STUDIES ON THE USE OF SPENT FERMENTATION LIQUOR FOR THE PRODUCTION OF GENTAMICIN

BY

GREGORY BLANK

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGMENTS

The author wishes to acknowledge the valuable direction, advice and encouragement offered by Dr. R.R. Pereira. He also wishes to express his appreciation to Dr. R.A. Gallop for his suggestions during this research, for the provision of financial support and to Dr. W. Bushuk and Dr. F.W. Hougen for their suggestions in the preparation of this manuscript.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Blank, Gregory. Ph.D., The University of Manitoba. October, 1977. Studies On The Use Of Spent Fermentation Liquor For the Production Of Gentamicin. Major Professor: Ronald R. Pereira.

Gentamicin is a wide-spectrum antibiotic produced from several species of microorganisms belonging to the genus Micromonospora. This antibiotic was produced in a submerged fermentation process utilizing spent fermentation liquor obtained from primary gentamicin fermentations. Calcium carbonate but not cobalt was required as a necessary supplement in these fermentations in order to realize optimum yields of gentamicin. Fortifying the spent liquor fermentation with glucose and yeast extract did not appreciably increase the yields of gentamicin. Studies indicated that the pH of spent liquor fermentations was critical in achieving maximum gentamicin yields; a pH of 8.9 or higher, resulting during the fermentation process, invariably decreased the growth of the organism and the accumulation of gentamicin. Spent fermentation liquor obtained from primary gentamicin fermentations was shown to inhibit the accumulation of gentamicin in the spent liquor when certain levels of spent liquor were surpassed.

Batch fermentation studies indicated that spent liquor was more efficient in producing gentamicin when automatic pH control was used. Continuous fermentation processes using fresh and spent liquor showed lower yields of gentamicin accumulation than did batch fermentations.

INTRODUCTION

The microbial production of antibiotics is fast becoming a major industry. Since the end of World War II, the production of antibiotics has radically changed the pharmaceutical industry. The nature of the operations carried out in this industry as well as the problems associated with handling, processing and disposal of fermentation by-products has become very complex.

One particular problem, in this industry, is the disposal of fermentation by-products in a manner that is economical and efficient. Large quantities of residual waste consisting of mycelium, extracted broth and wash water from equipment contribute to a very high biological oxygen demand placed on the environment by these wastes. It has been shown, for example, that fermentation wastes can contain from 10,000 to 50,000 parts per million B.O.D. as contrasted to 200 to 300 parts per million B.O.D. from normal domestic or municipal wastes (Fried and Stockton, 1973). The problem of efficiently and economically removing these wastes is important, especially in the industry today, where more and more constraints are being placed upon the quality of discharge water.

The ratios of waste to production, which are of considerable importance in the fermentation industry, are difficult to evaluate especially in the antibiotic industry. A major reason is the high potency and small yield of the active product. Modifications in the fermentation process, which may be of little significance in relation to product yield, may be very important with respect to material concentration and recovery, and pollution control of wastes.

In this study an attempt has been made to evaluate the feasibility

of using spent fermentation liquor with and without nutrient fortification for the fermentative production of a wide-spectrum antibiotic known as gentamicin, produced by Micromonospora purpurea. It was intended that this fermentation study be made with a representative type of fermentation process so that it could be conceivably extended to other types of similar fermentations where the antibiotic produced is largely confined to the mycelia.

The use of spent fermentation liquor for the additional production of an active product would have the initial advantage of drastically reducing the volumes of fermentation broth. This reduction in volume would not only ease the discharge waste problem but also increase the efficiency of antibiotic removal and purification. End products of metabolism, as well as accumulation of toxic compounds such as antibiotics will, no doubt present formidable problems. However, in an age where fermentation technology is advancing so rapidly, the problems presented should be investigated if only for a better understanding of the interactions taking place during the fermentation process.

REVIEW OF LITERATURE

Antibiotics

An antibiotic is a chemical substance, produced by a living organism, that demonstrates inhibitory or germicidal activity towards microorganisms *in vivo* or *in vitro* (Prescott and Dunn, 1959). Generally, antibiotics inhibit the normal growth and cell division of organisms which can result in the complete autolysis of the cell.

The number and variety of secondary effects which can be exhibited by the action of antibiotics is frequently associated with the amount of antibiotic administered, the time of exposure and the amount adsorbed by the cell. All these factors are greatly influenced by the state or condition of the organism and its general environment.

Classification and Mechanism of Antibiotic Action

No exact classification exists for antibiotics. In general terms they are divided either into broad or narrow spectrum antibiotics. In some cases, they are classified according to their chemical composition or according to the family or genus name of the producing organism. In other cases they are grouped according to their host susceptibility, that is, antimicrobial, antiviral and antifungal antibiotics.

Newton (1965), in describing the mode of action of antibiotics, has grouped the antibiotics according to their general mechanism (Table 1). Additional information is furnished by Schonfeld *et al.* (1971), Barber and Garrod (1963), Gottlieb and Shaw (1967) and Berdy (1961).

Table 1. Types and mode of action of some antibiotics.

General mechanism	Antibiotic	Mode of action
Inhibition of cell wall synthesis.	Penicillin	Blocks some stage in the biosynthesis of the cell wall mucopeptide.
"	Cycloserine (oxamycin)	A structural analogue of D-alanine.
"	Griseofulvin	Interferes with the synthesis and organization of the cell wall membrane.
"	Bacitracin	
"	Novobiocin	
"	Vancomycin	
"	Ristocitin	
Interference with cell membrane permeability.	Tyrocidin	Changes the structure of cell membranes and hence specific permeases. This causes interference with amino acid and sugar transport.

Table 1. (continued)

General mechanism	Antibiotic	Mode of action
"	Gramicidin	
"	Polymyxins (circulin, colistin)	
"	Polynes: (nystatin, filipin, condicidin)	Causes a rapid leakage of intracellular constituents.
Uncoupling agents and inhibitors of electron transport.	Antimycin A	Blocks electron transport chain specifically between cytochromes B and C.
"	Gramicidin	Inhibits phosphate uptake and causes uncoupling of oxidative phosphorylation in mitochondria.
"	Valinomycin	
"	Oligomycin	
"	Streptomycin	

Table 1. (continued)

General mechanism	Antibiotic	Mode of action
Chelation and inhibition of metalloprotein synthesis.	Tetracycline	Antibiotics have an affinity for metallic cations; these are important in enzyme activation and maintenance of cell integrity such as ribosomes.
"	Streptomycin	
"	Usinic acid	
"	Aspergillic acid	
"	Novobiocin	
Inhibition of purine and purine nucleotide synthesis.	Azaserine	Acts as an analogue of glutamine.
"	DON	
"	Cordycepin	Analogue of adenosine.
"	Halacidin	Analogue of aspartic acid; inhibits the synthesis of adenylic and deoxyadenylic acid.
"	Psicofuranine	Structural analogue of adenosine.

Table 1. (Continued)

General mechanism	Antibiotic	Mode of action
Inhibitors of DNA synthesis.	Mitomycin	Inhibits DNA synthesis by the formation of cross-links between complementary DNA strands.
"	Porfiromycin	
"	Phleomycin	Binding of antibiotic to DNA primer.
"	Edeine	Inhibits DNA polymerase.
Inhibitors of protein synthesis.	Puromycin	Inhibits induced enzyme formation; blocks some stage in protein synthesis after the formation of amino acyl sRNA.
"	Chloramphenicol	Blocks the transfer of amino acids from sRNA to ribosomes.
"	Streptomycin group:	Precipitates nucleic acid *in vivo*; inhibits protein synthesis *in vivo*.

Table 1. (continued)

General mechanism	Antibiotic	Mode of action
"	(streptomycin, kanamycin, neomycin, viomycin, paromomycin, streptothrincin)	
"	Tetracycline group:	Inhibits enzymes; chelating agents.
"	(chlortetracycline, aureomycin, oxtetracycline, terramycin)	

Bacterial Resistance to Antibiotics

Bacterial resistance to antibiotics is a phenomenon well known but not well understood. Many bacterial species appear to be unaffected by the action of antibiotics, either through a natural or induced mutational process. Antibiotics are basically inhibitors; their site of action may vary, and their method of inhibition can be explained by their interaction with a specific cell component such as the cell wall or cell membrane.

Gale et al. (1972) has explained bacterial resistance to antibiotics by four main methods:

(1) Modification of the Antibiotic Target. In this case the antibiotic target is modified so that it becomes insensitive to the action of the antibiotic, yet is still capable of carrying out its metabolic functions. The majority of targets in microbial cells are the enzymes associated with the various cellular functions; in most cases the antibiotic interacts with the active site of the enzzyme. In these cases, there is a competition between the antibiotic and the normal cell enzyme substrate. The affinity of the antibiotic relative to the cell substrate must be very high if any antibiotic action is to be noted. However, in those cases where enzyme mutations occur, a modified gene may be produced which shows a lower affinity for the specific antibiotic; consequently, the antibiotic cannot compete actively with the cell enzyme substrate.

(2) Reduction in the Physiological Importance of the Targets. Certain inhibitory growth mutations may be by-passed by adding the product of the inactivated biosynthetic pathway to the growth medium. Similarly,

chemical inhibition of certain enzymes in the bacterial cell may be by-passed by an exogenous supply of the product to the pathway. The net effect is a reduced physiological need for the inhibited pathway.

(3) Prevention of Access. Bacterial resistance may arise by the establishment of a permeability barrier against an antibiotic; this "molecular overcoat" could prevent the build-up of sufficient amounts of antibiotic within the cell to cause inhibition.

(4) Resistance by Inactivation. Bacterial resistance to antibiotics by inactivation can occur in two ways: (a) the antibiotic is destroyed by the opening of one or more covalent bonds in its structure or (b) the antibiotic is inactivated by the substitution of chemical residues. The specific mechanisms which can cause antibiotic inactivation include: antibiotic-destroying enzymes such as the β-lactamases (penicillinases and cephalosporinases), which are in essence peptidases acting on specific peptide bonds of antibiotics; adenylation enzymes, which are specific for only a small portion of the antibiotic molecule, that is, adenylation of the OH groupings in some aminoglycoside antibiotics; and phosphorylation enzymes. Two distinct phosphorylation enzymes are known, both of which are R-factor mediated. R-factor or resistance transfer factors are genetic structure or sex factors which carry specific, separable determinants of resistance to as many as four different antibacterial drugs (Hayes, 1965; Smith, 1969). The first enzyme acts against streptomycin alone, and the other, against neomycin, kanamycin and those gentamicin components that carry a 3' - OH group in the sugar ring (Davies et al., 1969). In addition, acetylation enzymes which can acetylate the free $-NH_2$ group of neomycin and kanomycin can also acetylate

the free $-NH_2$ groups of the gentamicin components, although this does not inevitably inactivate the molecule as an antibiotic (Davies, 1971). The study of adenylation, phosphorylation and acetylation is more complicated with gentamicin since it is actively composed of at least four components. Franklin and Snow (1971) indicated that resistance of the aminoglycoside antibiotics, to which gentamicin belongs, by gram-negative bacteria bearing the R-factor, is due to enzymatic inactivation of the antibiotic.

Uses of Antibiotics

The value of antibiotics in the treatment and prevention of infectious diseases of man, and the impact they have had on public health is well known and commonplace. Antibiotics, however, have not only been a great gift to medicine per se, but also a great gift to man's ability to feed both himself and his livestock. The more recent uses of antibiotics in agriculture and its related fields further demonstrates the ubiquity of antibiotics and their potential uses.

Antibiotics are used to promote the growth of pigs and chickens, to prevent spoilage of vegetables and sea foods, to delay deterioration of beef and even to preserve food by the incorporation in the ice used to chill it (Pramer, 1955). Much of the current understanding concerning protein synthesis and other biochemical reactions, such as enzyme regulation, is due to the use of specific investigative antibiotics. Molecular biology, and all its related disciplines, utilize antibiotics for the illumination of a variety of intricate mechanisms.

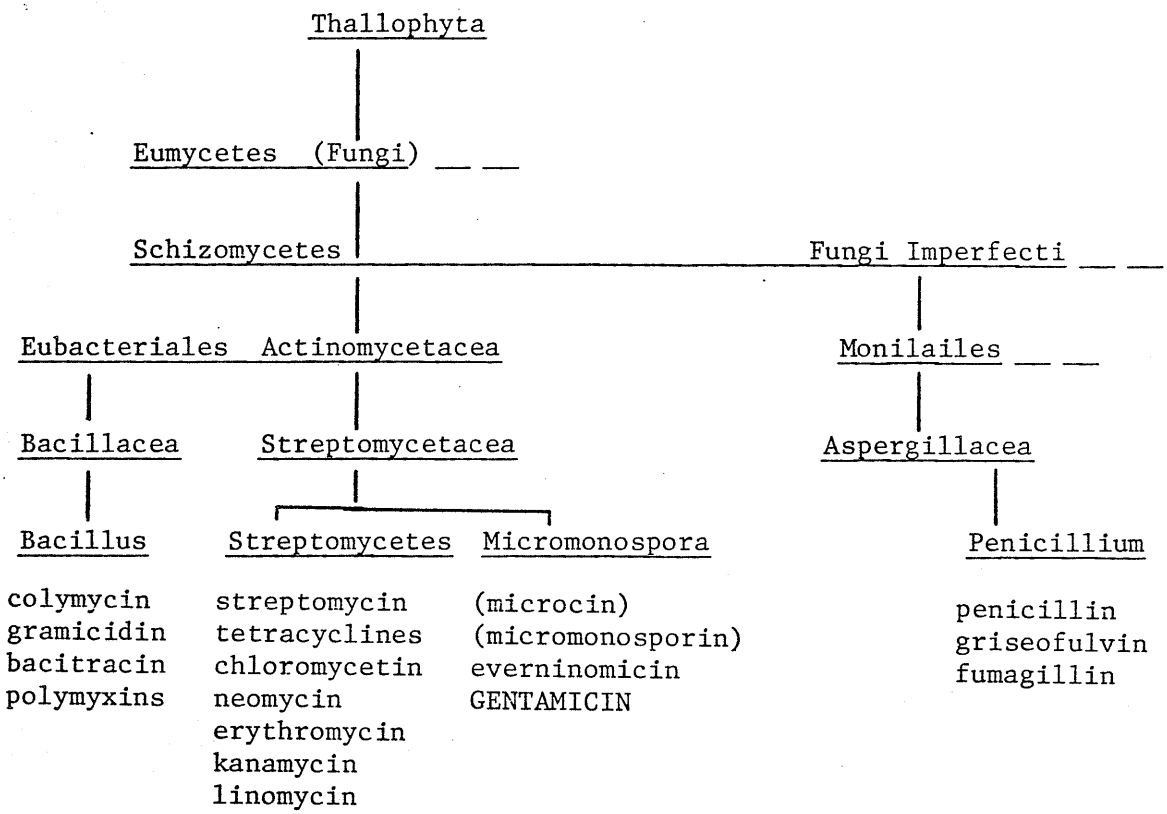
Micromonospora and the Antibiotics

The genus Micromonospora, in the family Actinomycetales (Table 2), has been extensively reviewed taxonomically by Luedemann et al. (1963, 1969), Waksman (1961) and Weinstein et al. (1967). Several species in this genus have been shown to produce a variety of antibiotics, some of which are chemically related. Some of the more important antibiotics isolated from this genus include:

(1) Verdamicin. Verdamicin is an aminoglycoside antibiotic produced by M. grisea. This is a compound which consists of at least one sugar component to which is attached one or more amino groups; the sugar component is joined to another fragment through a glycosidic linkage. The active fermentation product, found both in the fermentation broth and in the mycelia, consists of two major antibiotic components. One of the components was named verdamicin, while the other component showed hydrolytic patterns similar to the antibiotic sisomycin. The latter component was later shown to be identical to sisomycin. Verdamicin is a broad-spectrum antibiotic, especially active against Pseudomonas species (Weinstein et al., 1975).

(2) Sisomycin. Sisomycin is a broad-spectrum, aminoglycoside antibiotic produced by M. invoensis. This antibiotic has an in vitro spectrum similar to gentamicin with a potency equal to, or twice that of, gentamicin against Pseudomonas (Waitz et al., 1970). The antibiotic is substantially produced as a single component under aerobic, submerged fermentation conditions (Wagman et al., 1970). Weinstein et al. (1970) reported that the antibiotic contains 2-deoxystreptamine and resembles gentamicin components C_{1a}. In vitro studies with sisomycin

Table 2. Microbial sources of some antibiotics.



have been reported by Crowe and Sanders (1973).

(3) Megalomicin. Megalomicin is a macrolide antibiotic complex isolated from two strains of M. megalomicea. Macrolide antibiotics contain a large lactone ring consisting of 12 to 16 carbon members having few double bonds with no nitrogen atoms; moreover, the ring is substituted with one or more sugar residues, some of which may be amino sugars (Gale et al., 1972). The antibiotic complex consists of four compounds or components designated as A,B,C_1, and C_2. Like other macrolides, it was shown to be primarily active at an alkaline pH. The antibiotic shows only minimal activity against gram-negative bacteria. Studies performed clearly indicate that the antibiotic is a novel deosamine containing macrolide (Weinstein et al., 1968). The structure of the components making up the magalomicin complex have been investigated by Jaret et al. (1973).

(4) Everninomicin. Everninomicin is a solvent-extractable antibiotic complex that acts against gram-negative bacteria. It is produced by M. carbonacea sp. n. The complex, which consists of five components, was found only in the broth filtrate and not in the mycelium (Wagman et al., 1964). The components referred to as A,B,C,D and E can be separated by adsorption chromatography. Everninomicin D was found to have the highest specific activity when compared to the other components. Degradation studies have indicated that the derivative of everninic acid is an important constituent of at least two of the components making up the complex (Weinstein et al., 1965b). The purification and biological studies of everninomicin have been performed by Weinstein et al. (1965b), while the taxonomic studies of the producing

organism have been studied by Luedemann and Brodsky (1965).

