is reached at which growth rate is affected (James and Edwards, 1991). This was observed for every carbon source used and can explain the finding that the inflexion point for the change in growth rate occurred at a similar biomass concentration. Most rapid growth occurred in the presence of olive oil. The different growth rates allowed by the different carbon sources, showed that the faster the growth rate of the second phase of growth, the greater the production of volumetric lipase activity and extracellular protein. Campbell, (1984) suggested that regulation of primary or secondary metabolism is not strictly regulated at a particular locus. This can explain the variation in lipase activity and extracellular protein when different carbon sources were used which induce or repress metabolism to various degrees.

The use of batch culture fermentation demonstrated the important effect of culture pH on the parameters tested. The pH optimum for biomass yield was different for lipase activity, which was lower between pH 5.0-7.0, where biomass was at its maximum. The optimum pH for lipase activity was 8.0. This coincided with the findings for the lipase activity from *Rhodotorula glutinis* (Papaparaskevas, *et al.*, 1992) and *Galactomyces geotrichum* (Phillips and Pretorius, 1991) which also showed an optimum pH of 8.0. Stöcklein, *et al.*, (1993) on lipase activity from *Penicillium expansum*, reported that the optimum pH was higher, at 9.0. Overall, pH of 8.0 was higher than the reported optimum pH for other lipase producing microorganisms (Nahas, 1988). On lipase activity from thermophilic bacteria, Handelsman and Shoham, (1994) suggested that lipase activity from a thermophilic *Bacillus* sp. showed an optimum pH of 7.0 Extracellular protein levels exhibited a similar pH profile to biomass yield and had an optimum pH of 7.0.

The effects of different temperatures on growth of *Streptomyces thermoviolaceus* in olive oil batch fermenter, revealed that volumetric lipase activity is growth associated. There is a direct relationship between cell yield and lipase activity over the temperature range 30 to 58°C. Preference for biomass and volumetric lipase activity is shown at 30 and 50°C, while at the intermediate temperatures there is a decrease in both biomass yield and volumetric lipase activity. Earlier work by Edwards and Ball, (1987) revealed that *Streptomyces thermoviolaceus* has a thermosensitive respiratory chain and that there is a significant loss of NADH oxidase activity above 40°C. This suggests that other factors may also become limiting towards the maximum growth temperature of 58°C which can explain the reduced growth yields and low lipase activity between 50 and 58°C. When specific lipase activity was taken into account it was found that the ideal temperature for the secretion of lipase activity was 45°C.

Previous work by Gowland, *et al.*, (1987) isolated a number of lipase producing thermophilic bacteria and suggested that they produce the maximum level of lipase

activity at 55°C. Sugihara, *et al.*, (1991) reported that lipase activity from mesophilic sources have an optimum temperature which in most cases is between 30 and 40°C.

The temperatures of 30 and 50°C were proved to be the most efficient for volumetric lipase activity and extracellular protein secretion and as a result these parameters were studied in more detail in these temperatures. For both temperatures, volumetric lipase activity and extracellular protein became detectable during the second slower phase of growth which occur at a time when substrate levels are still in excess. Studies by Gibb, *et al.*, (1989) suggested slow growth rate rather than substrate repression as the prime inducer of enzyme synthesis, fact which was observed in the case of *Streptomyces thermoviolaceus* and *S. thermodiastaticus.* When specific values were taken into account it was revealed that lipase activity showed its maximum point at the beginning of the logarithmic phase of growth. Additionally, specific activity reached its lowest point at the beginning and during the second phase of growth. When specific values were taken into consideration results are the opposite of the volumetric data and this can be explained that by dividing the total activity produced with biomass the secretion of extracellular protein is not taken into consideration

At 30°C, the stationary phase of growth led to a decrease in volumetric lipase activity whereas at 50°C it remained constant after growth ceased, proving that lipase activity was thermostable at the higher temperature after the initiation of the stationary phase of growth. The regulation of hydrolytic enzyme activity has been studied in other *Streptomyces* (Matsue, *et al.*, 1982) and it has been found that the extracellular enzymes are produced during the stationary phase of growth (Gusek, *et al.*, 1988). In this case, it was clear that primary metabolism commences in actively growing cultures.

For both set of temperatures volumetric extracellular protein followed a similar pattern, increasing with cell growth and showing a stationary period after its maximum peak was reached. Cultures were growing at a constant growth rate and no obvious

stress was imposed on the mycelium. As a result, it was concluded that protein secretion was not occurring through some cultural perturbation and is an important element in secondary metabolism of *Streptomyces thermoviolaceus* and *S. thermodiastaticus*. Overall it was concluded that 50°C was the optimum temperature for all the parameters tested. In the case of specific extracellular protein the same pattern of result with the secretion of specific lipase activity were observed. It was concluded that 30 rather 50°C was the optimum temperature for lipase activity since at the later temperature a rapid decrease in the amount of activity produced was observed.

Hale, *et al.*, (1992) expressed and cloned an esterase gene from *Streptomyces scabies* FL1 in *Streptomyces lividans* and found that the esterase gene was expressed during later stages of growth and was regulated by zinc. The same results were found when the gene was expressed in *Streptomyces scabies* (Babcock, *et al.*, 1992). Wang and Saha, (1993) suggested that divalent ions such as Ca^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} enhanced extracellular esterase activity from a thermophilic *Bacillus* A30-1, whereas Zn^{2+} and Fe^{2+} acted as inhibitors of the enzyme. EDTA, p-chloromercuribenzoate and N-bromosuccinimide were found not to affect the enzyme activity. Stöcklein, *et al.*, (1993) on lipase activity from *Penicillium expansum*, suggested that lipase proved to be insensitive to some metal ions such as Ag^{2+} and Mn^{2+} but severely inhibited by $HgCl_2$ and p-chloromercuribenzoate (pCMB). They suggested that the inhibition by EDTA could be reversed by the addition of $CaCl_2$. PMSF had non significant effect on the enzyme activity although, a serine is supposed to be involved in the catalytic triad of some lipases (Schrag, *et al.*, 1991).

In the case of thermophilic *Streptomyces* extracellular lipase activity proved to be activated by Mg^{2+} and Ca^{2+} ions and inhibited by EDTA (a metallo inhibitor) and PMSF (a serine inhibitor) suggesting the presence of two types of lipase, a metallo-lipase and a serine-lipase.

Lipase activity was higher at 70°C than at 80 and 90°C in accordance with the findings from flask culture experiments. Currently commercial lipase exhibit high lipase activity at 40°C but much lower at 70 and very low at 80°C (Omar, *et al.*, 1987; Sugihara, *et al.*, 1991). That indicates that the lipase from thermophilic *Streptomyces* is more thermostable than the lipases derived from mesophilic sources. This can be used were there is an increased demand for more heat stable forms of lipases.

From the results obtained when *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown in batch fermenters, it became obvious that the lipase activity, produced at 30°C, was thermolabile since a considerable amount of the initial activity was lost at a very short time. By contrast, at 50°C lipase was fairly thermostable. In both cases inhibition never reached 100% which suggest the presence of more than one lipase activity.

Chapter 5

Preliminary separation and characterisation of lipases
Cloning of proteolytic enzyme from *S.thermoviolaceus* into *S. lividans*

5.1 Introduction

Various microorganisms possess lipase or esterase systems based on the existence of several enzymes that can be separated by electrophoresis or chromatography (Lambrechts, *et al.*, 1995; Smith, *et al.*, 1992; Murase, *et al.*, 1991). The different enzymes can differ from each other in terms of molar masses, pH and temperature optima, heat stability, sensitivity to inhibitors or substrate specificity. Lipases have generally been known to have dimeric or monomeric structures. Enzyme bands can therefore be the result of different combinations of monomeric or dimeric structures. As a result, lipids and esters are usually split not by one but by several enzymes. Lipases with a marked specificity can be of special importance in biotransformation and inter-esterification reactions as the lipase specificity could be exploited to yield better products (Phillips and Pretorius, 1991).

Chapters 3 and 4 dealt with general properties of lipase activity from a range of thermophilic *Streptomyces* species. Results suggested the possibility that more than one kind of lipase activity was present. The next series of experiments involved methods developed to separate and characterise different lipase activities of *Streptomyces thermoviolaceus* present.

In the *Streptomyces* and other Gram-positive organisms, enzymes that are targeted for secretion are released directly into the culture medium. Enzymes targeted for secretion are typically synthesised in a precursor form which is processed during secretion (Silhavy, *et al.*, 1983). Processing involves the removal of a signal peptide from the amino terminus (von Heijne and Abrahmsen, 1989). In general, there is a hydrophilic stretch of amino acids at the amino terminus of the signal sequence followed by a hydrophobic core of amino acids that precedes the cleavage site. Most of the streptomycete secreted enzymes studied thus far have been reported to contain signal peptides which show properties

similar to those of signal sequences in other organisms. Examples of this include lipase from *Streptomyces* sp. M11 (Perez, *et al.*, 1993), α-amylase from *Streptomyces limosus* (Virolle and Gagnat, 1994) and cholesterol esterase from *Streptomyces lavendulae* (Nishimura and Sugiyama, 1994). Common characteristics between these *Streptomyces* signal sequences have been noted (Doran, *et al.*, 1990).

Positive regulatory genes affecting enzyme synthesis and/or secretion can be cloned by searching for increased secretion of extracellular enzymes in a poor producer species such as *Streptomyces lividans* (Daza, *et al.*, 1990). When genes that encode secreted enzymes from a variety of *Streptomyces* species are cloned and expressed in *S. lividans*, the gene products are typically secreted (Chang, *et al.*, 1990; Koller and Riess, 1989; Martin, *et al.*, 1989). In some cases, the site for signal sequence processing in *Streptomyces lividans* was demonstrated to be identical to that for processing in the native host strain (Chang, *et al.*, 1990). The isolation of transcriptional control signals in streptomycetes can provide an opportunity for analysis of regulation of gene expression in an organism that undergoes both morphological differentiation and regulation of secondary metabolism.

The final part of this project was to attempt cloning of genes responsible for lipase activity from *Streptomyces thermoviolaceus* into *S. lividans*, in order to study the properties of lipase activity within a mesophilic host.

5.2 Concentration of lipase activity

In order to separate and characterise lipase activities of *Streptomyces thermoviolaceus* by SDS-PAGE zymogram analysis, concentration of lipase activity was required. Two methods were tested for their efficiency in order to concentrate the lipase activity: the acetone precipitation method and the polyethylene glycol (PEG) precipitation method. The

results are summarised in Tables 5.1 and 5.2 respectively. In the case of PEG precipitation it was found that PEG 6000 lead to more satisfactory results for lipase activity recovery than PEG 4000. In both cases best recovery was observed when the ratio between PEG and cell-free culture supernatant was 1:2.3. Nevertheless, for both PEG types it was concluded that poor recovery of lipase activity was obtained since only 29 or 23% recovery was achieved.

The precipitation of lipase by acetone treatment gave satisfactory recovery as compared with the concentration of activity by PEG treatment. Precipitation by acetone gave best results at the ratio of 1 volume of acetone to 1.5 of the cell-free supernatant. In this ratio, the recovery was 80%. Addition of the divalent metal ions, Mg^{2+} and Ca^{2+} was found to stabilise thermotolerance of lipase activity. As a result, these cations (at a concentration of 4mM) were added to samples treated with acetone, in order to test if they had any effect in the recovery of lipase activity. Results showed that Mg^{2+} and Ca^{2+} did not improve the efficiency of recovery.

5.3 Separation and characterisation of lipases

SDS-PAGE zymogram analysis was used in order to separate and identify lipases of *Streptomyces thermoviolaceus,* as described by Westergaard, *et al.,* (1980). Samples were prepared in 2x SDS-SB without 2-mercaptoethanol. Electrophoresis was carried out on a 10% (w/v) acrylamide gel for SDS-PAGE. In order to visualise the lipase bands the electrophoretograms were stained with Coomassie Brilliant Blue R or silver salts as described before. Figure 5.1 shows the molecular weight markers used in order to determine the molecular weight of the lipase bands observed. Figures 5.2 and 5.3 showed that two distinct bands were observed: one was estimated to be 36 kDa and the second 49

Table 5.1 Percentage recovery of lipase activity of *Streptomyces thermoviolaceus* by using the acetone precipitation method. In each case the total volume was kept constant. Data represent 3 separate determinations (mean±SD, n=3).

% (v/v) Acetone	% Recovery of lipase activity	% Recovery after addition of $MgCl_2$ (4mM)	% Recovery after addition of $CaCl_2$ (4mM)
96	12±0.12	12.5±0.09	12.3±0.15
98	16±0.16	17.7±0.13	17.13±0.13
90	18±0.13	20.5±0.16	19.76±0.09
85	23±0.19	25.7±0.16	24.79±0.17
80	30±0.09	33±0.09	32.81±0.15
70	40±0.15	42±0.19	41.34±0.16
60	53±0.11	55.16±0.20	55.08±0.11
50	75±0.18	77.24±0.11	76.47±0.21
40	80±0.14	81.63±0.13	81±0.19
30	79±0.11	79.98±0.15	79.56±0.16
20	65.5±0.13	67.84±0.18	66.98±0.14
10	39±0.08	40.1±0.16	40.13±0.09

Table 5.2 Percentage recovery of lipase activity of *Streptomyces thermoviolaceus* by using PEG precipitation method. In each case the total volume was kept constant. Data are representative of 3 separate determinations (mean±SD, n=3).

% (v/v) PEG 6000	% Recovery of lipase activity	% (v/v) PEG 4000	% Recovery of lipase activity
0	100	0	100
80	7±0.19	80	3±0.18
60	10±0.17	60	6±0.20
50	15±0.21	50	11±0.19
40	29±0.22	40	23±0.21
30	24±0.21	30	19±0.17
20	15±0.20	20	14±0.16
10	9±0.19	10	11±0.23

Figure 5.1

Molecular weight markers from an SDS-PAGE electrophoresis gel used to determine the molecular weight of lipase bands.

Figure 5.1

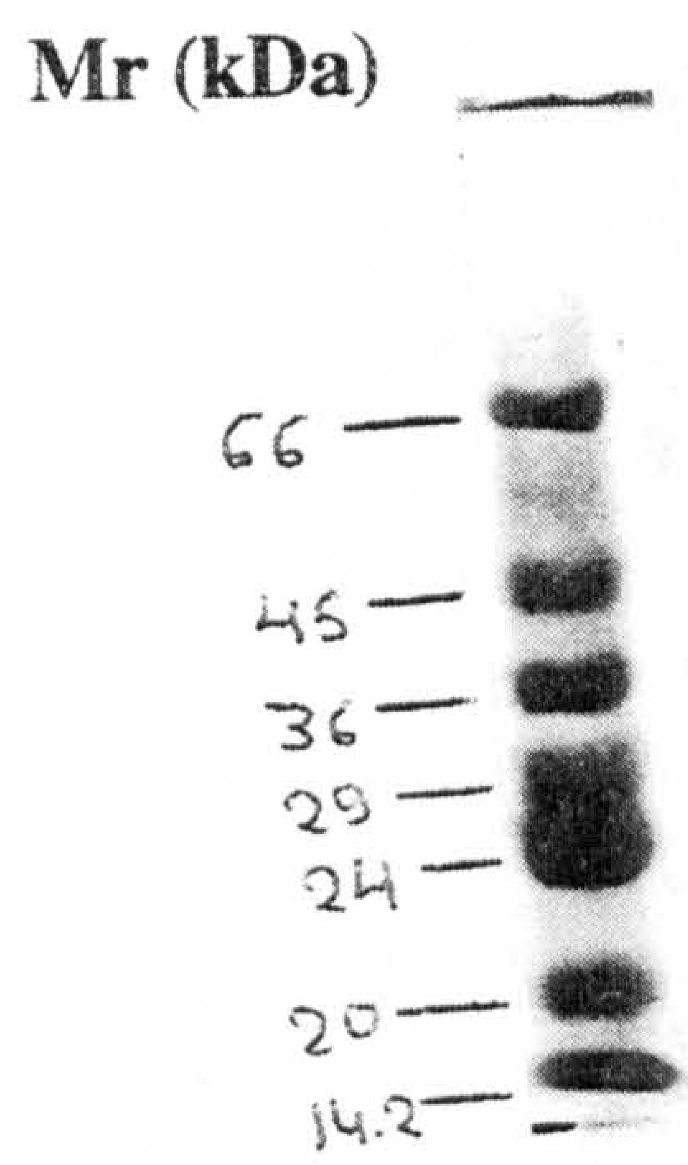
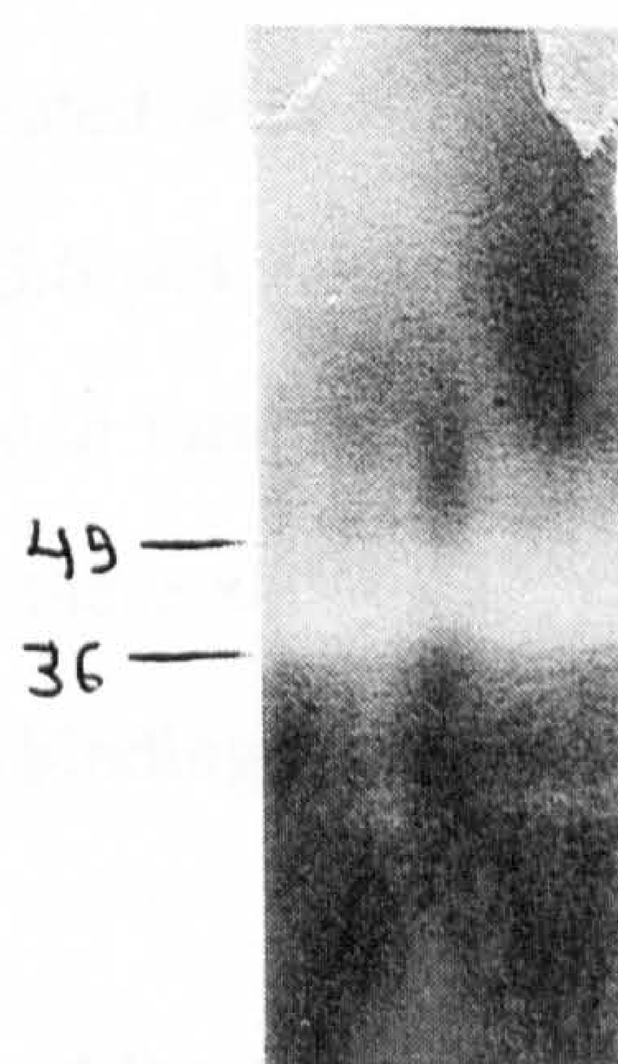


Figure 5.2 and 5.3

Zymogram analysis of *Streptomyces thermoviolaceus* lipases. Cultures were grown in the presence of olive oil as the sole lipid source. Acetone precipitated 48 hours culture supernatants were mixed with an equal volume of 2x SDS-SB and incubated at 50°C for 30 mins in order to bind the proteins with SDS. After cooling samples were resolved by SDS-PAGE zymogram analysis.