(5) Other Antibiotics. Other antibiotics produced by the genus Micromonospora include rosamicin, produced by M. rosaria, a macrolide antibiotic (Crowe and Sanders, 1974; Reimann and Jaret, 1972), halomycin and antibiotic 460, produced by M. halopytica and M. chalea, respectively. In addition, it has been found that a species of Micromonospora was capable of producing neomycin, hitherto produced only by the Streptomycetes. This antibiotic produced by Micromonospora sp. 69 - 683 was shown to possess similar biological properties to those of the neomycin produced by Streptomycetes (Wagman et al., 1973). Micromonosporin, discovered by Waksman et al. (1967), is perhaps the first antibiotic isolated from this genus. The antibiotic was found to be effective against gram-negative bacteria.

Gentamicin

Gentamicin is a broad-spectrum, basic, water-soluble antibiotic mixture isolated from several species belonging to the genus Micromonospora (Oden et al., 1964; Rosselet et al., 1963). The gentamicin producing species are: M. purpurea (Weinstein et al., 1963; Luedemann et al., 1963) and M. echinospora sp. n., M. echinospora var. n. palladia, and M. echinospora sp. n. ferruginea (Luedemann and Brodsky, 1965). All these species and varieties have produced gentamicin in submerged fermentation conditions.

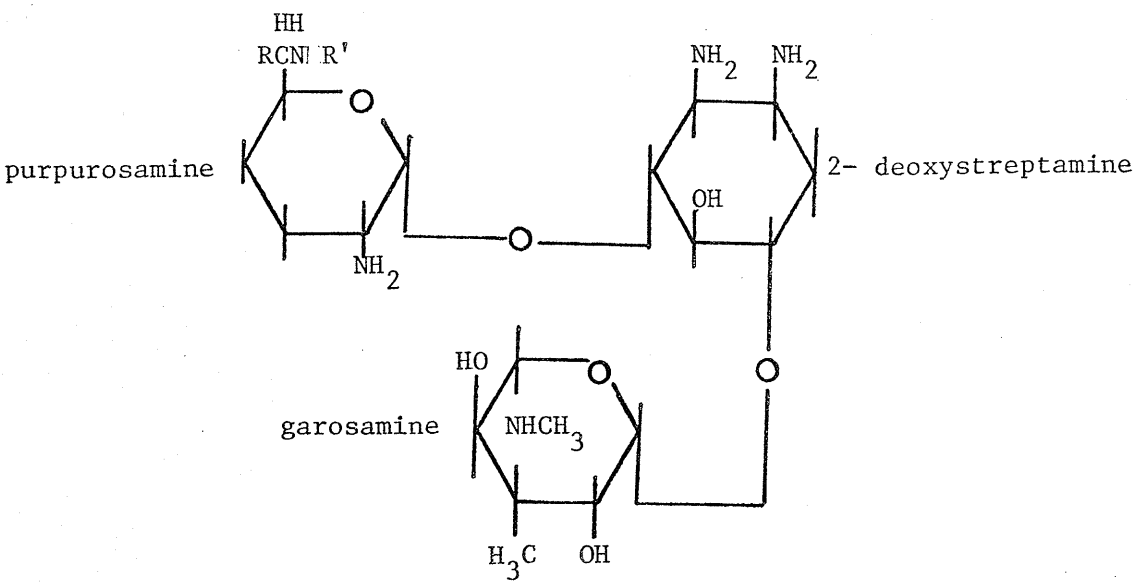
(1) The Gentamicin Complex. Initially, gentamicin was considered to be a complex consisting of two closely related components referred to as gentamicin C_1 and gentamicin C_2 (Weinstein et al., 1963; Kantor and Selzer, 1968). Both of these were established as being pseudo-oligo-

saccharides (Rosselet et al., 1963). It was also shown that these organisms produced additional basic substances with broad-spectrum antibiotic activity and a mycelial pigment which possessed activity against gram-positive bacteria (Weinstein et al.,1963). These additional, basic antibiotics have since been referred to as gentamicin A and gentamicin B (Weinstein et al., 1965a).

It has now been reported that M. purpurea produces a gentamicin complex consisting of three antibiotic components designated as C_1, C_{1a} and C_2 (Weinstein et al., 1967). Latest reports indicate that an additional aminoglycoside antibiotic is also produced as a minor component in gentamicin fermentations and is temporarily referred to as Sch. 14342 (Waitz et al., 1972).

The minor, co-produced antibiotics, known as gentamicin A and B (Wagman and Bailey, 1968), have since been characterized as consisting of at least four related antibiotics indentified as A,B,B_1, and X. Separation of these components, using paper or ChromAR® chromatography in various solvents, has been reported by Wagman et al. (1972). Their chemical composition and properties are given by Price et al. (1974).

(2) Physical and Chemical Properties of the Gentamicin Complex. The gentamicin C complex, produced by M. purpurea, is composed of three components: C_1, C_{1a} and C_2. These components are classified as methylaminoglycosides, and with the following structures (Lee et al., 1973; Cooper et al, 1969):



The components making up the complex, i.e., C_{1a}, C_1 and C_2, are found in approximately equal proportions; they contain the amino sugar deoxystreptamine, and two additional amino sugars, purpurosamine and garosamine. The difference in the three gentamicin components reside in the presence and number of methyl groups attached to the carbon 6 of the amino sugar purpurosamine, i.e.:

		R	R'
gentamicin	C_{1a}	H	H
"	C_2	CH_3	H
"	C_1	CH_3	CH_3

Gentamicin is distinguished from other chemically related antibiotics in the same family by its paper chromatography patterns (Rinehart, 1964). Chromatography performed by Weinstein et al. (1963) showed that the gentamicin complex moved as a single entity. Paper chromatographic resolution of the acid hydrolysis products of gentamicin when compared to products of other similar antibiotics indicated differentiating ninhydrin-positive components.

The antibiotic has a molecular weight of 543 and is extremely soluble in water and polar media such as pyridine and dimethylformamide. The activity of gentamicin is not significantly altered when an aqueous solution of the antibiotic is subjected to a temperature of 100°C for 30 minutes through a pH range of 2 to 12 (Luedemann et al., 1963). Gentamicin, being a moderately strong base, forms salts with any strong organic or inorganic acid. These salts are extremely water soluble; the most common salts of the antibiotic are the hydrochlorides and the sulfates.

A recent publication by Wagman et al. (1975) reported that several aminoglycoside antibiotics, including gentamicin, could be absorbed by various filtering agents. Thirty to one hundred percent adsorption of the aminoglycoside antibiotic to cellulose was observed, the percentage adsorbed being dependent on the ratio of adsorbent to antibiotic. It was also shown that the total quantity of adsorbed antibiotic could not be removed by acidification. Gentamicin was also adsorbed onto diatomaceous earth and Seitz filter sheets. The data presented indicate that unless extreme care is taken to evaluate results properly, errors in reporting the antibiotic titer may arise through losses of the antibiotic adsorbed by the various filtering agents.

(3) Biological Properties. Gentamicin is a complex of antibiotics belonging to the 2-deoxystreptamine family of antibiotics (Schaffner et al., 1964). The amino sugar, deoxstreptamine, is common to gentamicin, kanamycin and paromycin; however, the two additional amino sugars appear to be unique to the gentamicin complex (Weinstein et al., 1967).

Gentamicin is classified as being a wide- or broad-spectrum antibiotic; in addition it possesses a wide anti-rickettseal spectrum. It effectively inhibits the growth of Staphylococcus aureus and Klebsiella pneumoniae. It has been used for the treatment of cattle mastitis, and as a chemotherapeutic agent for a wide variety of microbial host infections caused by gram-negative bacteria, such as urinary infections brought about by Proteus sp. and Pseudomonas sp. (Luedemann et al., 1963). Gentamicin is the only broad-spectrum antibiotic that shows significant activity against strains of Pseudomonas, Proteus, Staphylococcus and Streptococcus (Weinstein et al., 1963).

The data presented by Weinstein et al. (1967) indicate no detectable differences in the biological activities of the components making up the gentamicin C complex. Wagman et al. (1968) reported these same results. With other antibiotics that contain deoxystreptamine, however, biological differences have been reported between various components, such as kanamycin A and B (Umezawa, 1964), and between the neomycins A, B and C (Waksman, 1958).

Waitz et al. (1970) reported that sisomycin, an aminoglycoside antibiotic, showed a similar antibacterial spectrum to that of gentamicin, both in vivo and in vitro. Verdamicin, another aminoglycoside antibiotic, also possesses a spectrum similar to gentamicin, although it does exhibit a higher activity against Pseudomonas sp. (Weinstein et al., 1975). Table 3 shows a comparative spectrum of the known aminoglycoside antibiotics;

Table 3. Minimum inhibitory concentrations (µg/ml) of some aminoglycoside antibiotics on various bacterial species.

Index organism	No. of strains	S*	N	K	F	P	G
S. aureus	29	2	0.5	1	0.5	1	0.125
S. faecalis	32	64	64	32	64	64	8
E. coli	22	8	8	4	8	8	1
Klebsiella sp.	20	4	2	2	2	2	1
Aerobacter sp.	10	4	2	2	2	2	0.5
P. mirabilis	6	8	8	4	8	8	2
P. vulgaris	6	4	4	4	4	4	1
P. morgani	10	8	8	4	8	4	1
P. rettgeri	7	4	8	2	8	4	1
Ps. aeruginosa	31	32	32	128	32	512	4
Salmonella sp.	14	16	2	2	2	2	1
Shigella sp.	17	8	8	4	8	8	2

* S - streptomycin K - kanamycin P - paromomycin
N - neomycin F - framycetin G - gentamicin

it should be noted that for all bacterial species listed, gentamicin shows the best activity (Garrod et al., 1973).

(4) Mode of Action of Gentamicin. Studies performed by Hahn et al. (1969) and Milanesi and Clifferi (1966) indicate that the mode of action of gentamicin is similar to that of streptomycin and other aminoglycoside antibiotics. Studies performed by these groups have shown that gentamicin effectively inhibits the uptake of C^{14} - labelled phenylalanine and lysine into the protein of E. coli. Gentamicin generally inhibits protein synthesis with a bacteriocidal effect as opposed to other antibiotics, such as chloramphenicol, which also inhibits protein synthesis but with a bacteriostatic effect.

Davies (1971, 1969) has indicated that gentamicin acts by irreversibly inhibiting protein synthesis in susceptible cells. Specifically, gentamicin acts by binding to the 30S ribosomal subunit, thereby interfering with the functions of the A site on the ribosome. This is also the case with kanamycin, neomycin, paromomycin, nebramycin and streptomycin.

Fermentation Production of Antibiotics

One of the first antibiotics produced on a commercial scale was penicillin, discovered by Fleming in 1929. With the advent of the Second World War, the need for increased amounts of antibiotic prompted the establishment of commercial fermentation plants producing feasible amounts of antibiotics. The initial type of fermentation procedure used was a surface culture method. The procedure used a sterile container which could provide a shallow (2 cm) layer of medium. This layer of medium was inoculated by spraying aqueous suspensions of the

spores into the container or by simply adding a few milliliters of the spore suspension to the container from a pipette. After incubation the containers were emptied and the broth collected after it had been separated from the vegetative mold (Sylvester and Coghill, 1954). Specific problems, other than low yields of antibiotic, were encountered. The maintenance of absolute sterility was formidable. Unless the strictest measures were taken to ensure sterile conditions, contamination would take place. This contamination could eventually inhibit or reduce the antibiotic production as well as interfere with the extraction and purification of the antibiotic.

Although many different designs for fermentors are now being used, most operate on a submerged fermentation technique. The fermentors are usually made of stainless steel with capacities in the range of 2 to 20,000 gallons and operate by a totally automated, or semi-automated method.

Gentamicin Fermentation

Gentamicin is produced using an industrial, aerobic, submerged culture method of fermentation. It employs suitable carbon and nitrogen sources in addition to trace elements and other growth promoting agents. A detailed description concerning the commercial production of gentamicin is outlined by Luedemann et al. (1963) and Charney (1964). The number of fermentation steps outlined by Charney (ibid.) is believed necessary for a particular enzyme to be synthesized; this enzyme is believed to be a requisite in the production of gentamicin.

Suitable carbon sources for the production of gentamicin include: maltose, soluble starch, glucose, corn starch, sucrose and dextrin.

For commercial production, however, dextrose, dextrin and starch are the main carbohydrates (Luedemann et al., 1963). Wagman and Weinstein (1966) reported various carbohydrates which supported the growth of M. purpurea. Maltose was shown to give the best growth results. No mention as to the gentamicin titer was reported, however. Growth and gentamicin production were shown to be unrelated. Abou-Zeid et al. (1974) reported that glucose as a carbon source gave maximal growth and antibiotic accumulation.

Several nitrogen sources include both organic (soybean meal, peptones, etc.) and inorganic (NH_4NO_3, NH_4Cl, $NaNO_3$) sources, although the most suitable sources for gentamicin production were those of organic nature. A comparison of the various media which contained different nitrogen sources indicated that yeast extract was the most suitable for gentamicin accumulation (Abou-Zeid et al. 1974).

The final fermentation stage described in a patent by Luedemann et al. (1963) consists of aerobically fermenting a dextrose based medium at 35°C for 24 hours. The fermentation vessel is aerated at 5 p.s.i. and 15 cubic feet per minute. At the end of this period the titer of gentamicin reached its peak and substantially remained constant in the pH range of 6.6 to 7.0.

Included in the fermentation medium are $CaCO_3$ and $CoCl_2$. Wagman and Weinstein (1966) have reported that the carbonate ion was a requisite for the growth of the organism. Peterson and Peterson (1954) indicated that in the production of aureomycin, a 1% $CaCO_3$ solution should be added to help maintain the desired pH of the fermentation. $CaCO_3$ also helped to increase the yields of antibiotic since the calcium ion reacted with the antibiotic to give a precipitate. This

promoted the production of additional antibiotic. The use of $CaCO_3$ is also included in the production of tetracyclines, penicillins and macrolide antibiotics (Hockenhull, 1963). Abou-Zeid et al. (1974) reported that gentamicin yields increased with larger concentrations of $CaCO_3$, reaching their optimum at 1 gram per liter. Wagman and Weinstein (1966) reported that the concentration of $CaCO_3$ in a synthetic fermentation medium was dependent upon the concentration of magnesium. This synthetic medium, however, was not used to produce gentamicin quantitatively.

Charney (1974) reported that the amount of gentamicin produced by M. purpurea could be enhanced considerably by the addition of cobalt into the fermentation medium. The cobalt, being in the form of a water soluble salt, was effective in increasing the antibiotic titre in concentration ranges from 2.5×10^{-9} to 1.25×10^{-5} grams per milliliter. Studies indicated that the cobaltic ion enhanced the production of gentamicin to a smaller degree than the cobaltous ion. Abou-Zeid et al. (1976) recently reported the cobalamin (B_{12}) had a stimulatory effect upon gentamicin accumulation. The specific effects of other vitamins and trace elements were also reported.

(1) Extraction of Gentamicin from Mycelia. Reiblein et al. (1973) found that the best method for releasing gentamicin bound to the mycelium was either acid or alkaline extraction. Extraction of mycelia-bound gentamicin was poor as the pH approached neutrality. Both alkaline and acid pH extremes were found to release the majority of bound antibiotic. However, clear supernatants obtained after centrifugation were obtained only with the acid extracts. The alkaline extracts were very turbid. Studies indicated that acid extraction at a pH of 2.0 for one

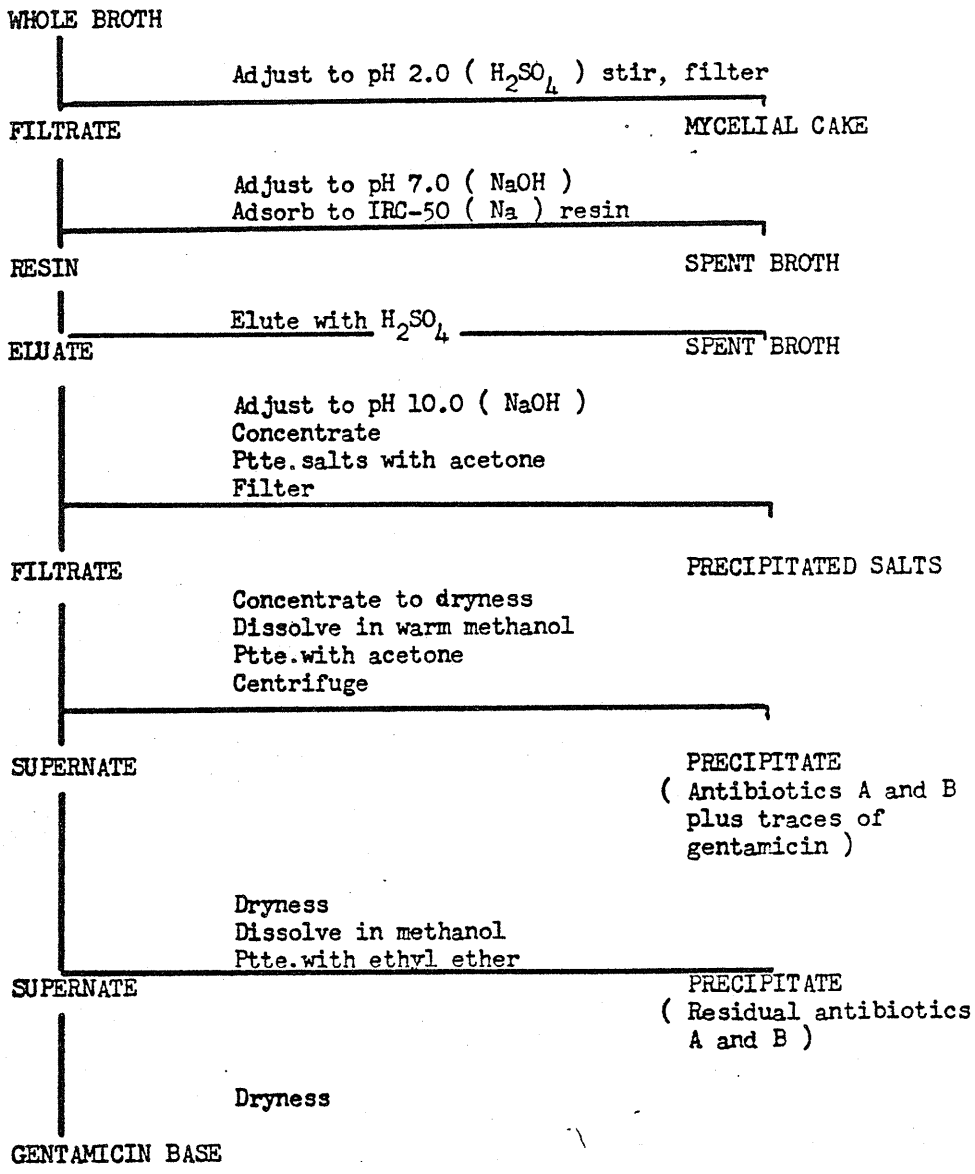
or more minutes was favorable for gentamicin release. Acid extraction also indicated no differences in the proportions of the gentamicin components liberated.