Samples were taken from shake flask cultures at the point where lipase activity showed maximum volumetric value.

Figure 5.2 and 5.3



kDa. It was concluded that two different lipases were present. Since the acetone precipitation method was used to concentrate lipase activity, samples concentrated with different % (v/v) acetone were loaded onto the gel in order to test any effect of acetone concentration on lipase bands. Figure 5.4 showed that at 40% (v/v) acetone the bands were brighter. Bands from samples diluted in more acetone appeared to faint, which suggested that dilution of samples with 40% (v/v) acetone resulted in better recovery of lipase activity.

Two additional experiments were performed in order to test the effect of temperature and divalent metals on lipase bands. Samples were heated at different temperatures: room temperature, 37, 50, 60,70,80 and 90°C prior of loading onto the gel. Figure 5.5 showed that as the temperature increased the 49 kDa band disappeared. This indicated that two lipases were present one thermolabile (49 kDa) and one thermostable (36 kDa). From previous experiments it was concluded that Mg^{2+} and Ca^{2+} enhanced the lipase activity and as a result samples were treated with these divalent metals to test for any possible effects on lipase bands. Figures 5.6 and 5.7 showed the results for Mg^{2+} and Ca^{2+} treated samples respectively. Two untreated samples were also loaded onto the gel. In both cases, bands from samples treated with these cations appeared to be clearer and this can provide another indication of the specific binding sites that the enzyme has for these two metals.

5.4 Selection of mesophilic host for cloning experiments

Streptomyces lividans TK64 was chosen as the mesophilic host for subsequent cloning experiments. It is used extensively as a host for cloning and expressing genes from *Streptomyces* species (Bahri and Ward, 1993) and being a streptomycete, it should allow expression from the natural promoter of the *Streptomyces thermoviolaceus* lipase gene.

Figure 5.4

Effect of acetone concentration on zymogram analysis of *Streptomyces thermoviolaceus* lipases. Cultures were grown in the presence of olive oil as the sole lipid source. Acetone precipitated 48 hours culture supernatants were mixed with an equal volume of 2x SDS-SB and incubatd at 50°C for 30 mins in order to bind the proteins with SDS. After cooling samples were resolved by SDS-PAGE zymogram analysis.

Lane 1	**40% (v/v) acetone**
2	**50% (v/v) acetone**
3	**60% (v/v) acetone**
4	**70% (v/v) acetone**
5	**80% (v/v) acetone**

Figure 5.4

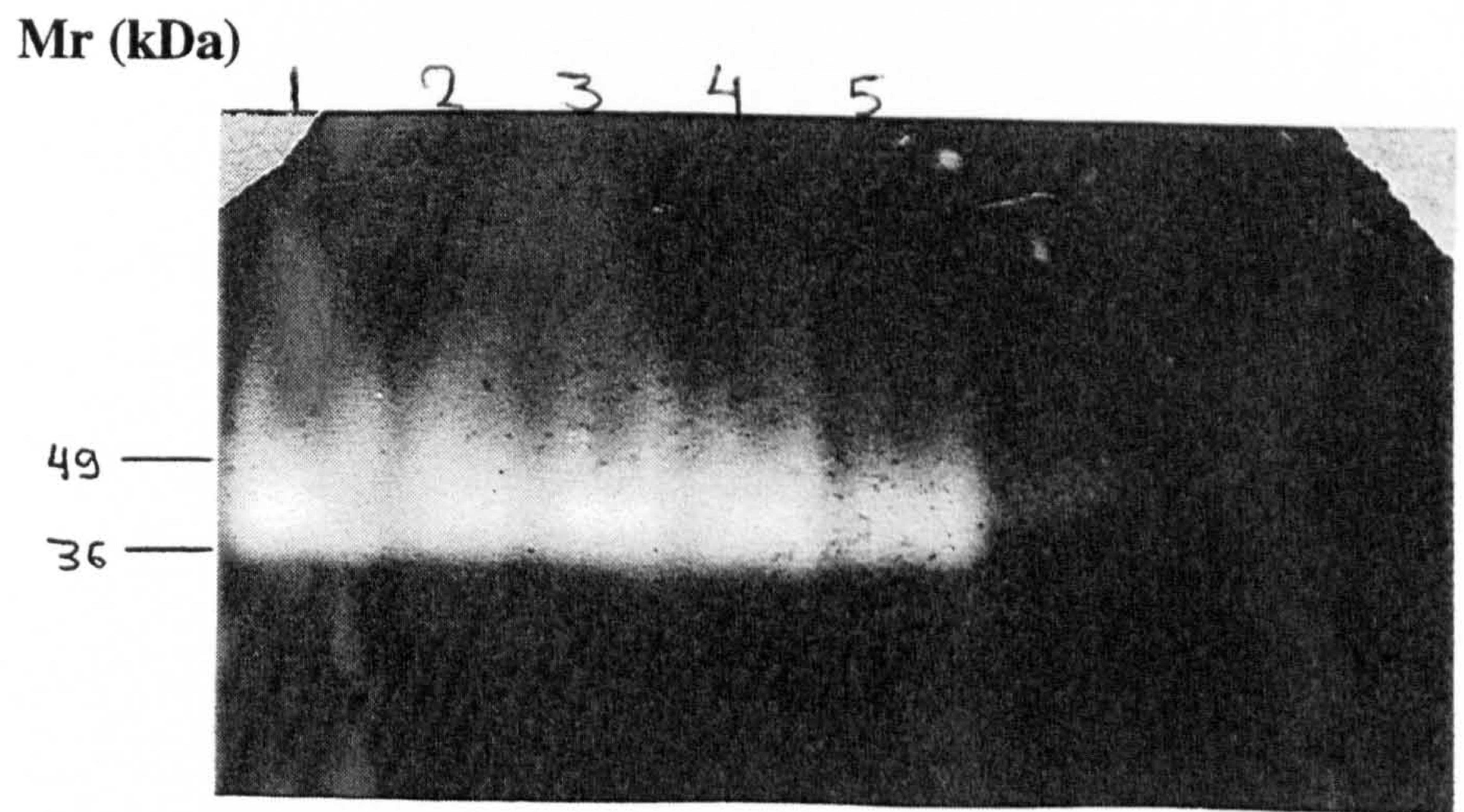


Figure 5.5

Effect of temperature on zymogram profile of *Streptomyces thermoviolaceus* lipases. Cultures were grown in the presence of olive oil as the sole lipid source. Acetone precipitated 48 hours culture supernatants were mixed with an equal volume of 2x SDS-SB and incubated at given temperature for 7 mins. After cooling samples were resolved by SDS-PAGE zymogram analysis.

Lane 1	**room temperature**
2	**37°C**
3	**50°C**
4	**60°C**
5	**70°C**
6	**80°C**
7	**90°C**

Figure 5.5

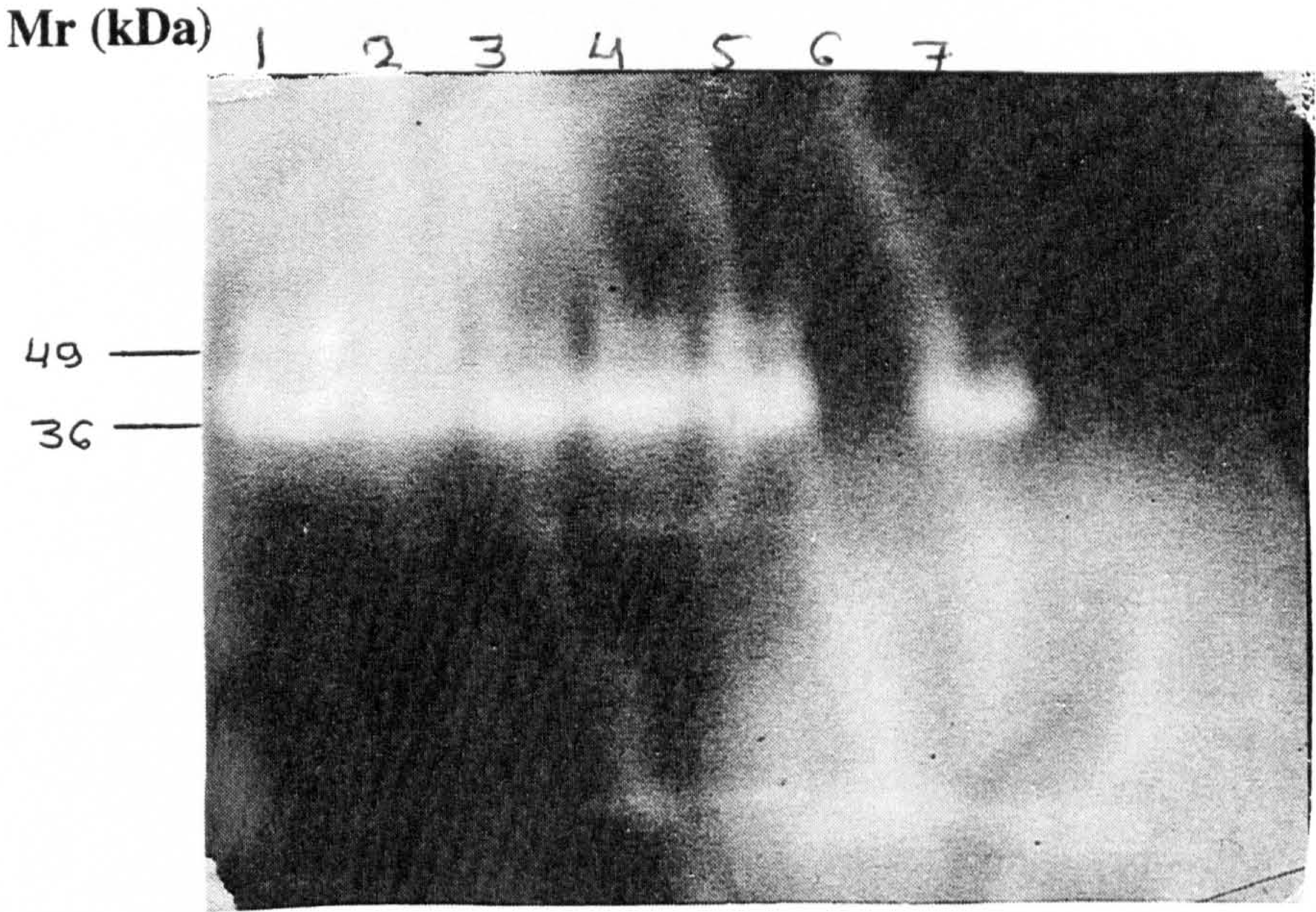


Figure 5.6 and 5.7

Effect of divalent metal ions on zymogram analysis of *Streptomyces thermoviolaceus* lipases. Samples were treated with $MgCl_2$ or $CaCl_2$ (both at a concentration of 4mM) for 30 mins at 37°C and mixed with an equal volume of 2x SDS-SB and incubated at 50°C for 30 mins, then resolved by SDS-PAGE zymogram analysis.

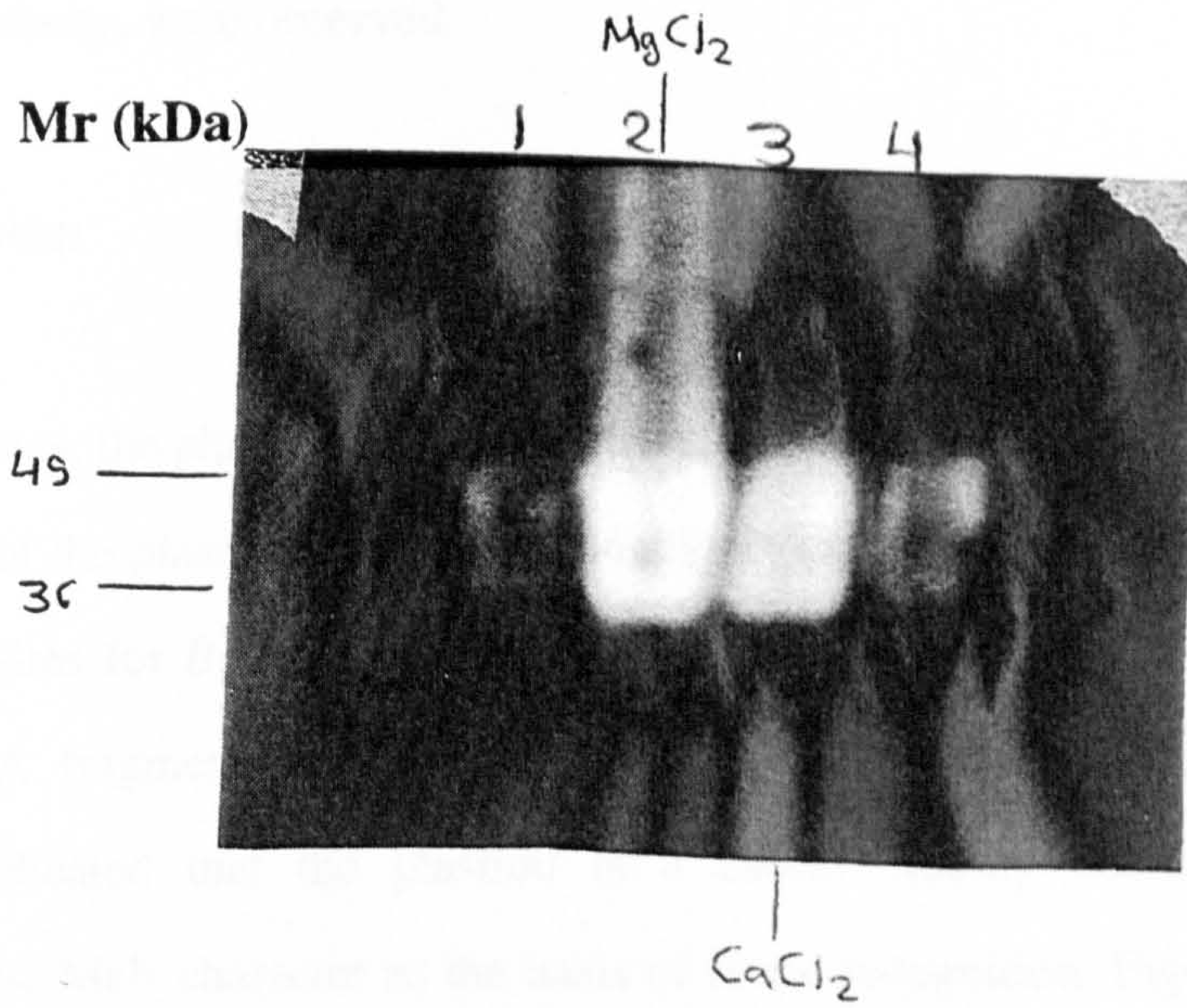
Figure 5.6

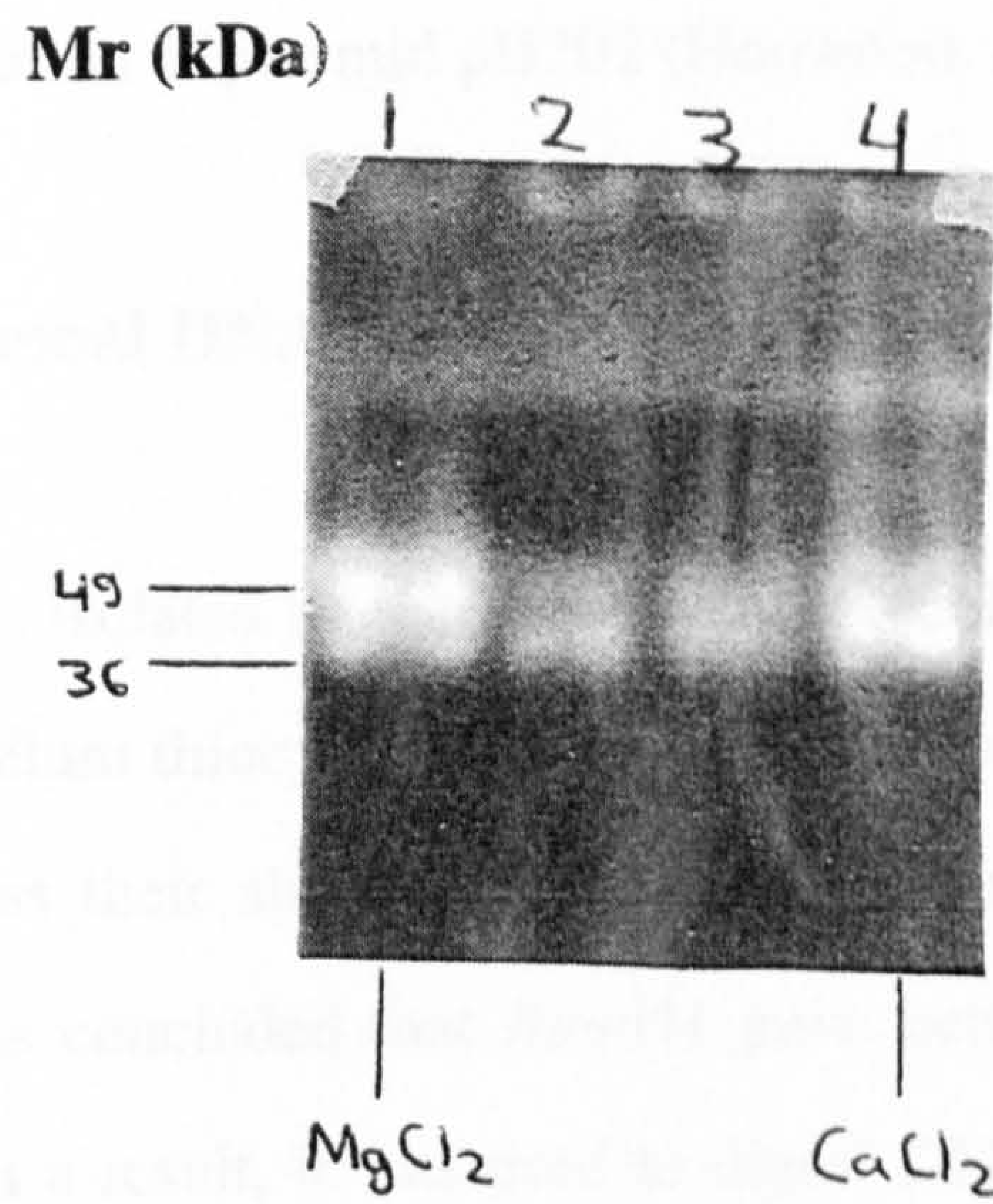
Lane	
Lane 1	untreated sample
2	treated with Mg^{2+}
3	treated with Ca^{2+}
4	untreated sample

Figure 5.7

Lane	
Lane 1	treated with Mg^{2+}
2	untreated sample
3	untreated sample
4	treated with Ca^{2+}

Figure 5.6 and 5.7





The organism consistently gave negative results for lipid hydrolysis since, during qualitative assays using agar plates, no white zones around the wall perimeter of the agar, which indicate hydrolytic activity, were observed.