Earlier studies, performed by Rake and Donovick (1946) with streptomycin, indicated that the addition of NaCl to fermentation broths prior to sterilization realized higher titers of the antibiotic in the fermentation broths. Reiblein et al. (1973) found no such effects when NaCl was added to neutral and acid extracts of gentamicin. Perlman (1953) indicated that the addition of NaCl to fermentations only effected the release of the bound antibiotic from the mycelia. The overall effect, however, decreased the total amount of antibiotic produced.

(2) Isolation of Gentamicin from Fermentation Broths. Once the peak antibiotic titer has been reached, the whole fermentation is acidified to a pH of 2.0 with 6 N H_2SO_4. The acidified broth is then filtered with the aid of Celite Super-Cel. The resulting clear, acidic filtrate is neutralized with 4 N NaOH; 1.56 kg. oxalic acid per 1.0 kg. of $CaCO_3$ originally used in the fermentation medium is then added to the filtrate. The pH is then adjusted to 7.0 with 2 N NaOH. After a 12 hours aging period, the precipitated calcium oxalate is removed by filtration and the clear filtrate purified by ion exchange. (Figure 1; Rosselet et al., 1963; Weinstein et al., 1963; Luedemann et al., 1963; Schaffner et al., 1964).

(3) Purification of the Gentamicin Complex. The clear filtrate is passed through a column containing Amerlite IRC-50 (Na^+). The initial resin effluent is discarded. The resin is then washed with water and

Figure 1. Isolation of gentamicin from fermentation broths.



the antibiotic mixture is eluted with 2 N H_2SO_4. The acid eluate is neutralized to a pH of 6.8 and treated with 70% sodium dodecylbenzenesulfonate (Santomerse S) for complete precipitation of the active material. The suspension is then stirred for 15 minutes and filtered again with Celite. The filter cake is washed exhaustively with distilled water and air dried. The partially dried cake is extracted with methanol. The methanol extracts are combined and passed through a 401S anion exchange Amberlite resin column, in its OH cycle, in methanol.

The basic eluate is concentrated until all the methanol has evaporated, leaving an essentially aqueous solution. The pH of the concentrated solution is adjusted to a pH of 4.5 with 2 N H_2SO_4. Then the solution is treated with Darco-G-60 and filtered.

The clear, nearly colorless filtrate is then added to 10 volumes of methanol while being agitated, and the precipitate, gentamicin sulfate, is collected by filtration, washed and finally dried at 60°C in a high-vacuum oven (Rosselet et al., 1963). Another isolation procedure has been described by Luedemann et al. (1963) in regard to tank fermentations using 500 gallon fermentations. Lee et al. (1973), working with radioactive gentamicins, has described an isolation method using Amberlite IRC-50 (NH_4^+) in a batch process. This method of isolation was especially suitable for a large number of small samples.

Other aminoglycoside antibiotics - neomycin, sisomycin, and verdamicin - are isolated and purified in a similar manner (Wagman et al., 1973; Weinstein et al., 1970, 1975).

(4) Isolation and Purification of the Gentamicin C Complex. The gentamicin complex, which is isolated by ion exchange chromatography,

consists of the gentamicin C complex (C_1, C_{1a} and C_2) and other minor co-produced antibiotics. The purification and isolation of the components making up the gentamicin complex are largely accomplished through paper and thin layer chromatographic techniques (Wagman et al., 1968). Chromatographic separation, and bioassay of the gentamicin complex, have been reported by Kantor and Selzer (1968), and Wagman et al. (1968) who used a solvent system composed of chloroform-methanol-17% ammonium hydroxide (2:1:1 v/v). Marquez et al. (1968) described, in part, the methods used for the separation of the gentamicin C components and discussed their novel structures. The separation of the C components has also been described above. Oden et al. (1968) give a detailed method for the chromatographic bioassay for the gentamicin complex.

SCOPE OF INVESTIGATION

The purpose of this study was to investigate the feasibility of using spent fermentation liquor, obtained from primary gentamicin fermentations, for the additional production of the same antibiotic. The primary objectives to be studied in this investigation included:

1. To use spent liquor obtained from primary gentamicin fermentations for the additional production of gentamicin.

2. To evaluate the parameters of shake-flask fermentations, i.e., inoculum level, duration of fermentation, distributions of gentamicin in mycelia and broths, effect of cobalt and calcium carbonate, accumulation of gentamicin and pH and other factors which could influence the optimum production of gentamin in spent liquor fermentation.

3. To evaluate the beneficial effects of gentamicin production obtained through shake-flask fermentations, in batch fermentation studies using a 14-liter fermentor.

4. To compare the yields of gentamicin produced and the volumes of fermentation broth required in batch and continuous fermentation systems, employing spent fermentation liquor.

MATERIALS AND METHODS

Materials

The bacterial cultures used throughout this investigation were:

(1) Micromonospora purpurea N.R.R.L. 2953, Northern Regional Research Laboratory, Peoria, Illinois, U.S.A.

(2) Staphylococcus aureus A.T.C.C. 6538P, American Type Culture Collection, Rockville, Maryland, U.S.A.

The microbiological analysis of gentamicin was carried out by using:

(1) U.S.P. reference standard, gentamicin sulfate, United States Pharmaceutical Commission Inc., Rockville, Maryland, U.S.A.

(2) Grove and Randall No. I and No. II antibiotic medium, Difco Laboratory, Detroit, Michigan, U.S.A.

(3) Bacto concentration disks (½" - sterile blanks), Difco Laboratory, Detroit, Michigan, U.S.A.

General Analytical Methods

Nitrogen

Nitrogen determination of the fermentation broths was determined using a Kjeldahl procedure, performed by the Department of Plant Science, University of Manitoba. Prior to the actual determination of nitrogen, the fermentation samples were filtered using a 0.2 μ Millipore filter. The mycelia-free filtrate was then analyzed for nitrogen and reported as mg/ml Kjeldahl nitrogen.

Calcium and Cobalt

Calcium and cobalt were both determined by atomic absorption spectroscopy, performed by the Department of Plant Science, University of Manitoba. Prior to their actual determination, a wet ashing procedure was employed to completely oxidize all organic material. This wet ashing procedure consisted of placing 20 ml of filtered fermentation broth in a crucible and adding 20 ml of concentrated nitric acid and 10 ml of concentrated perchloric acid. The crucible was gently heated until the contents were evaporated to near dryness. Further additions of 20 ml of concentrated nitric acid were made and evaporated until a clear, light-colored solution appeared (Parker, 1972). The solution was then made up to a final volume of 5.0 ml with de-ionized water and further diluted if necessary.

A Perkin-Elmer model 403 atomic absorption unit was used for these analyses. Calcium was read at 422.7 nm and cobalt was read at 240.7 nm.

Cell Weight

The cell growth of M. purpurea was determined as a change in cell weight at intervals by determining the dry mycelial weight of the organism expressed as mg/ml of fermentation broth. Ten milliliters of sample broth was filtered using a 0.2 μ, 47 mm Gelman filter in association with a Millipore filtration unit. The filter was previously weighed on an analytical balance after it had been dried to a constant weight in an air oven at 100°C. Once the whole broth was filtered, the filter was dried in a similar fashion and reweighed. The difference in weight was recorded as the mycelial weight.

To avoid erroneous results in the calculation of the mycelial weight, due to $CaCO_3$ in the fermentation broth, 1.0 N HCl was added

to the broth prior to the filtration step in order to ensure complete dissolution of $CaCO_3$. Methyl orange was used as the indicator to ensure proper acid conditions (Wagman and Weinstein, 1966).

Residual Glucose

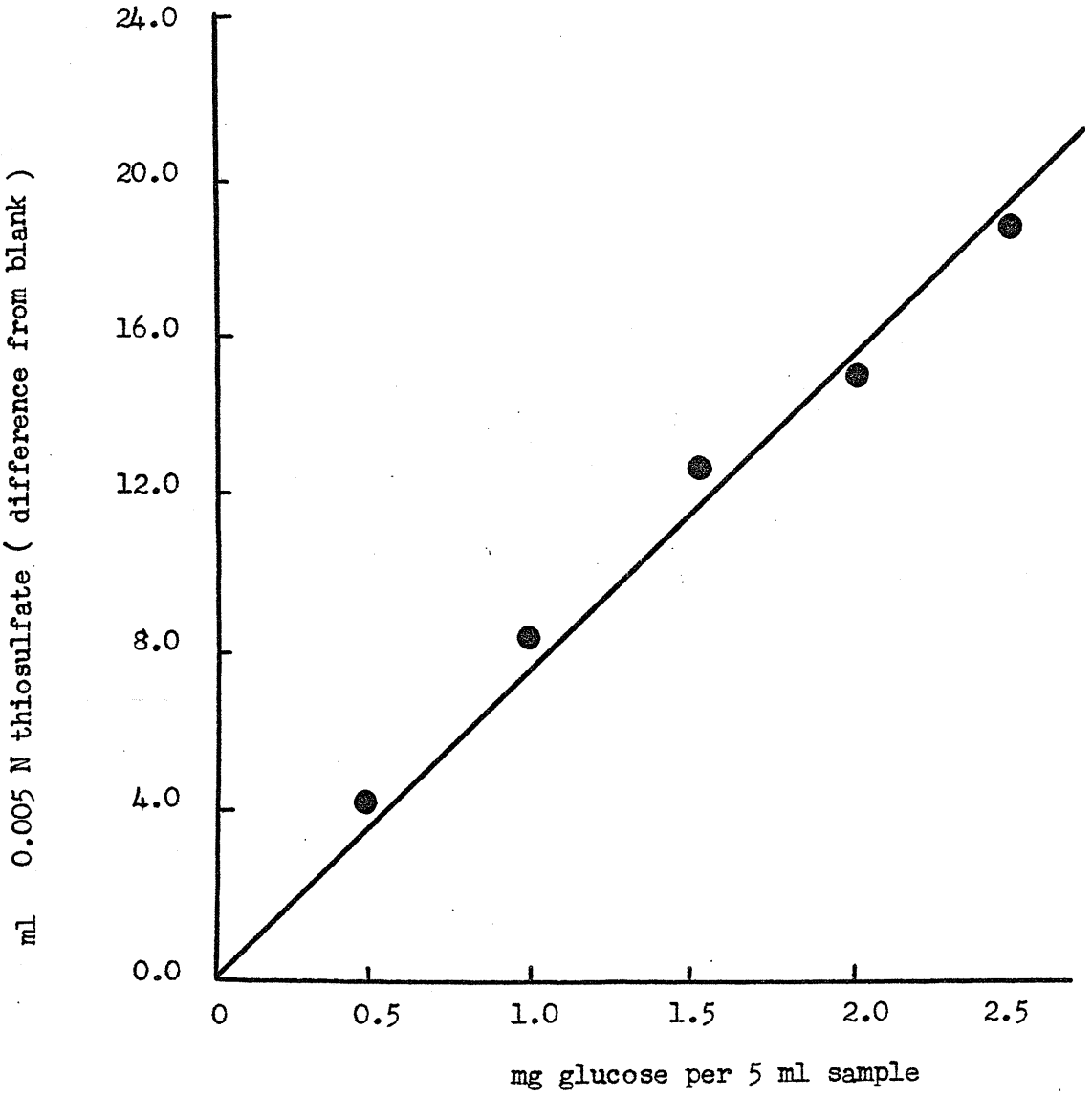
Residual glucose was determined according to the Shaffer-Somogyi micro method as outlined in the A.O.A.C. (1965). A standard curve, with glucose concentration versus ml of 0.005 N thiosulfate, was plotted and used to determine residual glucose in fermentation broths. Results were expressed as mg/ml of fermentation broth.

A 25 ml fermentation sample, diluted with distilled water when necessary, was mixed with an equal volume of 10% neutral lead acetate and made to a final volume of 100 ml with distilled water. The sample was then centrifuged for 10 minutes at 3,000 r.p.m. (3,050 x g). Fifty ml of the resulting supernatant was mixed with 12.5 ml of a 10% potassium oxalate solution. The sample was once again made to a volume of 100 ml and similarly centrifuged. An aliquot of this supernatant was used to determine residual glucose. Titration values were compared to the standard curve (Figure 2).

Residual Starch

Residual starch was determined using an acid hydrolysis method. Starch concentration was determined in terms of glucose concentration by using the glucose standard curve. The glucose value obtained was then converted to starch by the formula: wt. of dextrose (mg/ml) x 0.90 = wt. of starch (mg/ml) (A.O.A.C., 1965). The acid hydrolysis method consisted of mixing a ten-ml fermentation sample with 20 ml of concentrated HCl. It was then made to a volume of 50 ml with distilled water and refluxed for one hour in a 500-ml flat bottom boiling flask. After

Figure 2. Standard curve for glucose: Shaffer-Somogyi micro method.



acid hydrolysis, the contents of the flask were neutralized with 6 N NaOH and treated in a fashion similar to that described for glucose.

Concentration of Fermentation Broth

Concentrated spent fermentation liquor was prepared by freeze-drying; a Virtis Freeze-Mobile lyophilizer was employed. Five-hundred-ml batches of spent fermentation liquor obtained from fermentations at 96 hours were Millipore-filtered and then evenly distributed in 250-ml round-bottom flasks; each contained approximately 25 ml of fermentation liquor. The flasks were partly immersed in liquid nitrogen and swirled constantly until the contents had frozen as a film on the walls of the flasks. Lyophilization was carried out for 24 hours using a condenser temperature of -40°C in a vacuum of 100 microns of mercury.

The freeze-dried samples were then scraped into crucibles and ground into a fine powder. The powder was stored in vials at -10°C until used. The powder was reconstituted with tap water. A final volume of 200 ml was made to contain 25 µg/ml gentamicin. The hydrated powder was sterilized by Millipore filtration using a 0.2 µ filter.

Gentamicin Assay Method

Test Organism

An agar-diffusion, disc-plate assay was used for the determination of gentamicin titers in the fermentation broth, as outlined by Oden *et al.* (1964, 1968). *Staphylococcus aureus* A.T.C.C. 6538P was maintained by weekly transfers to fresh slants of Grove and Randall No. 1 agar. The inoculated slants were incubated for 24 hours at 35°C and then stored at 5°C.

Inoculum Preparation

A sterile slant of S. aureus was washed with 3.0 ml of sterile 0.85% saline. The cell suspension was then transferred to a 500-ml Roux bottle slant containing 100 ml of Grove and Randall No. 1 agar. The cell suspension, aided by the use of sterile glass beads, was then distributed evenly over the agar slant. The Roux bottle was subsequently incubated for 24 hours at 35°C.

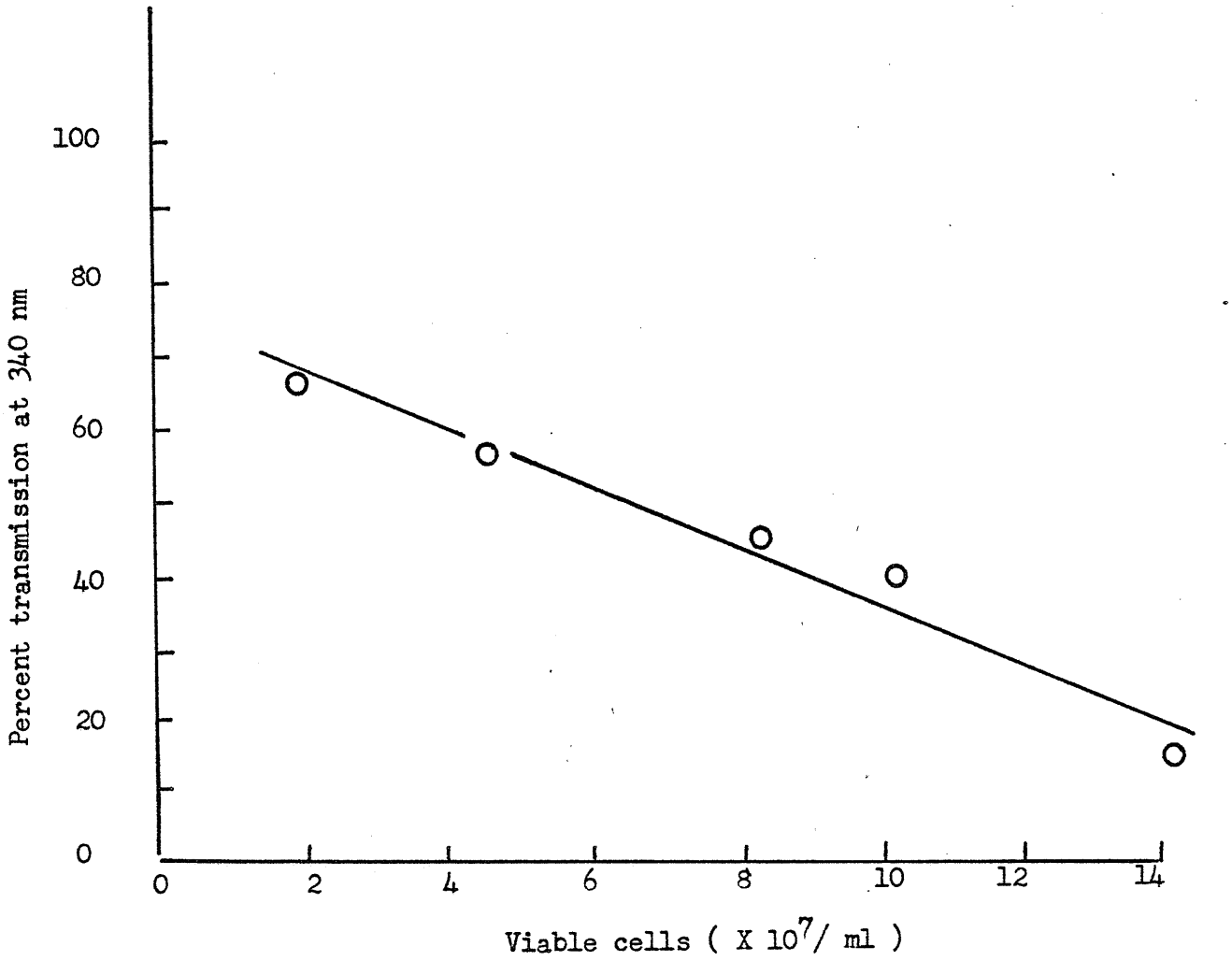
The resulting cell growth was harvested by washing the surface of the slant with 50 ml of 0.85% sterile saline. The stock inoculum was stored at 5°C for no longer than one week.