5.5 Plasmid vector selection

In this series of experiments, the plasmid pIJ702 was used. As described by Katz, *et al.*, (1983) restriction mapping of the plasmid revealed endonuclease cleavage sites for several enzymes, including single sites for *Bgl*II and *Sst*I that are absent from the parent vector (pIJ350). Insertion of DNA fragments at any one of these sites abolished the Mel^+ phenotype. The results indicated that the plasmid is a useful cloning vector with insertional inactivation of the Mel^+ character as the basis of clone recognition. Figure 5.8 shows a schematic configuration of plasmid pIJ702 (Hopwood, *et al.*, 1985).

5.6 Isolation of chromosomal DNA from *Streptomyces thermoviolaceus*

Chromosomal DNA was isolated by the Maxiprep protocol-Alkali lysis method or by rapid extraction with guanidium thiocyanate as described before. Different endonucleases were used in order to assess their ability to digest the chromosomal DNA. Results are shown in Figure 5.9. It was concluded that *Bam*H1 gave better separation of the DNA bands on agarose gel and as a result, it was used to digest DNA for ligation experiments with plasmid DNA.

Figure 5.8

Restriction map of plasmid pIJ702 (Hopwood, *et al.,* 1985).

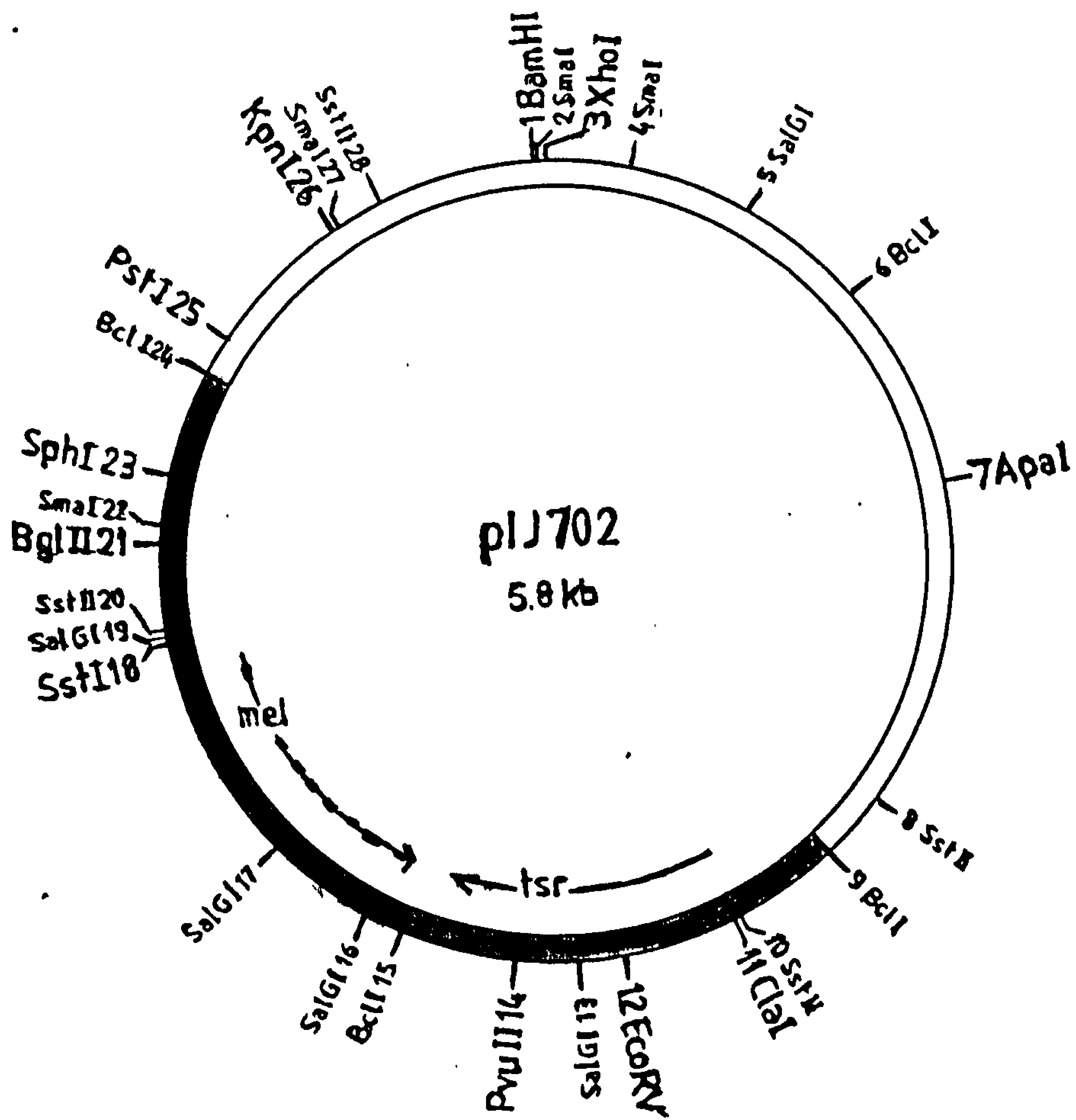

pIJ702
5.8 kb
1 BamHI
2 SmaI
3 XhoI
4 SmaI
5 SalGI
6 BclI
7 ApaI
8 SstII
9 BclI
10 SstII
11 ClaI
12 EcoRV
SalGI 13
PvuII 14
BclI 15
SalGI 16
SalGI 17
SstI 18
SalGI 19
SstII 20
BglII 21
SmaI 22
SphI 23
BclI 24
PstI 25
KpnI 26
SmaI 27
SstII 28
mel
tsr

Figure 5.9

Digestion of chromosomal DNA from *Streptomyces thermoviolaceus* with restriction endonucleases.

Lane		
	1	Molecular weight marker
	2	Chromosomal DNA undigested
	3	Digested with *Eco*RI
	4	------------- *Bam*H
	5	------------- *Bgl*II
	6	------------- *Xho*I
	8	------------- *Sac*I
	9	------------- *Eco*RI
	10	------------- *Pst*I
	11	Molecular weight marker

Figure 5.9

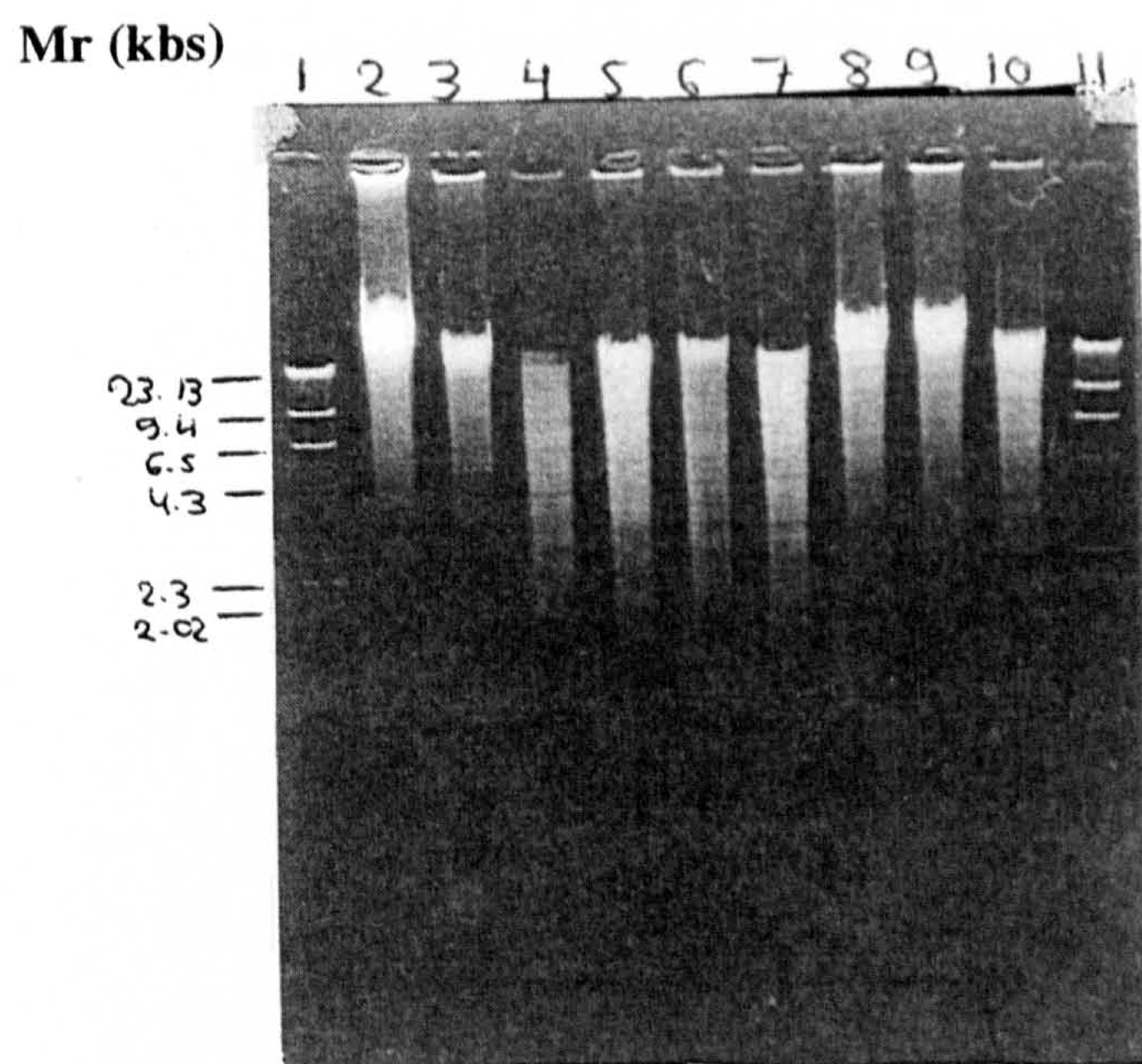
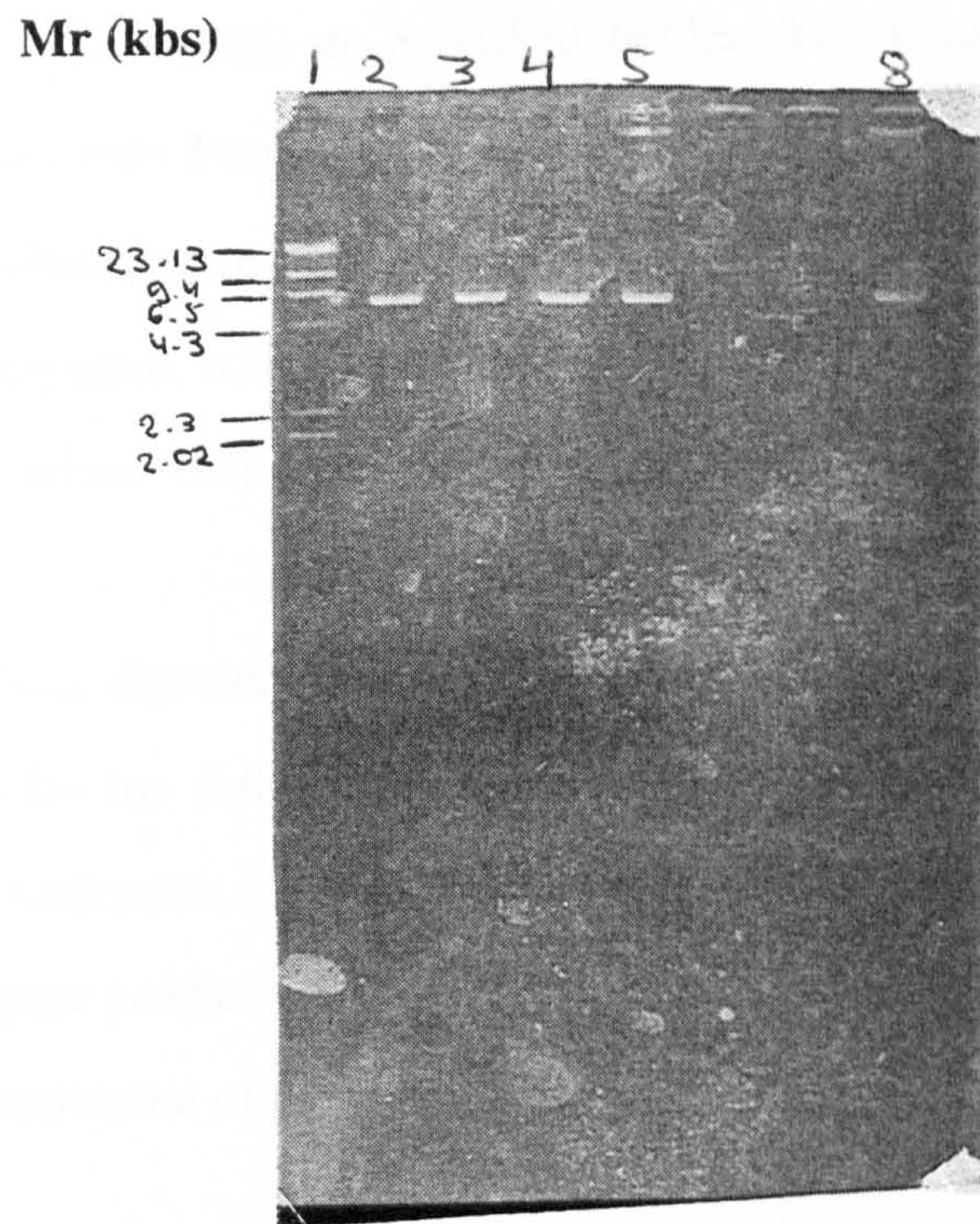


Figure 5.10

Digestion of plasmid pIJ702 with single restriction site endonucleases.

Lane		
	1	Molecular weight marker DNA digested with *Hind*III
	2	Plasmid pIJ702 DNA digested with *Bam*H1
	3	------------------------------------ *Bgl*II
	4	------------------------------------ *Cla*I
	5	------------------------------------ *Sst*I
	8	------------------------------------ *Pst*I

Figure 5.10



5.7 Digestion of plasmid DNA

The plasmid was isolated from a plasmid-containing *Streptomyces lividans* strain by the alkaline lysis method (Ish-Horowicz and Burke, 1981) or the mini plasmid preparation method (Hopwood, *et al.*, 1985) as described before. The plasmid was linearised by endonuclease digestion with *Bam*H1, *Sst*I, *Bg*II, *Pst*I and *Cla*I, all of which have a single cutting site and was characterised using an agarose gel. The molecular size was estimated by running λDNA digested with *Hind*III as standard marker. The molecular size was estimated at 5.8 kbs which is in agreement with published values. Results are shown in Figure 5.10.

Plasmid pIJ702 was digested with *Bgl*II in order to linearise the plasmid DNA. This enzyme was chosen for the following reasons: a) its sticky ends were compatible with chromosomal DNA fragments digested with *Bam*H1 and b) detection of possible clones carrying genes for lipase production would be detected as non-melanin producing colonies due to insertional inactivation of the melanin gene.

5.8 Preparation of protoplasts

Protoplasts from *Streptomyces lividans* TK64 (non-plasmid bearing strain) were prepared. Samples were diluted in parallel P buffer and 0.01% SDS and plated on R2YE plates. This was done in order to subtract colonies arising from non protoplasted units from the total number of protoplasts. The regeneration frequency was found to be 7.69%±0.56.

5.9 Transformation of protoplasts and detection of transformants

An aliquot of protoplasts/ml ($4x10^9$) was transformed with $1\mu g$ of total DNA (vector+insert) by using 25% (v/v) PEG 1000. Transformation plates were incubated at 30°C for 17 hours and overlaid with a sucrose solution containing thiostrepton. Non melanin producing colonies were considered as true transformants. Transformation frequency was determined as $1.7x10^5$ transformants per µg DNA and 36% of these were recombinants calculated after subtracting melanin producing colonies.

5.10 Screening for clones carrying inserts

Non melanin producing colonies, due to insertional inactivation of the melanin gene, were screened for lipase production on olive oil agar plates. Clear zones around the colonies provided an indication of lipase production. From one series of experiments one transformant was isolated that produced a clear zone. The lipase producing colony was named colonyA. Unfortunately due to plate damage, there is no plate photograph to support the above statement.

5.11 Isolation of plasmid carrying insert

Plasmid preparation from recombinant clone, colonyA, named as pIJ702A, showed that the colony carried insert along with plasmid DNA as shown in Figure 5.11. The molecular size of the cloned DNA was found to be ~6 kbs.

Figure 5.11

Molecular size of cloned DNA from a lipase producing colony named colonyA.

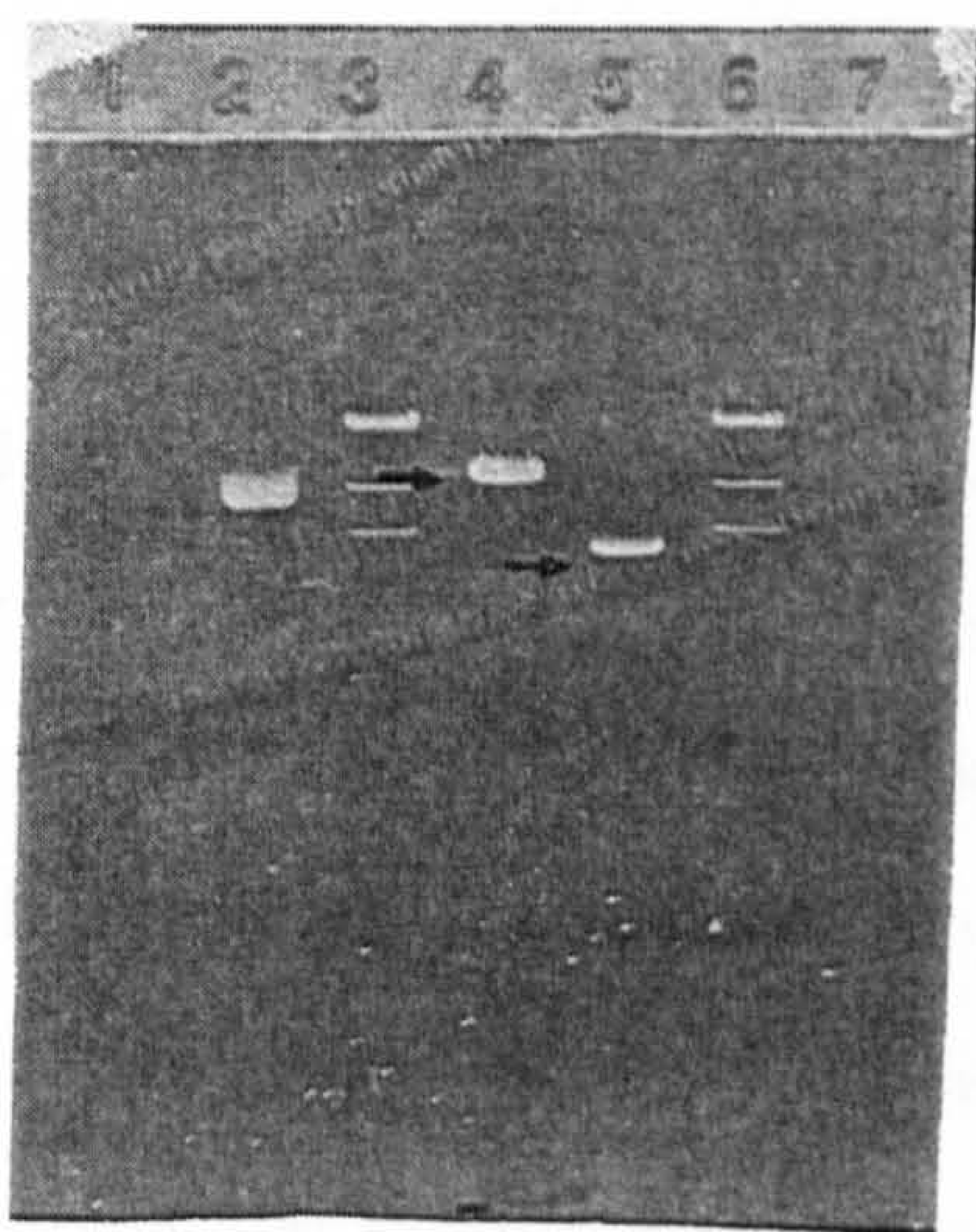
Lane		
	2	Plasmid pIJ702A non linearised
	3	Molecular weight marker λDNA digested with *Hind*III
	4	Plasmid pIJ702A linearised with *Bam*H1
	5	Plasmid pIJ702 linearised with *Bam*H1
	6	Molecular weight marker λDNA digested with *Hind*III