(1) Inoculum Standardization. A reference curve for the standardization of S. aureus was prepared by plotting the viable cell count versus the % transmission at 340 nm. A Coleman model 111 spectrophotometer was used for the transmission readings, using saline as the blank. A serial dilution technique, employing Grove and Randall No. 1 agar, was used for the determination of viable cell counts at each transmission level. Petri plates were incubated at 35°C for 24 hours and then counted. A recommended level of 1×10^8 cells per ml was used as the standardized inoculum for the plate assay (Figure 3).

(2) Assay Plate Preparation. The gentamicin assay plates were prepared by distributing evenly 21 ml of Grove and Randall No. 11 agar (basal layer) into each 100 x 15 mm petri plate. When the basal layer had solidified, 4.0 ml of the seed layer was distributed evenly over the top of the hardened basal layer and allowed to harden. The seed layer consisted of 0.5 ml standardized S. aureus inoculum in 100 ml liquified and tempered (45°C) Grove and Randall No. 11 agar.

Sterile, ½" Difco blank disks, held by means of sterile tweezers,

Figure 3. Inoculum standardization for S. aureus .



were used to gently dip the blank disk edges into the gentamicin sample until capillary action had soaked them completely. The loaded disks were then carefully laid on the surface of the prepared plates. Previously, the petri plates were sectioned into four equal parts by means of a China marker; one disk occupied each quarter of the plate. Sample assays were performed in triplicate on the same plate, with the fourth quarter being occupied by a disk containing a known titer of gentamicin. This last disk was used as a reference dose. Plates were incubated at 35°C for 24 hours.

(3) Response Measurement. The diameters of the zones of inhibition were measured on both axes through the center of the disk by means of precision calipers. The average of the diameters in mm was then used as the final value for the dose response. This dose response was then compared to a standard curve which correlated the zone of inhibition with gentamicin potency.

(4) Standard Curve. The working standard, gentamicin sulfate (650 µg/mg anhydrous powder), was dried at 110°C under a pressure of 5 mm Hg or less for three hours.

Ten mg of the dried standard (6,500 µg gentamicin) was then dissolved and brought to a final volume of 250 ml with 0.1 M phosphate buffer, pH 8.0. The gentamicin stock solution, containing 26 µg/ml, was stored at 5°C for no longer than one month. A standard curve for determining gentamicin was prepared by suitably diluting the stock solution with 0.1 M phosphate buffer, pH 8.0, as shown.

Gentamicin stock solution (ml)	Phosphate buffer (ml)	Total volume (ml)	Gentamicin potency (µg/ml)
1.0	25	26	1.0
2.0	24	26	2.0
3.0	23	26	3.0
4.0	22	26	4.0
5.0	21	26	5.0
6.0	20	26	6.0

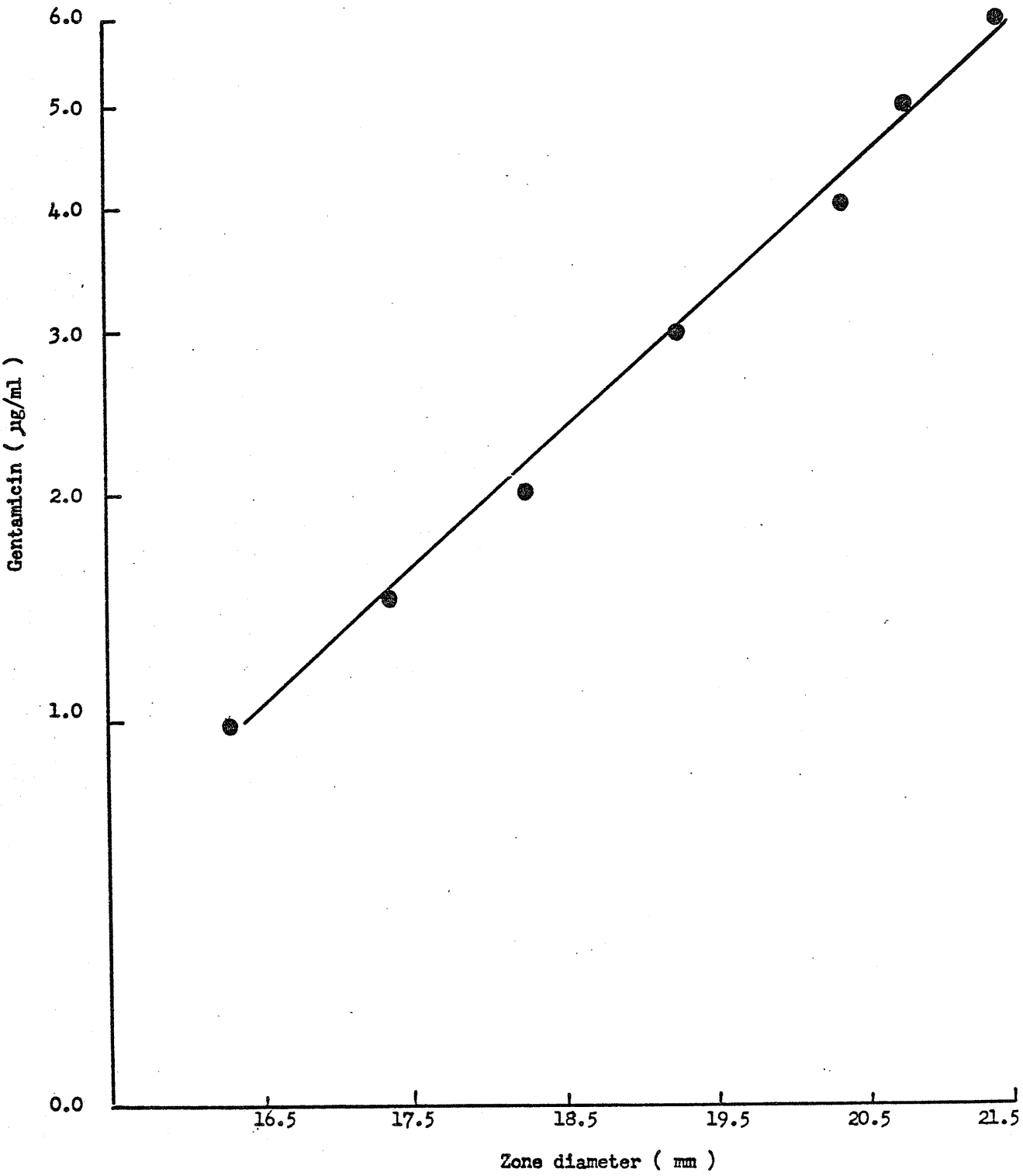
A standard curve (Figure 4) plotting the gentamicin potency, in a semilog form, versus the zone of inhibition, was then set up. The standard curve was derived from two sets of triplicate results which were averaged.

Preparation of Sample for Gentamicin Testing

(1) Total Gentamicin. Fermentation samples to be tested for gentamicin potency were prepared in the following manner: ten-ml samples of whole fermentation broth were acidified to a pH of 2.0 with 6 N H_2SO_4 and shaken for 30 minutes on a gryatory shaker at room temperature. After shaking, the acidified, whole broth was centrifuged for 20 minutes at 10,000 r.p.m. (12,100 x g). Oxalic acid, in quantities of 1.56 gram per 1.0 gram of calcium carbonate, originally present in the fermentation medium, was then added to the supernatant in a 50-ml flask. The pH of the supernatant was re-adjusted to 7.0 with 2 N NaOH and the supernatant was allowed to age after 12 hours at 5°C.

After the aging period, the supernatant was centrifuged once more in a similar manner. The supernatant was subsequently adjusted to a final pH of 7.9 to 8.0 with 2 N NaOH; it was made to a volume of 25 ml using phosphate buffer, 0.1 M, pH 7.9. In some cases, higher dilutions

Figure 4. Standard curve for the determination of gentamicin.



were performed in order that the zones of inhibition would fall along the linear portion of the plotted gentamicin standard curve. These dilutions also employed a similar buffer.

(2) Free Gentamicin. Determination of free gentamicin in the fermentation broth was prepared in the following manner: samples of whole broth were centrifuged for 20 minutes at 10,000 r.p.m. (12,100 x g). Ten ml of the resulting supernatant was then used for subsequent testing, after the sample had been treated with oxalic acid as described.

(3) Bound Gentamicin. The bound gentamicin was empirically formulated by using the equation: total gentamicin - free gentamicin = bound gentamicin.

Fermentation Methods

Shake-Flask Fermentations

(1) Inoculum Preparation. Fresh nutrient agar slants of M. purpurea were inoculated into 500 ml flasks containing 100 ml of the following medium, hereafter referred to as medium (A):

Bacto-beef extract	3.0 g
Tryptone	5.0 g
Dextrose	1.0 g
Soluble potato starch	24.0 g
Yeast extract	5.0 g
Tap water	1000 ml

The flasks were then incubated on a New Brunswick rotary shaker (300 r.p.m., 3/4" circular orbit) for 96 hours at 35°C.

The inoculum preparation was harvested by transferring it to sterile

stainless steel Sorvall centrifuge tubes where it was centrifuged for 20 minutes at 10,000 r.p.m. (12,100 x g). The supernatant was discarded and the mycelial pellet washed twice with sterile 0.85% saline solution. The washed inoculum was then brought up to its original volume with 0.85% saline.

(2) Primary Fermentation. A 5% (v/v) inoculum from the inoculum preparation stage was used to seed 1000-ml flasks containing 200 ml of the following medium, hereafter referred to as medium (B):

Yeast extract	10.0 g
Dextrose	10.0 g
$CaCO_3$	1.0 g
$CoCl_2$	0.0013 g
Tap water	1000 ml

Flasks were subsequently incubated on rotary shakers, such as described for the inoculum preparation, for 96 hours at 30°C. In all cases, flasks were adjusted to a pH of 6.8 to 7.0 following sterilization using 2 N NaOH and 2 N H_2SO_4; all flasks were routinely sterilized for 15 minutes at 121°C. Calcium carbonate and cobalt chloride were autoclaved separately and added into the fermentation flasks upon cooling. Those fermentation studies examining the effect of pH had their initial pH values re-adjusted every 24 hours with 2 N H_2SO_4 and 2 N NaOH.

Individual flask fermentation studies were performed simultaneously in duplicate, from freshly prepared medium (B). The broth was prepared in bulk and then evenly distributed to the fermentation flasks in order to reduce the medium composition among the flasks.

(3) Spent Liquor Fermentation. Once the optimum potency of gentamicin from the primary fermentation stage was reached, the contents of the fermentation flasks were filtered using a Whatman No. 40 filter (12.5 cm). The collected mycelia were either analyzed or discarded, depending upon the experiment. The filtrate was centrifuged in stainless steel Sorvall centrifuge tubes for one hour at 10,000 r.p.m. (12,100 x g) with a Sorvall superspeed centrifuge. The supernatant was recollected and a portion of it tested for residual glucose, free gentamicin and total nitrogen. The bulk of the supernatant was stored at 5% during the time these analyses were performed. In those fermentations where the effect of calcium carbonate and cobalt chloride was being studied, these chemicals were also analyzed. The supernatant was then sterilized using a 0.2 μ Gelman (47 mm) filter in conjunction with a Millipore unit. The sterile broth was aseptically pipetted into previously sterilized 1000-ml flasks. Those studies which were investigating the effects of fortifying spent liquor had their nutrients added to the fermentation flasks in such a manner that both the original volume and nutrient concentration were approximately achieved. Since the volume of spent broth from any one primary fermentation flask did not yield 200 ml after it had been filtered, it was necessary to include extra primary fermentations. These extra primary fermentations were not analyzed; however, their filtered broth was used to adjust the spent fermentation liquor back to 200 ml in each flask. Nutrients to be supplemented into fermentation flasks were dissolved in the extra spent liquor which was used to achieve a final volume of 200 ml.

Fermentor Fermentations

(1) Inoculum Preparation. The inoculum preparation was carried out in two stages. The first stage consisted of inoculating 500-ml flasks containing 100 ml of medium (A) in the manner as described for the shake-flask inoculum. The second stage consisted on inoculating five-1000 ml flasks containing 100 ml of fresh, sterile medium (A) with a 5% (v/v) washed inoculum preparation from the first stage. Flasks were incubated for 72 hours at 30°C on rotray shakers operating at 300 r.p.m. After incubation the contents of the flasks were pooled and centrifuged for 20 minutes at 10,000 r.p.m. (12,100 x g). The mycelial pellet was washed twice in sterile 0.85% saline and brought up to one quarter its original volume with the addition of more sterile 0.85% saline.

(2) Fermentor Design. A Chemap, p.e.c. 14-liter fermentor, type GF 014-141, was used for the fermentor fermentation studies. The conditions for fermentation, as outlined below, were used throughout the various fermentor studies, unless otherwise specified.

The pH of the fermentation broth was adjusted to 6.8 - 7.0 after sterilization by the addition of 3 N NaOH and 3 N H_2SO_4. A one-station Ingold auto pH controller, using an in situ, autoclavable glass reference electrode, was used to monitor the pH of the fermentation medium when the maintenance of the pH was desired. pH integrated, peristalic pumps, using 3 N NaOH and 3 N H_2SO_4, were used for the auto pH controller.

A temperature of 32-35°C was used in these fermentor studies. The temperature of the fermentation was controlled automatically by a thermistor control which was integrated with the fermentor unit.

Filtered air was fed into the fermentation vessel at a rate of 1 ml/minute/ml medium by means of glass wool, plug-in, air line filters. Aeration was controlled by the use of a flow meter and an air line pressure gauge.

Two impellers, with four flat, stirring paddles, were used to agitate the fermentation medium in a radial fashion. Agitation was carried out at 1,5000 r.p.m.

Foam was controlled by the addition of antifoaming agents such as Mazola oil added prior to sterilization.

(3) Primary Batch Fermentation. The fermentor studies employed medium (B), using a total fermentation volume of seven liters. A 5% (v/v) inoculum, obtained from the inoculum preparation stage, was used to seed the fermentation. Fermentation studies were carried out for 96 hours and then terminated. Samples were withdrawn every 24 hour period and analyzed. The fermentor and its contents were sterilized in situ, for two hours at 121°C.

(4) Primary Continuous Fermentation. Continuous fermentation studies employing medium (B) used a total volume of 7.2 liters. All other conditions remained the same. Once the optimum potency of gentamicin was reached, the continuous feed of fresh, sterile medium (B) was begun. Two Masterflex peristalic pumps were used to feed the fermentation and withdraw fermentation broth. Thus, a fixed, constant volume of fermentation broth remained in the fermentation vessel at all times. Pumps were adjusted so that the inflow of fresh medium equalled the outflow of fermentation broth. The outflow of fermentation broth was continuously collected in a fraction collector housed in a refrigerator at 5°C. Samples were analyzed every six hours.

A dilution rate, corresponding to 5 ml medium per minute was used ($D = 0.0416\ hr^{-1}$). The dilution rate refers to the amount of incoming medium in liters per hour divided by the fermentation volume.

(5) Spent Liquor Batch Fermentation. In this case, once the optimum potency of gentamicin was reached, the whole fermentation broth was harvested by means of a Sorvall KSB continuous flow system, using a SS-34 rotor head operating at 15,000 r.p.m. The collected broth was then analyzed; it was fortified with essential nutrients, if required. The spent fermentation liquor was then filtered into a previously sterilized fermentation jar using a 0.2μ Millipore filter. The sterile, spent liquor was then pumped back into the fermentor by using pressurized, sterile air. The fermentation volume used for these studies was 5 liters. This volume was used because the filtered, spent liquor never equalled 7 liters. The agitation of the fermentor remained the same; however, the air input was adjusted to yield the same air flow per minute per ml of fermentation medium.

(6) Spent Liquor Continuous Fermentation. Two types of continuous fermentation methods were investigated by using spent fermentation liquor. The first method, called method A, involved the batch fermentation of spent liquor for 72 hours after which the maximum accumulation of gentamicin was produced. Thereafter, fresh, spent fermentation liquor was infused to initiate the continuous process. Samples were collected every 6 hours in a fraction collector housed in a refrigerator at 5°C. A dilution rate of D = 0.0416 was used, with a total fermentation volume of 7.2 liters for the initial batch fermentation. In order to obtain sufficient amounts of spent fermentation liquor, primary fermentations, using fresh medium (B), were performed

in 12 liter fermentation jars magnetically stirred and aerated as described for the batch fermentations.

The second method of continuous fermentation, method B, involved the continuous fermentation of spent liquor from the outset of fermentation. In this process, spent liquor was infused into the fermentor vessel at the beginning of the fermentation without waiting for maximum gentamicin accumulation to take place. The conditions used in this process remained the same as those used for method A.

RESULTS

Preliminary studies carried out during this investigation focused upon shake-flask fermentations which employed M. purpurea in an effort to determine the fermentation patterns and yields of gentamicin obtained through primary and spent liquor media. Parameters obtained through shake-flask fermentations that showed beneficial effects were applied to fermentor fermentation studies; conversely, those parameters showing deleterious effects were omitted.

Part 1: Shake-Flask Fermentation Studies

The Vegetative Growth of M. Purpurea in a Starch Based Medium (A) at 35°C

The vegetative growth of M. purpurea, employing a recommended starch based medium (A) at 35°C for 96 hours, showed a maximum accumulation of gentamicin - 4.5 µg/ml - at 96 hours (Figure 5). Accumulation of gentamicin increased progressively after 48 hours of growth with a concomitant increase in the pH of the medium. Very little gentamicin could be detected during the first 48 hours of growth. Approximately 22% of the initial starch was utilized during the vegetative growth.

The Fermentative Growth of M. Purpurea in a Glucose Based Medium (B) at 30°C

The fermentative growth of M. purpurea carried out at 30°C showed a maximum yield of gentamicin - 44.0 µg/ml - at 96 hours of fermentation (Figure 6). Mycelial weight and pH steadily increased as the fermentation progressed; no lag of gentamicin production was noted prior to 48 hours of fermentation. Approximately 38% of the initial glucose was utilized during the fermentation.

Figure 5. The vegetative growth of M. purpurea in a starch based medium (A) at 35°C.

(cf. Appendix table 1)

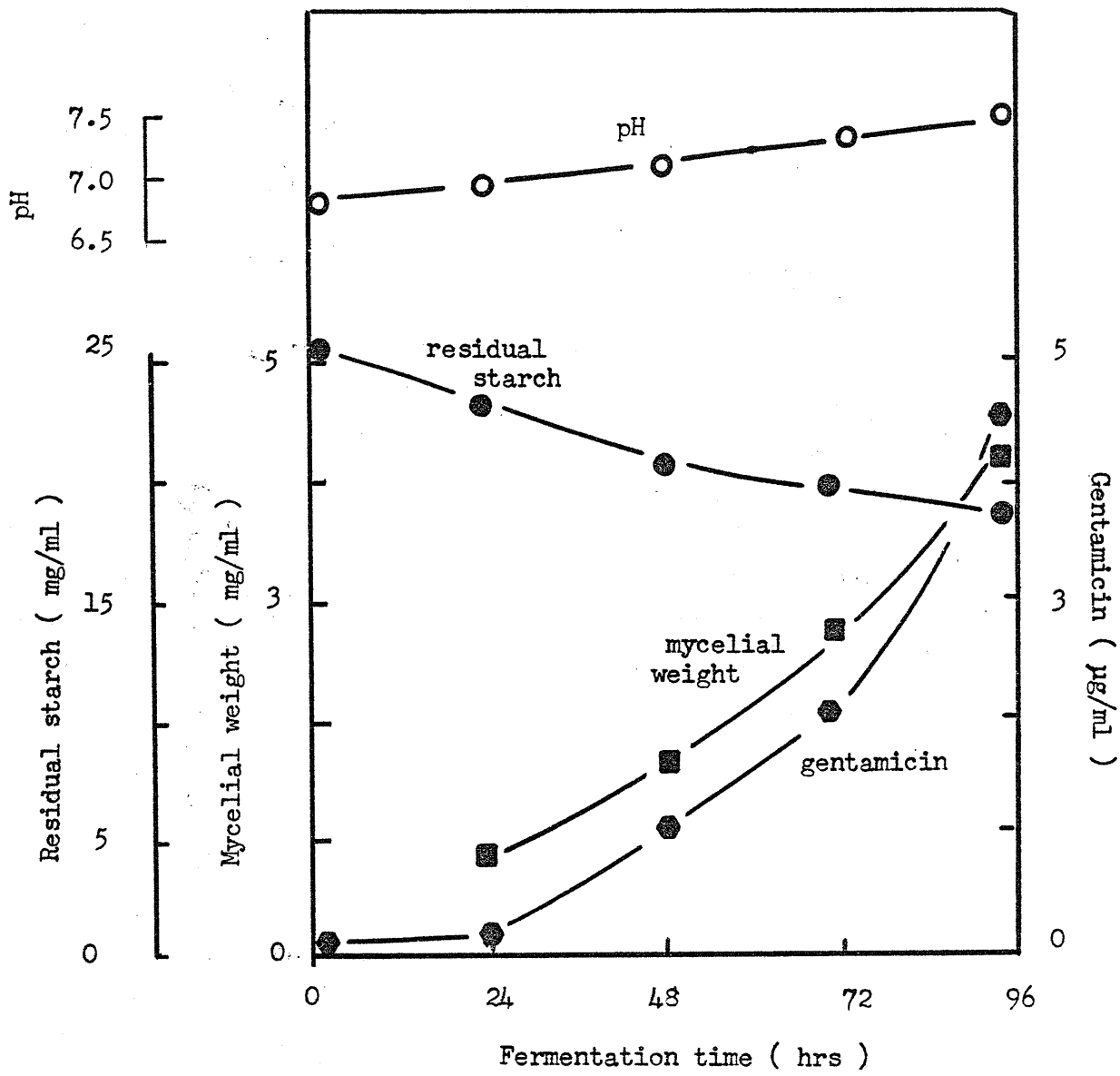
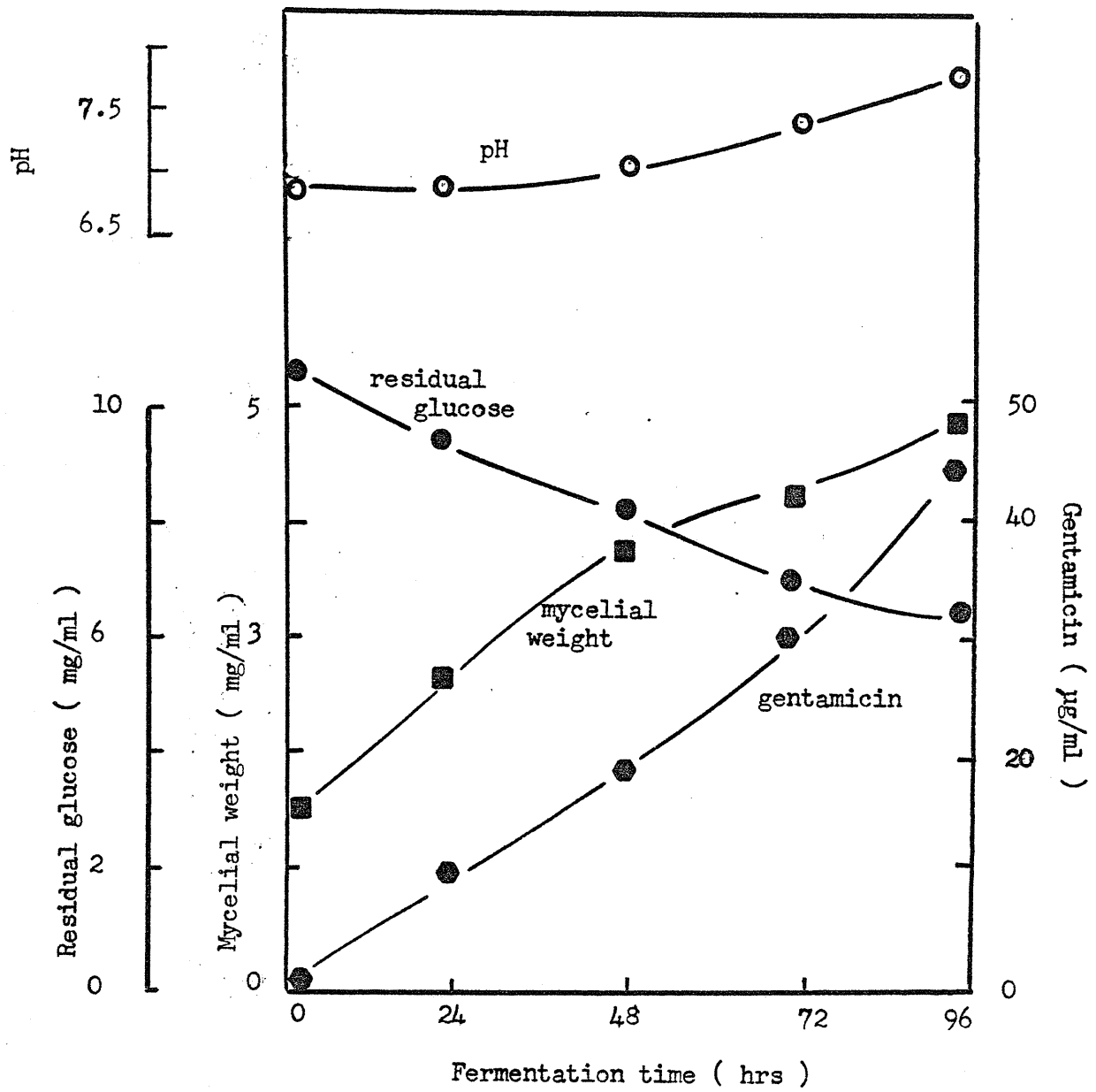


Figure 6. The fermentative growth of M. purpurea in a glucose based medium (B) at 30°C.

(cf. Appendix table 2)



The Effect of Increasing the Time of Fermentation on the Production of Gentamicin.

Subsequent studies of fermentation growth (Figure 7), in which the time of fermentation was extended to 144 hours, indicated that the maximum accumulation of gentamicin occurred at 120 hours of fermentation. The net increase in gentamicin production occurring from the 96th to 120th hour, however, was minimal when compared to the amount of gentamicin produced at 96 hours of fermentation. Further primary fermentation studies were henceforth conducted with a 96 hour fermentation period.

Size of Inoculum and Production of Gentamicin

As shown in Table 4, the maximum yield of gentamicin was 55.5 µg/ml using a 6% (v/v) inoculum (2.48 mg/ml). Increasing the size of the inoculum from 2% to 6% (0.82 to 2.48 mg/ml) progressively increased the maximum yield of gentamicin; this occurred in all inoculum levels at 96 hours of fermentation.

Inocula higher than 6% decreased the maximum yield of gentamicin. Those fermentations having an 8 and 10% inoculum (3.27 and 4.99 mg/ml) showed a decreased growth pattern after 72 and 48 hours of fermentation respectively. The yield of gentamicin, however, continued to increase in both cases. A 6% initial inoculum was henceforth used for further fermentation studies.

The Effects of Using Spent Liquor for the Production of Gentamicin.

Flask fermentations employing spent fermentation liquor are compared to primary fermentations employing fresh, sterile medium (B) (Figure 8). The results indicated that an additional 67.5% of gentamicin could be produced by using the spent fermentation liquor obtained

Figure 7. The effect of increasing the time of fermentation on the production of gentamicin.

(cf. Appendix table 3)

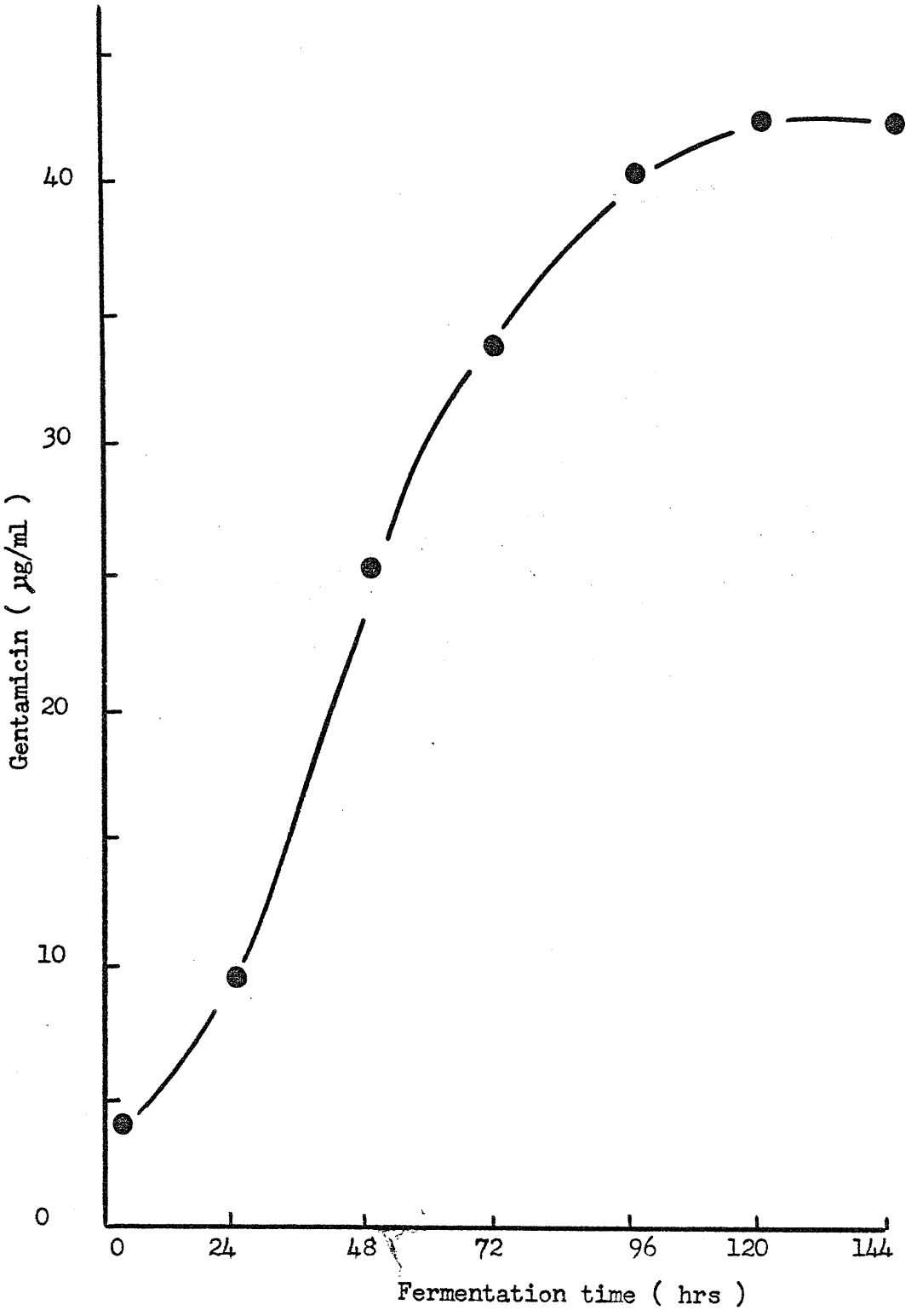


Table 4. Size of inoculum and production of gentamicin.

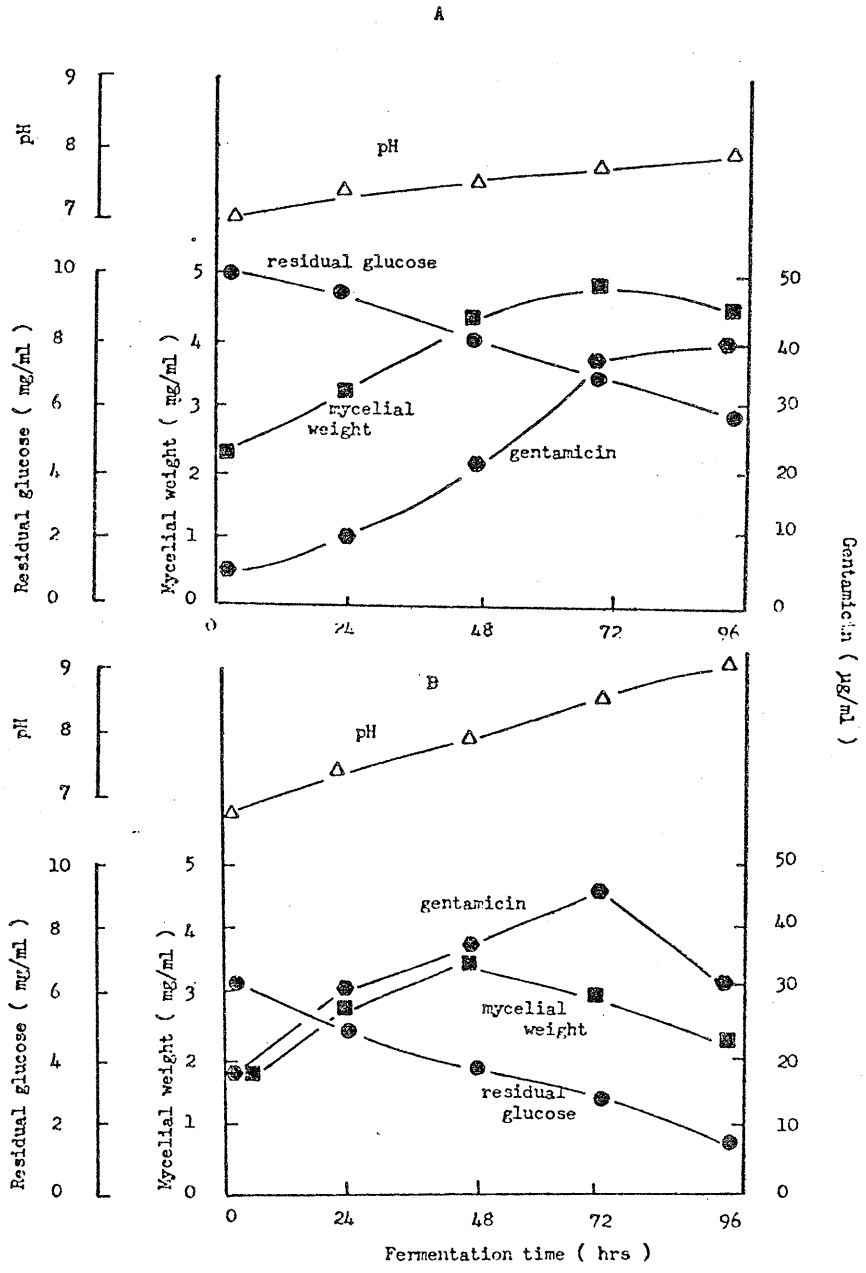
(cf. Appendix tables 4,5)

Initial inoculum (% v/v)		Fermentation time (hrs) 0	24	48	72	96
2	Mycelial weight (mg/ml)	0.82	1.61	2.50	3.09	3.76
	Gentamicin (µg/ml)	*	8	14.5	25.5	33.5
4	Mycelial weight (mg/ml)	1.49	2.11	3.12	4.10	4.87
	Gentamicin (µg/ml)	*	8.5	18.5	30.0	40.0
6	Mycelial weight (mg/ml)	2.48	3.14	4.30	5.27	6.35
	Gentamicin (µg/ml)	*	9.5	27.5	49.0	55.5
8	Mycelial weight (mg/ml)	3.27	4.72	5.73	6.21	5.76
	Gentamicin (µg/ml)	*	17.0	30.5	47.0	53.0
10	Mycelial weight (mg/ml)	4.09	5.49	6.29	5.58	5.00
	Gentamicin (µg/ml)	*	23.5	32.0	37.0	42.5

* Not determined.