Figure 5.11

Mr (kbs)

pIJ702A 12

pIJ702 5.8



5.12 Characterisation of colonyA lipase

Unsuccessful attempts were made to grow colonyA and non transformed *Streptomyces lividans* TK64 in salts media used for growth in *Streptomyces thermoviolaceus* with olive oil as the lipid source.

5.13 Discussion

Perez, *et al.,* (1993) suggested that lipase from *Streptomyces* species was a small protein of molecular mass 27.9 kDa. In the case of *Streptomyces thermoviolaceus* two different bands were observed: one at 36 kDa and one at 49 kDa. It was found that when samples were heated at different temperatures, the 49 kDa band disappeared as the temperature increased. This indicated that two lipases were present: one thermolabile (49 kDa) and one thermostable (36 kDa). Bands from samples treated with Mg^{2+} and Ca^{2+} appeared to be clearer and this can provide an indication of the specific binding sites that the enzyme has for these two metals. In addition, Mg^{2+} was found to have a greater effect over Ca^{2+} since the enzyme bands were brighter.

Plasmids have been identified in many *Streptomyces* species and have been implicated in the control of a range of phenotypic properties, including the transfer of chromosomal markers, antibiotic synthesis and resistance and differentiation (Bibb, *et al.*, 1981).
Cloning and analysis of transcriptional signals is greatly facilitated by the availability of promoter-probe vectors and the subsequent ability to isolate and characterise regions of DNA involved in the initiation of transcription. The initial results obtained from the use of some of these relatively simple vectors, have indicated considerable promoter sequence heterogeneity (Buttner and Brown, 1985). There are a few promoter-probe plasmids which

have been developed for the study of gene expression in *Streptomyces* based on antibiotic resistance genes (Ward, *et al.*, 1986), in the chromogenic identification of promoter activity (Forsman and Jaurin, 1987) or in luminescence (Shauer, 1988). Asturias, *et al.*,(1990) developed a promoter-probe vector, pULJA30 derived from plasmid pIJ486. Some important features of this new vector included: wide *Streptomyces* host range, a high plasmid copy number and a sequence with unique sites for *Bam*H1 and *Bgl*II for flexible cloning, fragment re-isolation and direct sequencing of promoter-active inserts. In this work plasmid pIJ702 was used since the plasmid is a useful cloning vector with insertional inactivation of the melanin character as the basis of clone recognition. The plasmid was linearised with *Bgl*II which gives ends compatible with chromosomal DNA fragments. Plasmid DNA can be introduced into *Streptomyces* cells by polyethylene-glycol (PEG)-assisted transformation. In this study the conditions and procedures as described for *Streptomyces lividans* transformations have been applied. Chromosomal DNA was linearised with *Bam*H1 which gave better separation of the DNA bands on agarose gel.

Streptomyces lividans TK64 was chosen as the mesophilic host for cloning experiments. Daza, *et al.*, (1990) have described *Streptomyces lividans* as being lipolytic but qualitative assays using agar plates proved otherwise.

Anné *et al.*, (1990) summarised the factors which can affect transformation of *Streptomyces* protoplasts and concluded that the factors which differed the most in obtaining satisfactory results for transformation were: the age of the mycelium, the concentration of lysozyme and the number of protoplasts in the regeneration plate. The physiological states of the cell, including the degree of digestion of the cell wall resulting in the formation of true protoplasts, influence both the capacity of regeneration and the uptake of DNA.

Streptomyces clavuligerus (Garcia-Dominguez, *et al.*, 1987) or *S. ambofaciens* (Baltz and Matsushima, 1983) gave more transformants with protoplasts from mycelia grown to the stationary phase. *Streptomyces venezuelae* protoplasts gave an optimum number of transformants from mycelium from the beginning of the exponential growth phase. Transformation frequency of *Streptomyces lividans* protoplasts was much lower compared with the protoplasts from other *Streptomyces* species (Matsushima and Baltz, 1985). Factors such as phase in the growth cycle, temperature in the regeneration medium and cell age could be responsible for the low frequency transformation observed.

Perez, *et al.*, (1993) cloned and expressed an extracellular lipase-encoding gene from *Streptomyces* sp. M11 in *S. lividans* 66. In their studies, total DNA from this strain was partially cut with *Sau*3AI, size fractionated, ligated to *Bam*HI-cut pIJ486 (Ward, *et al.*, 1986), and the ligation mixture used to transform *Streptomyces lividans*. Three lipolytic clones were identified and the plasmid with the smallest insert, named pB13, was chosen for more detailed analysis. They concluded that culture supernatants of *Streptomyces lividans* carrying pB13, which was absent from supernatants of *S. lividans* carrying the vector, showed large amounts of protein with molecular size 28 kDa. It was concluded that this protein was responsible for the lipolytic activity showed by *Streptomyces lividans*[pB13].

In this work one transformant, which provided an indication of lipase production, was detected and plasmid preparation from recombinant clone showed that the cloned DNA was found to be about 6 kbs. Attempts to grow recombinant clone colony and non transformed *Streptomyces lividans* in salts media used for *Streptomyces* were unsuccessful and no further characterisation of clone lipase was possible.

The final part of the project, concerned with cloning genes responsible for lipase activity from *Streptomyces thermoviolaceus* into a mesophilic host (*S. lividans*), was

unsuccessful even though, a considerable amount of time was spent in trying to achieve the required results. For future cloning experiments the possible use of mesophilic *Streptomyces* hosts other than *Streptomyces lividans* can be tested which can possibly lead to successful cloning. Also, the transformation and the screening methods can be revised to provide better results.

Chapter 6

Discussion

Thermophilic *Streptomyces* often rely on degradation of polymeric materials for their nutrition by secretion of a wide variety of extracellular hydrolytic enzymes. Edwards, (1993) suggested that a relationship exists between enzyme thermostability and the thermotolerance of the host bacterium. This can be significant to thermophilic *Streptomyces* which are involved in biodegradation of polymeric substances.

Bacterial lipases are mostly extracellular in nature and in some cases are inducible by the inclusion of lipid substrates in the growth media. While considerable data on enzymes of unusual properties have accumulated there is little information regarding the production of lipases by thermophilic *Streptomyces*. In this study five thermophilic *Streptomyces* species, *S. thermoviolaceus, S. thermodiastaticus, S. thermoflavus, S. thermovulgaris* and *S. thermonitrificans*, were screened for their ability to produce lipase.

In chapter 3, preliminary flask culture experiments investigated the physiology of lipase produced by thermophilic. *Streptomyces*. Previous experiments dealing with microbial lipases, used a set of different assays in order to characterise lipases. In this work the spectrophotometric assay with p-nitrophenyl palmitate as the enzyme substrate was used, as it is a time saving reproducible and easy to handle detection method.

Initially, a range of lipid sources (olive oil, vegetable oil, Tween 80, Tween 40 and Tween 60) was selected in order to determine their effect on growth and lipase production. Results suggested that greatest biomass production and secretions of lipase activity were recorded in olive oil followed by Tween 80 and vegetable oil media. Growth under the lipid sources examined, was satisfactorily reproducible and homogeneous. After an initial lag phase the cultures grew exponentially as determined by measurement of dry weight. It was suggested that olive oil or Tween 80 act as an inducer of lipase and there is a degree of specificity with respect to the chemical nature of the inducer.

Jaeger, *et al.,* (1994) concluded that extracellular lipases appear in the culture medium when the bacterial cells reach the end of the logarithmic growth phase. Hale, *et al.*, (1992) suggested that some factors required for the gene expression may not be available until later stages of growth or that a repressor of expression may be present during active mycelial growth. In order to assess the effect of growth phase on lipase activity, *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were grown in the presence of olive oil or Tween 80 at 50°C. From the results it was concluded that lipase activity was detected during exponential and stationary growth phases. Secretion of lipase activity reached its maximum value when biomass production was at its maximum level. In addition of lipase activity, secretion of extracellular protein was detected which followed the same pattern of secretion. It was also revealed that during stationary growth phase, constant biomass gave rise to the secretion of a constant amount of protein and lipase activity which is an indication of extracellular secretion of protein and lipase.