Figure 8. The effects of using spent fermentation liquor for the production of gentamicin.

(cf. Appendix tables 6,7)



A. Primary fermentation.
B. Spent liquor fermentation.

from primary fermentations. Maximum yields of gentamicin were noted at 72 hours of fermentation, decreasing thereafter. The mycelial weight increased for the first 48 hours of fermentation, decreasing thereafter as well. No decrease in the gentamicin yield, however, was observed during the first 72 hrs of fermentation. The pH in both fermentations increased as the fermentation progressed; higher pH values were observed, however, in those fermentations using spent fermentation liquor. pH increments after 24 hours of fermentation were observed to be greater in spent liquor fermentations than those in primary fermentations. The yields of gentamicin decreased as the pH of the spent liquor fermentation passed 8.6.

Approximately 42% of the initial glucose was utilized during primary fermentation. Fermentations using spent liquor utilized approximately 68% of that glucose remaining after 96 hours of primary fermentation.

The Distribution of Bound and Free Gentamicin in Flask Fermentations

The distribution of bound and free gentamicin in primary fermentations is shown in Figure 9. At the commencement of fermentation no free gentamicin could be detected. The total gentamicin present at the beginning of fermentation - 5.5 µg/ml - was found to be contained within the mycelia which was used as the inoculum. Approximately 35% of the gentamicin produced in the fermentation at 96 hours was free gentamicin; approximately 65% of the gentamicin produced in the fermentation at 96 hours was bound gentamicin.

The distribution of bound and free gentamicin in fermentations employing spent liquor are illustrated in Figure 10. In these fermentations the initial gentamicin potency was observed to be much higher than those potencies found in primary fermentations. Free

Figure 9. The distribution of bound and free gentamicin in primary fermentations.

(cf. Appendix table 8)

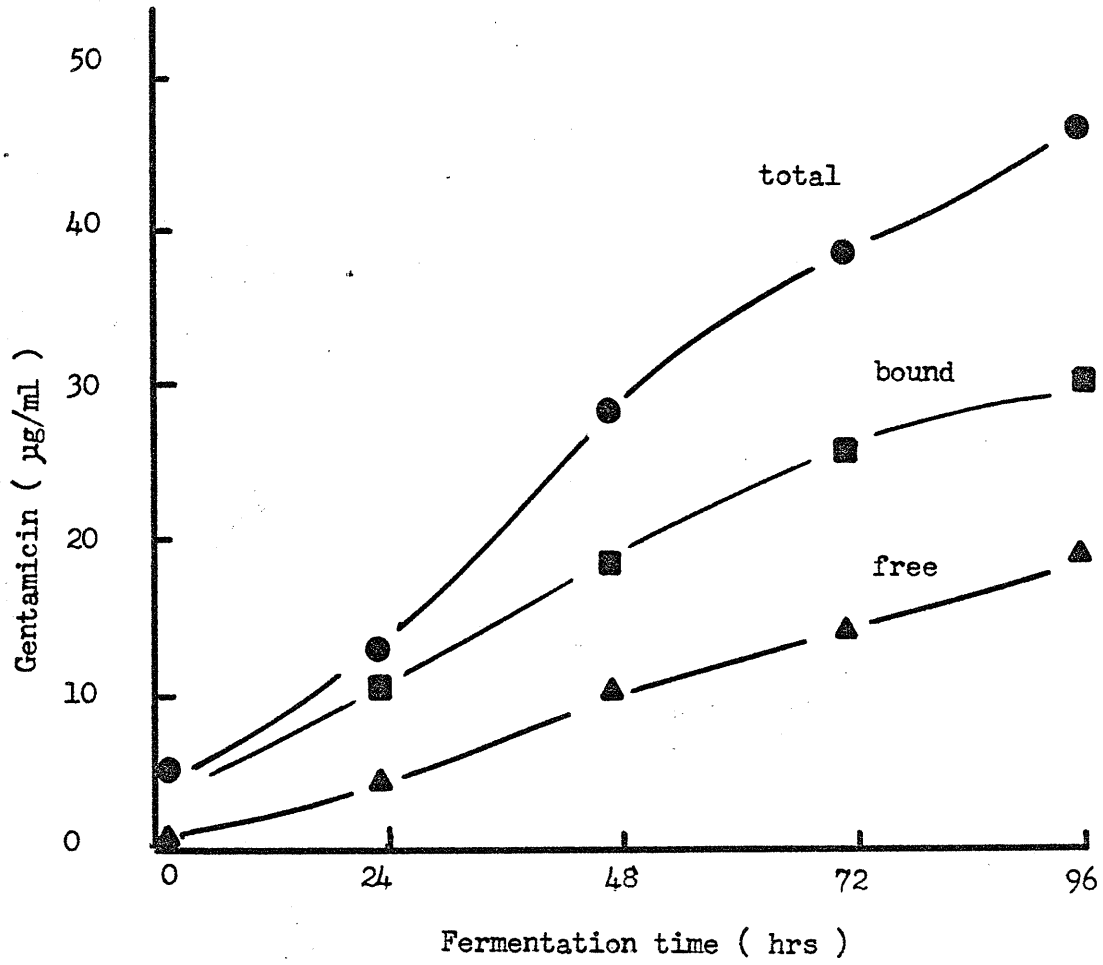
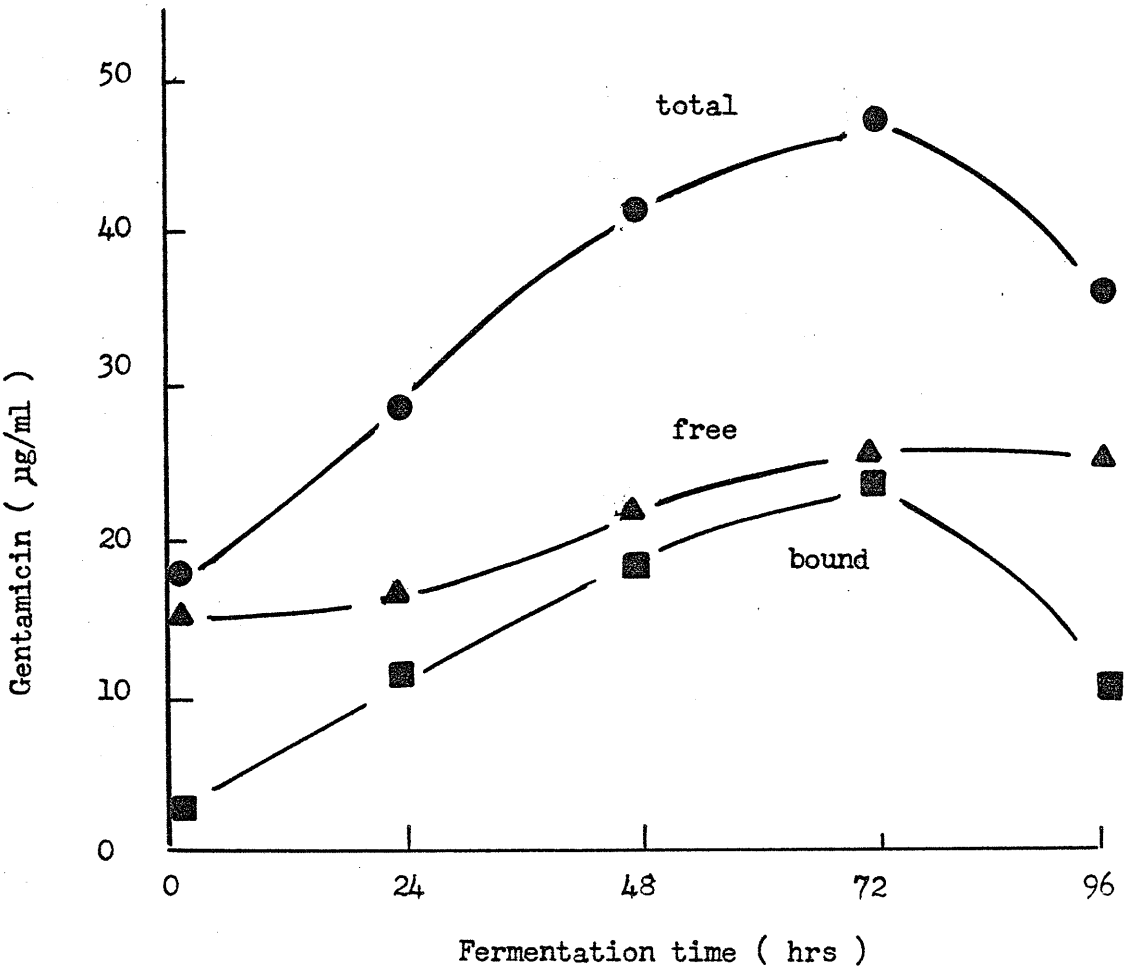


Figure 10. The distribution of bound and free gentamicin in fermentations employing spent fermentation liquor.

(cf. Appendix table 9)



gentamicin accumulation was noted to increase progressively in the fermentation broth for 72 hours; after this time it decreased. This same effect was observed for the bound gentamicin; however, the decrease in bound gentamicin at 72 hours of fermentation was much greater than the corresponding decrease in the free gentamicin concentration. The average accumulation of free gentamicin in the fermentation medium at 72 hours of fermentation was approximately 29%, while the average accumulation for the bound gentamicin was 71%. At 96 hours of fermentation the concentration of free gentamicin increased to approximately 65% while the concentration of bound gentamicin decreased to approximately 35%; the percent of free and bound gentamicin at 96 hours of fermentation indicated that the bound portion of the total gentamicin decreased much faster than the free portion of the total gentamcin concentration.

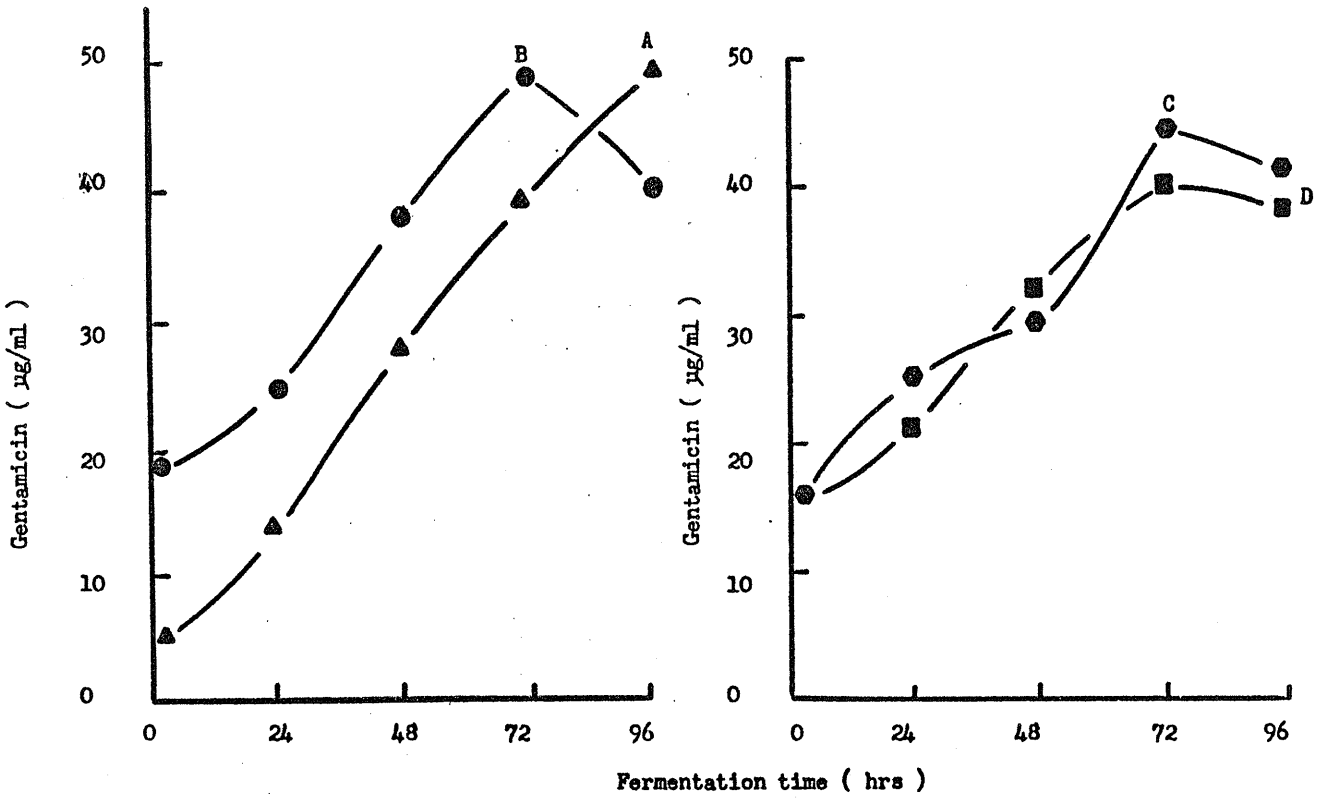
Influence of Cobalt on the Production of Gentamicin in Primary and Spent Liquor Fermentations.

Primary flask fermentations employing a recommended level of $CoCl_2 \cdot 6H_2O$ (0.0013 grams per liter or 0.32 µg/ml as Co^{++} - medium (B)) are compared to spent liquor fermentations containing various concentrations of cobalt (Figure 11).

The maximum accumulation of gentamicin was obtained in those primary fermentations using a recommended level of cobalt. Fermentations employing spent fermentation liquor indicated a progressive increase in the maximum yield of gentamicin as the level of cobalt supplementation was decreased; the greatest accumulation of gentamicin was produced when liquor fermentations were not supplemented with cobalt. Atomic absorption spectroscopy indicated the presence of residual cobalt in all fermentations

Figure 11. Influence of cobalt on the production of gentamicin in primary and spent liquor fermentations.

(cf. Appendix tables 10,11)



A. Primary fermentation containing recommended level of cobalt.
B. Spent liquor fermentation with no cobalt supplementation.
C. Spent liquor fermentation with 0.16 µg/ml cobalt supplementation.
D. Spent liquor fermentation with 0.32 µg/ml cobalt supplementation.

and further studies excluded cobalt supplementation in spent liquor fermentations.

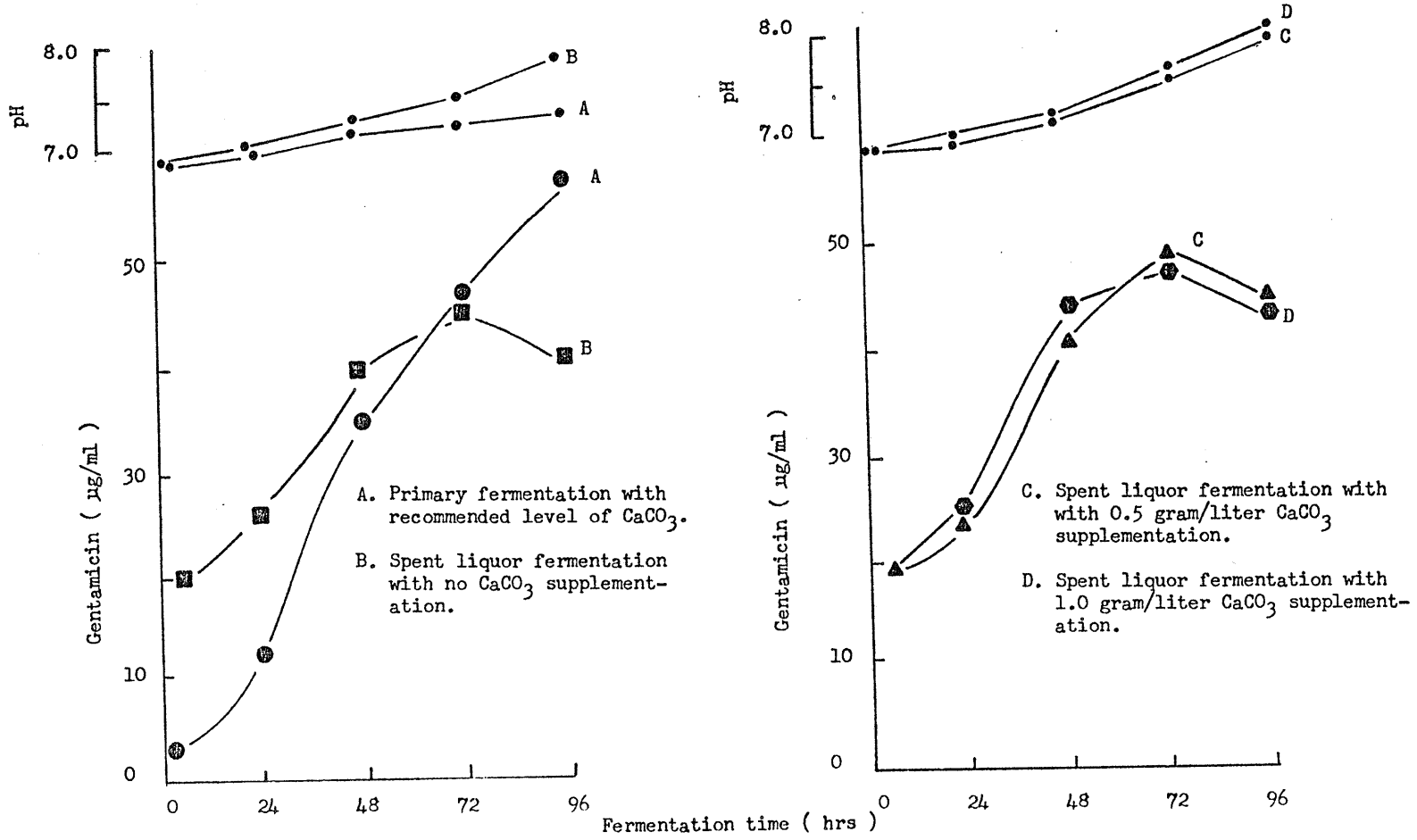
Influence of Calcium Carbonate on the Production of Gentamicin in Primary and Spent Liquor Fermentations.

Primary flask fermentations employing a recommended, (medium (B)), level of calcium carbonate, 1.0 gram/liter, are compared to fermentations using spent liquor with varying concentrations of calcium carbonate (Figure 12). The highest accumulation of gentamicin was noted in primary fermentations at 96 hours of fermentation. Spent liquor fermentatins, having no supplemented calcium carbonate, showed a decrease in gentamicin accumulation at 72 hours of fermentation. It was observed, however, that the total amount of gentamicin in both these fermentations were similar at 72 hours, although the net production of gentamicin in the primary fermentation was considerably greater. Supplementing spent liquor fermentations with 0.5 gram/liter calcium carbonate indicated a stimulatory effect on gentamicin production when compared to similar fermentations having no calcium carbonate supplementation. Increasing the level of calcium carbonate supplementation to 1.0 gram/liter showed adverse effects in gentamicin accumulation. In all cases, the maximum accumulation of gentamicin occurred at 72 hours of fermentation, with the exception of the primary fermentation; this occurred at 96 hours. The pH in those fermentations using spent liquor increased slightly as the level of calcium carbonate was increased. All spent liquor fermentations showed residual levels of calcium carbonate prior to calcium carbonate supplementation.

Calcium carbonate, in quantities of 0.5 gram/liter, was henceforth supplemented into spent liquor fermentation studies.

Figure 12. Influence of calcium carbonate on the production of gentamicin in primary and spent liquor fermentations.

(cf. Appendix tables 12,13)



The Effect of pH Adjustment on the Production of Gentamicin Using Spent Fermentation Liquor.

The use of various initial pH values for flask fermentations employing spent fermentation liquor were investigated (Table 5). Results indicated that an initial pH of 7.0, adjusted to 7.0 every 24 hours, was optimal for the accumulation of gentamicin in these studies. Initial pH values of 6.5, adjusted to 6.5 every 24 hours, showed comparable yields of gentamicin at 96 hours of fermentation. Initial pH values of 5.5 to 7.0 showed progressive increases in the yields of gentamicin at 96 hours of fermentation. The manual adjustment of pH every 24 hours was shown to be inadequate in order to maintain a constant pH. The pH within any one fermentation increased with the time of fermentation.

Gentamicin Production at pH 7.0 Using Spent Fermentation Liquor With/Without pH Adjustment.

The maximum yields of gentamicin occurred at 72 hours of fermentation when no pH adjustment was made (Figure 13). Those fermentations having pH adjustments every 24 hours exhibited maximum yields of gentamicin at 96 hours of fermentation. pH adjustment to 7.0 every 24 hours showed greater accumulation of gentamicin than did similar fermentations without pH adjustment. It was observed, however, that the yields of gentamicin accumulated at 72 hours without pH adjustment decreased during the next 24 hours of fermentation. This effect was not observed with those fermentations having pH adjustment. Relatively high alkaline pH values were observed in those fermentations having no pH adjustment, especially at 72 and 96 hours of fermentation.

Table 5. The effect of pH adjustment on the production of gentamicin using spent fermentation liquor.

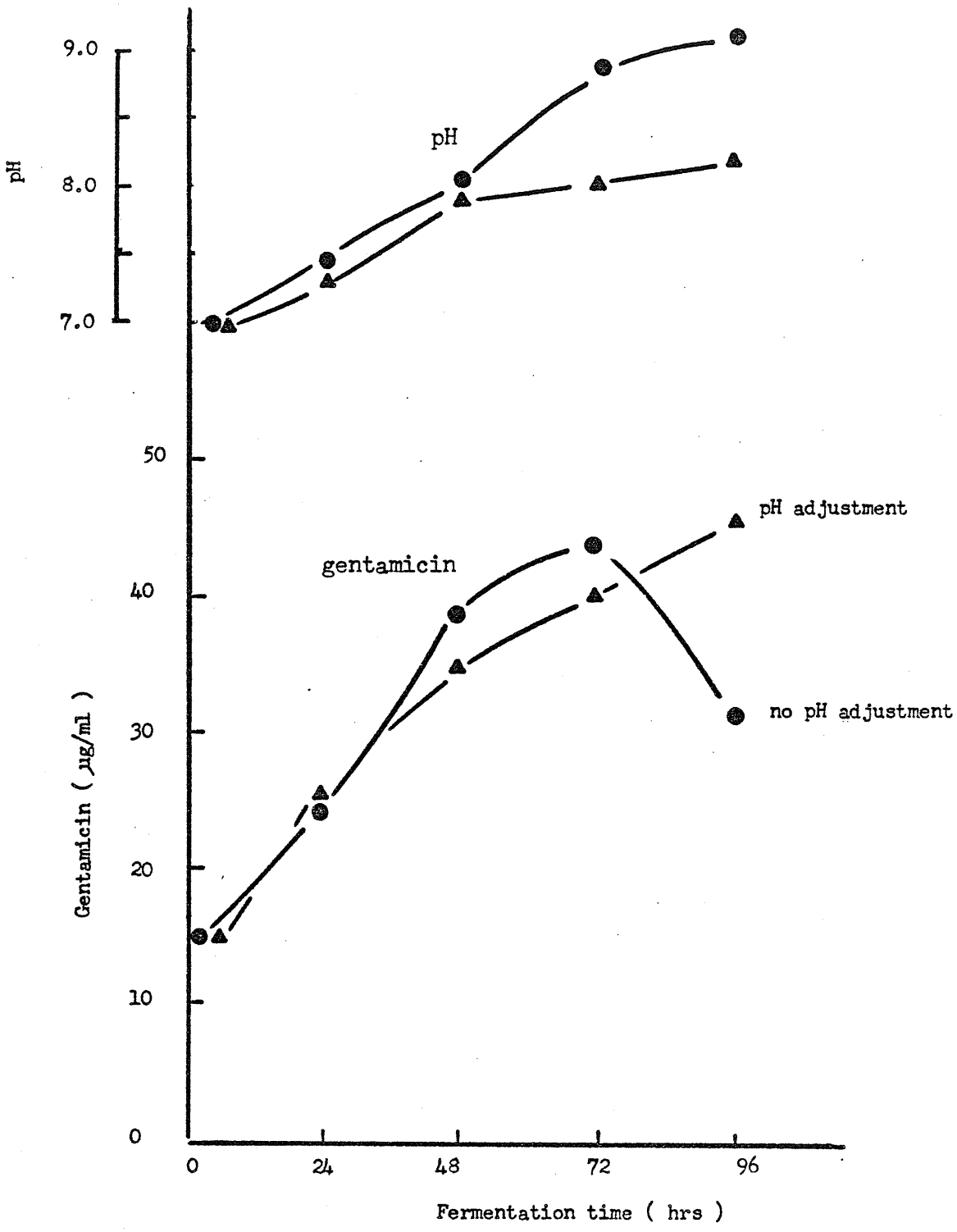
(cf. Appendix tables 14,15)

	Fermentation time (hrs)				
	0 *	24	48	72	96
pH	5.5	5.7	5.8	6.1	6.7
Gentamicin (µg/ml)	14	13	17	21	28
pH	6.0	6.5	6.6	6.9	7.1
Gentamicin (µg/ml)	14	15	19	27	35
pH	6.5	6.7	7.1	7.3	7.6
Gentamicin (µg/ml)	14	17	24	34	46
pH	7.0	7.5	7.8	8.0	8.3
Gentamicin (µg/ml)	14	19	28	40	48

* These values represent initial pH values of the media. pH values other than at the commencement of fermentation represent pH values of the fermentation prior to adjustment.

Figure 13. Gentamicin production at pH 7.0 using spent fermentation liquor with/without pH adjustment.

(cf. Appendix table 16)



The Effect of Gentamicin Sulfate on the Growth of M. Purpurea in Medium (B).

The addition of varying levels of gentamicin sulfate standard (1.0 to 20.0 µg/ml) to flask fermentations employing fresh medium (B) were compared to control fermentations having no added gentamicin sulfate (Table 6). It was observed that mycelial growth continued to increase in all flask fermentations containing added gentamicin sulfate. Those fermentations having an initial 0.0 µg/ml to 10.0 µg/ml concentration of added gentamicin sulfate, however, showed a decrease in growth after 72 hours of fermentation. The fermentation that had an initial concentration of 20.0 µg/ml added gentamicin sulfate continued to show mycelial growth through the entire period. Mycelial growth, and the utilization of glucose between individual fermentations, showed no definite correlation. It was observed, however, that the addition of gentamicin sulfate to flask fermentations did not decrease the general growth or affect the glucose metabolism of the organism when compared to control fermentations.

The Effect of Gentamicin Sulfate on the Production of Gentamicin by M. Purpurea in Medium (B).

The addition of 20.0 µg/ml gentamicin sulfate standard to fermentations employing fresh medium (B) indicated that there was a net decrease in the total amount of gentamicin produced at 96 hours of fermentation, when compared to flask fermentations containing no added gentamicin sulfate (Table 7). A net production of 26 µg/ml gentamicin was produced at 96 hours when 20.0 µg/ml gentamicin sulfate was initially administered. The control flask showed a net production of 44.0 µg/ml gentamicin during the same time interval. It was also observed that a slight decrease in gentamicin production appeared

Table 6. The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

(cf. Appendix tables 17,18)

Gentamicin sulfate added to medium (μg/ml)		Fermentation time (hrs) 0	24	48	72	96
0.0	Mycelial weight (mg/ml)	1.85	2.68	3.85	4.82	4.55
	Residual glucose (mg/ml)	9.56	8.11	7.06	5.91	5.10
1.0	Mycelial weight (mg/ml)	1.85	2.70	3.69	4.76	4.68
	Residual glucose (mg/ml)	9.56	8.17	7.18	6.17	5.66
5.0	Mycelial weight (mg/ml)	1.85	2.70	3.91	5.00	4.93
	Residual glucose (mg/ml)	9.56	8.30	7.11	5.98	5.34
10.0	Mycelial weight (mg/ml)	1.85	2.77	3.99	5.04	4.87
	Residual glucose (mg/ml)	9.56	8.23	7.11	5.81	5.11

Table 6 (continued). The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

(cf. Appendix tables 17,18)

Gentamicin sulfate added to medium (µg/ml)		Fermentation time (hrs) 0	24	48	72	96
20.0	Mycelial weight (mg/ml)	1.85	2.69	3.85	4.90	5.05
	Residual glucose (mg/ml)	9.56	8.32	7.53	5.50	5.03

Table 7. The effect of gentamicin sulfate on the production of gentamicin by M. purpurea in medium (B).

(cf. Appendix table 19)

Fermentation time (hrs)	Gentamicin * (µg/ml)	
	A	B
0	21	2
24	18	15
48	23	26
72	34	38
96	47	46

A. Gentamicin sulfate standard (20.0 µg/ml) added at the start of fermentation.

B. No addition of gentamicin sulfate at the start of fermentation.

* Refers to the total amount of gentamicin present at time of assay.

after 24 hours in those fermentations that had been administered gentamicin sulfate.

The Growth Pattern of M. Purpurea in Varying Concentrations of Spent Fermentation Liquor.

The growth patterns of M. Purpurea, using varying concentrations of spent fermentation liquor, are presented in Table 8. In all cases, glucose and Kjeldahl nitrogen were fortified to approximately 10.0 mg/ml glucose and 1.2 mg/ml nitrogen (yeast extract) before the start of fermentation. The data indicates that as the concentration of spent liquor was increased within any one fermentation, a concomitant decrease in mycelial weight was noticed; the poorest mycelial growth was observed in those fermentations containing concentrated spent fermentation liquor. The pH within a 24 hour period in any one fermentation increased as the concentration of spent liquor was increased. The elapsed time for maximum growth within any one fermentation also decreased as the level of spent liquor was increased. The fermentation which contained concentrated spent liquor showed its maximum growth at 24 hours, thereafter continually decreasing.

The Production of Gentamicin Using Varying Concentrations of Spent Fermentation Liquor.

Gentamicin production in flask fermentations which continued varying concentrations of spent liquor are presented in Table 9. Gentamicin production was shown to decrease as the concentration of spent liquor was increased. A net production of 32.0, 26.0, 24.0, 21.0, and 11.0 µg/ml gentamicin was produced in fermentations containing 25, 50, 75, 100% and concentrated spent fermentation liquor, respectively.

Table 8. The growth pattern of M. purpurea in varying concentrations of spent fermentation liquor.

(cf. Appendix tables 20,21)

		Fermentation time (hrs)				
		0	24	48	72	96
A.	pH	6.9	7.4	7.6	8.0	8.5
	Mycelial weight (mg/ml)	1.98	3.26	4.48	5.05	4.83
B.	pH	6.9	7.6	8.0	8.7	9.1
	Mycelial weight (mg/ml)	1.98	2.95	3.60	3.60	2.78
C.	pH	6.9	7.6	8.1	8.6	9.2
	Mycelial weight (mg/ml)	1.98	2.64	3.28	2.85	2.55
D.	pH	6.9	7.6	8.1	8.8	9.2
	Mycelial weight (mg/ml)	1.98	2.69	3.15	2.85	2.33
E.	pH	6.9	7.8	8.8	9.3	9.3
	Mycelial weight (mg/ml)	1.98	2.15	1.99	1.57	1.40

A. Fermentation using 75% fresh medium (B) and 25% spent fermentation liquor.
B. " 50% " 50% "
C. " 25% " 75% "
D. " 0% " 100% "
E. Fermentation using concentrated (lyophilized) spent fermentation liquor.

Table 9. The production of gentamicin using varying concentrations of spent fermentation liquor.

(cf. Appendix table 22)

Fermentation time (hrs)	Gentamicin (µg/ml)				
	A.	B.	C.	D.	E.
0	7	12	15	19	26
24	14	14	18	25	32
48	27	24	26	34	37
72	45	38	39	39	36
96	31	27	27	30	25

A. Fermentations using 75% fresh medium (B) and 25% spent fermentation liquor.

B. Fermentations using 50% fresh medium (B) and 50% spent fermentation liquor.

C. Fermentations using 25% fresh medium (B) and 75% spent fermentation liquor.

D. Fermentations using 0% fresh medium (B) and 100% spent fermentation liquor.

E. Fermentations using concentrated (lyophilized) spent fermentation liquor.

Fermentation Patterns of M. Purpurea Using Spent Fermentation Liquor With and Without Glucose and Yeast Extract Fortification.

Fermentations using spent liquor, with and without glucose and yeast extract fortification, are compared in Table 10. It was observed that those fermentations which had glucose and yeast extract fortification exhibited higher maximum mycelial weights and showed smaller increments in the pH within any 24 hour period of fermentation. Maximum growth was reached at 72 hours; thereafter it decreased. Approximately 4.8 grams of glucose (45%) was utilized during the fermentation. Kjeldahl nitrogen decreased slowly through the fermentation and increased slightly after 72 hours.

Those spent liquor fermentations without glucose and yeast extract fortification showed smaller maximum mycelial weights; these occurred at 48 hours of fermentation. The pH increments within any 24 hour period of fermentation, especially after 48 hours, showed greater increases than those observed in spent liquor fermentations having glucose and yeast extract fortification. Approximately 3.0 grams of glucose (62.6%) were utilized during the fermentation. Kjeldahl nitrogen decreased slowly during the first 72 hours of fermentation, thereafter slightly increasing.

The Production of Gentamicin Using Spent Fermentation Liquor With and Without Glucose and Yeast Extract Fortification.

Gentamicin production, using spent liquor with glucose and yeast extract fortification, were compared to similar fermentations with no glucose or yeast extract fortification (Figure 14). Those spent liquor fermentations having glucose and yeast extract fortification showed slightly higher amounts of accumulated gentamicin when compared to similar fermentations without glucose or yeast extract fortification.

Table 10. Fermentation patterns of M. purpurea using spent fermentation liquor with and without glucose and yeast extract fortification.