In order to find whether extracellular lipase was secreted by the mycelia or arose as a result of lysis of the mycelia, preliminary characterisation of any intracellular lipase activity of *Streptomyces thermoviolaceus* or *S. thermodiastaticus* took place. It was found that the amount of lipase activity found in cell-free supernatants was significantly higher than the amount found in cell-mass and it was concluded that the higher extracellular lipase activity was a result of secretion by the mycelial cells.

The optimum pH for lipase activity, for cultures growing with olive oil or Tween 80, was 8.0 but at pH 2-3, 36% of the activity could still be observed.

Profound thermostability at 70°C was observed for the thermophilic *Streptomyces* species under investigation. Since inhibition never reached 100% the residual activity suggested that a heat labile enzyme activity as well as a highly thermostable one could possibly be present.

The effect of potential lipase inhibitors and divalent metals on lipase activity was investigated. Previous studies suggested that various divalent metal ions and inhibitors, enhanced or inhibited the secretion of lipase or esterase by different microorganisms to a considerable degree (Babcock, *et al.*, 1992; Hale, *et al.*, 1992; Stöcklein, *et al.*, 1993).

Inhibitors were used in an attempt to resolve the type of lipases produced by thermophilic *Streptomyces* species. It was found that lipase activity was inhibited to a great extent by EDTA and o-phenanthroline and to a lesser extent by PMSF. This suggested the presence of two types of enzymes, a metallo-lipase and a serine-lipase. Extracellular lipase activity was found to be inhibited to divalent metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} whereas it was activated by Mg^{2+} and Ca^{2+} ions. By adding these two metal ions to EDTA treated samples, some of the lost activity was restored. It was suggested that the lipase activity produced by the thermophilic *Streptomyces* species examined, has a specific binding site for these two metals which when present make the molecule more compact and more thermostable.

Flask culture temperature shift experiments were performed in order to examine the properties of *Streptomyces* lipase with respect to growth temperature. It was shown that lipase activity produced its peak value earlier at 50°C than 30°C, which reflected the higher metabolic rate at the higher temperature. Extracellular protein concentration was unaffected by the change in temperature. Also, it was found that more thermostable lipase activity was secreted when growth was shifted to 50°C whilst at 30°C a proportion of the activity could be ascribed to a more thermolabile enzyme not detectable at 50°C.

In chapter 4 laboratory-scale fermentation experiments took place in order to examine lipase activity of thermophilic *Streptomyces* under a controlled environment. For these series of experiments, *Streptomyces thermoviolaceus* and *S. thermodiastaticus* were used

as they showed the best yields for lipase activity. Every experiment was performed with olive oil or Tween 80 as the lipid source.

The effect of pH on growth lipase activity and extracellular protein was examined and it was found that in accordance with flask culture experiments the optimum pH for lipase activity was 8.0. The optimum pH for biomass production and secretion of extracellular protein was found to be 7.0. Overall, pH of 8.0 was higher than the reported optimum pH for other lipase producing microorganisms (Nahas, 1988; Handelsman and Shoham, 1994).

Streptomyces species were grown at 50°C with different lipid sources and it was concluded that irrespective of the lipid source employed, growth was biphasic and the faster the growth rate of the second phase of growth the greater the production of volumetric lipase activity and extracellular protein. When specific lipase activity was taken into account it was concluded that the cessation of the more rapid growth phase coincided with a decrease in the lipase activity produced and the activity reached its lowest value at the second slower phase of growth.

The effect of different temperatures on growth of *Streptomyces thermoviolaceus* revealed that lipase activity is growth associated. Preference for biomass and volumetric lipase activity is shown at 30 and 50°C, with peak values achieved at the latter temperature. At the intermediate temperatures there is a decrease in both biomass yield and lipase activity. In the case of specific lipase activity, the ideal temperature was proven to be 45°C. Sugihara, *et al.,* (1991) reported that lipase activity from mesophilic sources has an optimum temperature which in most cases is between 30 and 40°C.

At 30°C the stationary growth phase led to a decrease in volumetric lipase activity whereas at 50°C it remained constant, proving that volumetric lipase activity was thermostable at the higher temperature. For both set of temperatures, it was found that

secretion of lipase activity started in actively growing cultures. Gusek, *et al.*, (1988) suggested that extracellular enzymes in other *Streptomyces* species are produced during the stationary phase of growth. Extracellular protein followed a similar pattern increasing with cell growth and remaining stable after its maximum peak was reached.

In the case of specific lipase activity, it was suggested that 30°C rather than 50°C, was the optimum temperature for lipase activity since at the later temperature a rapid decrease in the amount of lipase activity secreted was observed. Nevertheless, by calculating specific activity the secretion of extracellular protein is not taken into account and thus the results appear to be different from the ones obtained for volumetric activity.

Cell-free culture supernatants obtained from laboratory scale experiments showed identical behaviour with flask culture experiments towards divalent metal ions and potential lipase inhibitors and it was again demonstrated that Mg^{2+} and Ca^{2+} enhance the secretion of lipase activity by thermophilic *Streptomyces*.

The last part of the project involved the development of methods in order to separate and characterise lipase activities of *Streptomyces thermoviolaceus*. Also, an attempt was made to clone genes responsible for lipase activity from this organism into *Streptomyces lividans* in order to study the properties of lipase activity within a mesophilic host.

SDS-PAGE zymogram analysis was used in order to separate and identify lipases from *Streptomyces thermoviolaceus*. For these experiments concentration of lipase activity is required and it was found that satisfactory recovery of the activity was obtained with acetone precipitation. Two different lipase bands were observed: a 36 kDa and a 49 kDa band. When samples were heated at different temperatures it was found that the 49 kDa band disappeared as the temperature increased. This suggested that the 49 kDa band was thermolabile whereas the 36 kDa was thermostable at the higher temperatures.

For efficient cloning of *Streptomyces thermoviolaceus* chromosomal DNA, the regeneration and transformation frequency of protoplasts from a strain of *Streptomyces lividans* were examined. The regeneration frequency was above 7% in accordance with previously published data (Thompson, *et al.*, 1982). Because of factors such as cell age, phase in the growth cycle and temperature in the regeneration medium, transformation frequency was much lower compared with the protoplasts from other *Streptomyces* species (Matsushima and Baltz, 1996).

Cloning of chromosomal DNA from *Streptomyces thermoviolaceus* using the plasmid vector pIJ702 lead to isolation of a *Streptomyces lividans* recombinant clone which was transformed with pIJ702A, a recombinant plasmid which contained a 6.2 kbs fragment DNA. The molecular size of the cloned DNA was found to be ~6 kbs. Nevertheless, unsuccessful attempts were made to grow the recombinant clone and non transformable *Streptomyces lividans* in salts media used for growth of *Streptomyces*, and as a result no further characterisation of recombinant clone lipase was achieved.

The main reason for the steadily growing interest in lipases is the biotechnological versatility of these enzymes including their potential to catalyse the hydrolysis and also the synthesis of esters. The synthesis and secretion of lipases by bacteria are influenced by a variety of environmental factors like ions, carbon sources, or presence of non metabolizable polysaccharides. These parameters can stimulate or repress the production of lipases by Gram-positive or Gram-negative bacteria. Perez, *et al.*, (1993) concluded that the lipase of *Streptomyces* species is a small protein of molecular mass 27.9 kDa which may require a second gene product for high level expression. Nevertheless, while considerable data on proteases, carbohydrases and other enzymes of unusual properties have accumulated, there is little information regarding the properties of lipases in thermophilic *Streptomyces* species.

Table 6.1 reveals the properties of bacterial lipases as reviewed by Jaeger, *et al.*, (1994). As a main conclusion, lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, and hence presenting a fascinating field for future research.

Table 6.1 Properties of bacterial lipases as reviewed by Jaeger, *et al.*, (1994).

Source of lipase	Gene cloned and sequenced	Helper protein	Molecular mass (kDa)	Substrate specificity	Specific features
S. aureus	yes	no	76	broad	synthesised as preproprotein
S. hyicus	yes	no	71.4	broad	synthesised as preproprotein
S. epidermis	yes	no	77	n.d.	synthesised as preproprotein
B. subtilis	yes	no	19.4	1,3 position and C8-FA	stable at pH 12
Streptomyces species	yes	no	27.9	n.d.	no
Aeromonas hydrophila	yes	no	71.8	preference for C6-C8-FA	no
Xenorhabdus luminescens	yes	no	68.1	n.d.	no
Chromobacterium viscosum	yes	no	33	broad	active in organic solvents
Pseudomonas aeruginosa	yes	yes	30	broad	forming high Mr aggregates

P. fragi	yes	n.d.	30	broad	stable at pH 9 and 50°C
P. glumae	yes	yes	33	broad	contains Ca^{2+} binding site
P. cepacia	yes	yes	33	broad	no
P. species KW156	yes	yes	33	n.d.	no
P. fluorescens B52	yes	no	50.2	n.d.	no
P. putida	no	n.d.	45	n.d.	stable at 75°C
Moraxella species	yes	no	34.7	n.d.	active at 4°C
Propionibacterium acnes	no	no	41.2	broad	forming high Mr aggregates

Abbreviations : n.d., not defined

: C6-FA, fatty acid with a chain length of six carbon atoms

: C8-FA, fatty acid with a chain length of eight carbon atoms

Chapter 7

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