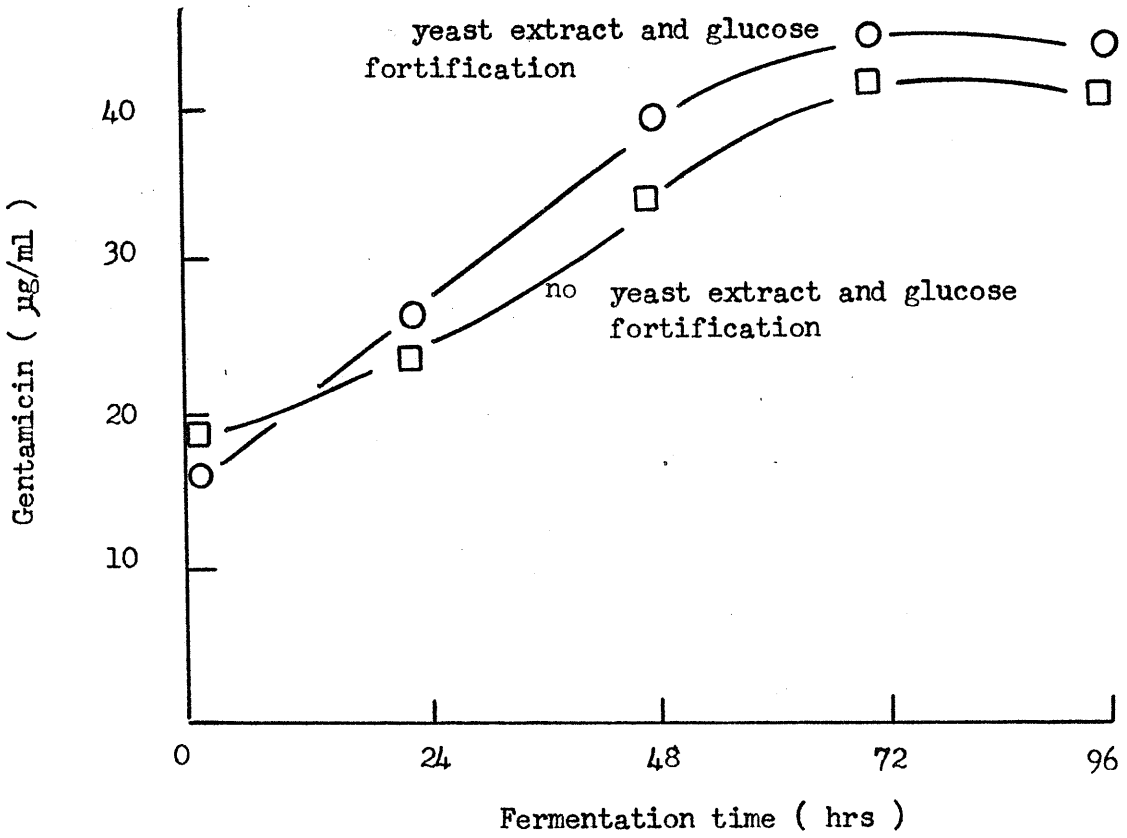
(cf. Appendix tables 23,24)

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)
		A.		
0	6.9	1.87	10.53	1.44
24	7.6	2.73	8.84	1.37
48	8.0	3.38	7.77	1.33
72	8.2	4.06	6.95	1.29
96	8.7	3.90	5.76	1.30
		B.		
0	6.9	1.84	4.90	0.87
24	7.5	2.62	3.51	0.80
48	8.0	3.63	2.23	0.75
72	8.6	3.16	1.95	0.71
96	9.3	2.56	1.83	0.72

A. Spent liquor fermentation with glucose and yeast extract fortification.
B. Spent liquor fermentation with no glucose and yeast extract fortification.

Figure 14. The production of gentamicin using spent fermentation liquor with and without glucose and yeast extract fortification.

(cf. Appendix table 25)



The maximum yields of gentamicin, in both types of fermentation, occurred at 72 hours. The net amount of gentamicin produced in fermentations with glucose and yeast extract fortification was 28.0 µg/ml; 22.0 µg/ml gentamicin was produced in those fermentations without glucose and yeast extract fortification.

Part II: Fermentor Fermentation Studies

Batch Fermentation Pattern of M. Purpurea in Medium (B).

Investigations using a 14-liter fermentor were initially carried out to determine the fermentation patterns exhibited by M. purpurea in medium (B) (Table 11). Maximum accumulation of gentamicin - 61.0 µg/ml - occurred at 120 hours of fermentation; the gentamicin accretion sharply increased after the first 24 hours of fermentation. The pH of the medium, initially adjusted to 7.0, showed a decrease for the first 24 hours; thereafter it increased gradually throughout the fermentation. Mycelial weight and glucose utilization progressively increased, although the most noticeable increase in mycelial weight and glucose utilization occurred during the first 24 hours of fermentation. Kjeldahl nitrogen decreased at a constant rate and increased slightly at 120 hours of fermentation. The maximum accumulation of gentamicin occurred during the time in which the mycelial weight decreased.

Batch Fermentation Pattern of M. Purpurea in Medium (B) with Automatic pH Control.

Fermentor studies, using fresh medium (B) with automatic pH control, indicated slightly lower values for accumulated gentamicin than those fermentor studies without pH control. Maximum yield of

Table 11. Batch fermentation pattern of M. purpurea in medium (B).

(cf. Appendix tables 26,27)

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	7.0	2.00	10.34	1.39	1.0
24	6.8	3.54	7.66	1.20	14.0
48	7.2	4.13	6.92	0.95	40.0
72	7.4	4.94	5.59	0.79	51.0
96	7.6	5.20	4.68	0.62	57.0
120	7.7	4.95	4.13	0.66	61.0

gentamicin - 57.0 µg/ml - occurred at 96 hours of fermentation. Mycelial weight was increased until 96 hours of fermentation; thereafter it decreased. The most noticeable growth occurred after 24 hours of fermentation. Glucose metabolism, however, was most noticeable during the first 24 hours of fermentation. Approximately 4.6 grams of glucose (45%) was utilized during this fermentation as compared to 6.2 grams (60%) of glucose utilized in those fermentor studies without pH control (Table 12).

Further studies in this investigation employed primary fermentations without automatic pH control using a fermentation period of 96 hours.

Batch Fermentation Pattern of M. Purpurea Employing Spent Fermentation Liquor.

Batch fermentations, employing spent fermentation liquor without pH control (Table 13), showed a maximum yield of 43.0 µg/ml gentamicin at 72 hours of fermentation. The net yield of gentamicin - 23.0 µg/ml - was accompanied by increases in the mycelial weight until 48 hours of fermentation; thereafter it decreased. A concomitant increase in the pH followed; it reached 8.3 at 72 hours of fermentation. Approximately 2.5 mg/ml of glucose was utilized during the fermentation; this represented approximately 51% of that residual glucose left from the primary fermentation. The pH of the fermentation medium increased as the fermentation progressed; it reached 9.0 at 96 hours of fermentation. Kjeldahl nitrogen was shown to decrease slowly, until 72 hours, thereafter it slightly increased. The Kjeldahl nitrogen increase was observed to coincide with a decrease in the mycelial weight.

A similar batch fermentation, using spent liquor with automatic

Table 12. Batch fermentation pattern of M. purpurea in medium (B) with automatic pH control.

(cf. Appendix tables 28,29)

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Gentamicin (µg/ml)
0	7.0	2.11	10.09	3
24	7.0	2.81	8.89	11
48	7.0	4.65	7.16	35
72	6.9	5.50	5.96	47
96	6.9	5.71	5.79	57
120	7.4 *	5.55	5.47	55

* pH was not maintained at this time period due to pH controller failure.

Table 13. Batch fermentation pattern of M. purpurea employing spent fermentation liquor.

(cf. Appendix tables 30,31)

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.18	4.97	0.90	20
24	7.4	3.31	4.14	0.92	23
48	7.8	4.45	3.23	0.75	35
72	8.3	4.08	2.74	0.56	43
96	9.0	3.11	2.44	0.62	41

pH control (Appendix Table 32), showed a maximum accumulation of 48.0 µg/ml gentamicin at 96 hours of fermentation, or, a net yield of 30.0 µg/ml gentamicin. Mycelial weight in this fermentation steadily increased until the termination of fermentation; approximately 3.0 mg/ml glucose was utlized, i.e., 58% of that residual glucose left from the primary fermentation.

Batch Fermentation Pattern of M. Purpurea Employing Spent Fermentation Liquor With Glucose and Yeast Extract Fortification.

Batch fermentations, employing spent fermentation liquor fortified with glucose and yeast extract, showed a maximum accumulation of gentamicin at 96 hours of fermentation (Table 14). Gentamicin yields obtained from spent liquor, fortified with glucose and yeast extract, and spent liquor fermentations without glucose and yeast extract fortification are compared in Figure 15. Higher yields of gentamicin were observed in those spent liquor fermentations not having glucose and yeast extract fortification. The net yield of gentamicin (initial gentamicin - gentamicin yield at each time period) was higher in those fermentations having no nutrient fortification for all time periods tested during the fermentation.

Batch fermentations, using glucose and yeast fortification (Table 14), indicated increasing pH and mycelial weight throughout the fermentation. Kjeldahl nitrogen decreased progressively through the fermentation.

Fermentation Pattern of M. Purpurea Using Medium (B) in a Continuous Culture Process.

Continuous fermentation of fresh sterile medium (B) in a 14-liter fermentor, at an infusion rate of 5.0 ml medium/minute ($D = 0.0416\ hr^{-1}$),

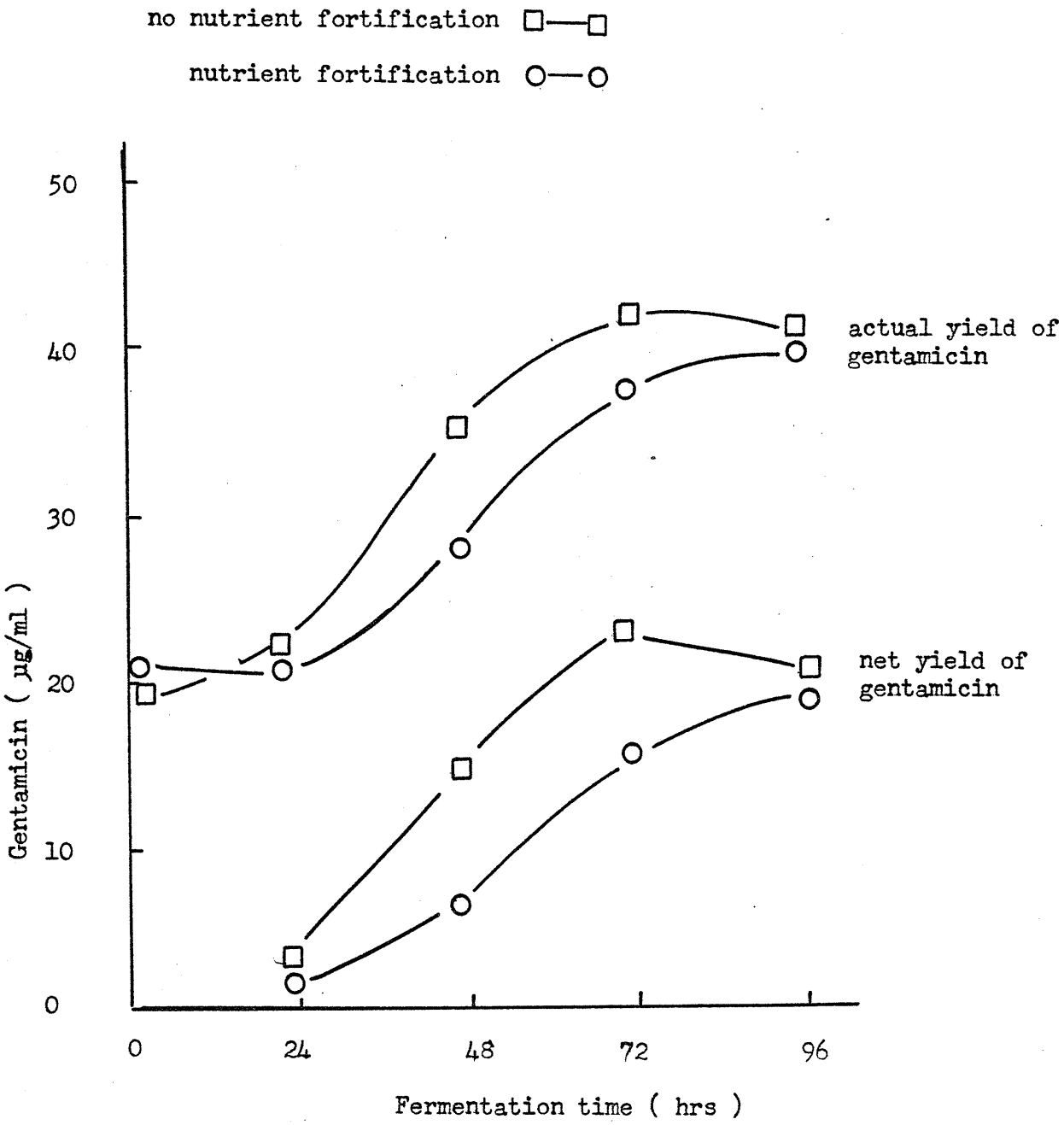
Table 14. Batch fermentation pattern of M. purpurea employing spent fermentation liquor with glucose and yeast extract fortification.

(cf. Appendix tables 33,34)

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.34	9.28	1.24	21
24	7.2	2.96	8.42	1.21	21
48	7.7	4.22	7.12	1.06	28
72	8.3	4.55	6.28	0.89	37
96	8.9	4.66	5.94	0.82	40

Figure 15. Batch fermentation pattern of M. purpurea employing spent fermentation liquor with and without glucose and yeast extract fortification.

(cf. Appendix tables 30,31,33,34)



was begun after 96 hours of primary fermentation. It was observed that the initial level of gentamicin at the outset of continuous fermentation - 58.0 µg/ml - immediately decreased as the continuous fermentation progressed. Gentamicin was observed to decrease continually for the first 30 hours of continuous fermentation; thereafter it increased slightly. The initial value of gentamicin at the commencement of continuous fermentation was never reached again during continuous fermentation. Mycelial weight decreased for the first 6 hours of continuous fermentation; thereafter it rapidly increased for 24 hours. Following the first 30 hour period, the mycelial weight decreased progressively until the termination of the continuous fermentation. Residual glucose accumulated steadily and showed maximum utilization during the period of peak mycelial weight. The pH of the fermentation medium was maintained at a pH of 7.2 to 7.6 by the infusion of fresh medium (B) which had a pH of 7.0.

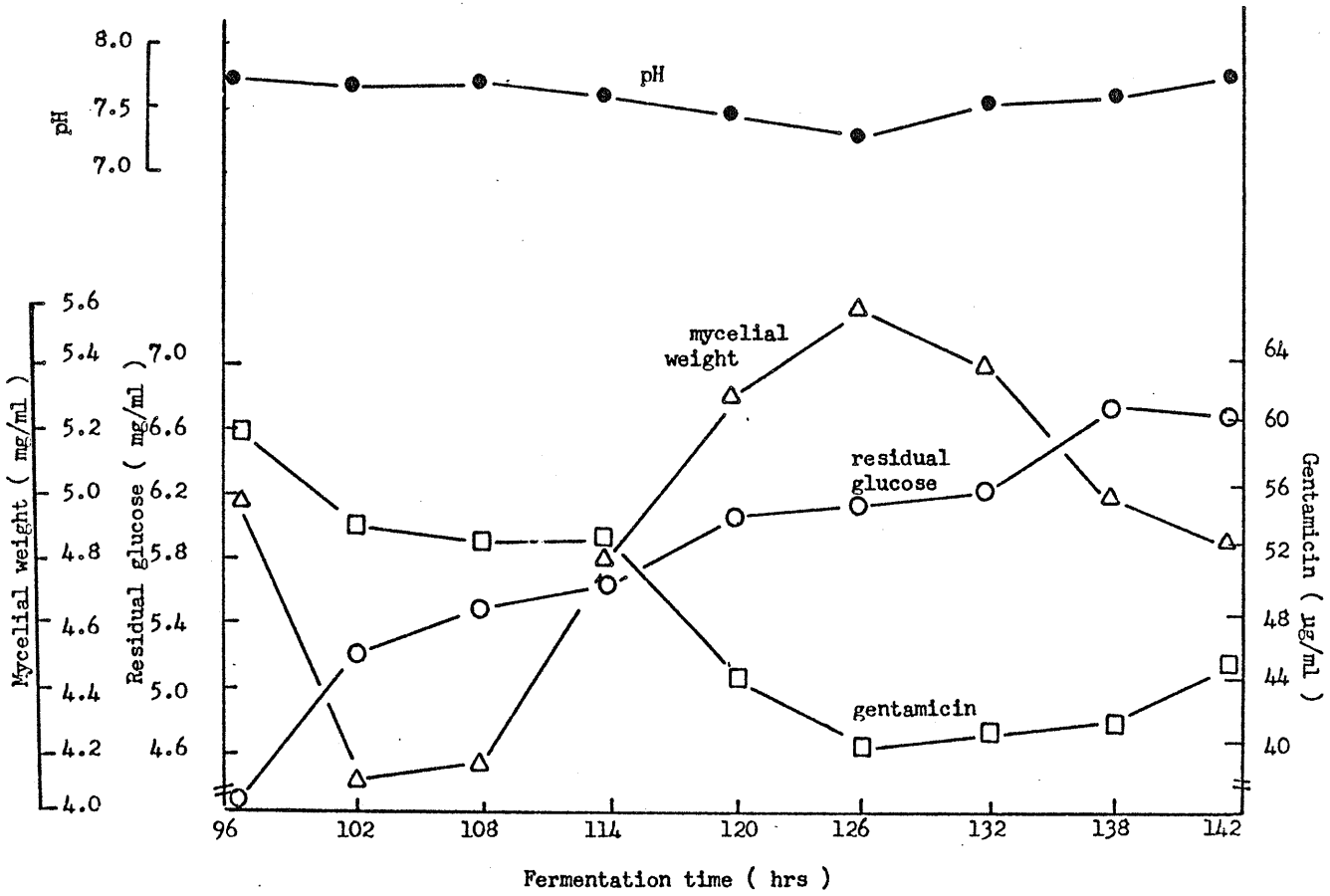
The data indicated that the lowest accumulation of gentamicin occurred at 30 hours of continuous fermentation; this corresponded to maximum mycelial weight (Figure 16).

Continuous Fermentation Method A and B Using Spent Fermentation Liquor with Automatic pH Control.

Gentamicin production, using spent fermentation liquor in a continuous culture, method A, indicated decreasing levels of gentamicin once the continuous fermentation process was initiated. The accumulated levels of gentamicin from the batch fermentation of spent liquor at 72 hours were shown to decrease steadily (Appendix Table 37). The data indicated that approximately 88% of the gentamicin which had accumulated at 72 hours of batch fermentation was lost during continuous spent

Figure 16. Fermentation pattern of M. purpurea using medium (B) in a continuous culture process.

(cf. Appendix tables 35,36)



liquor fermentation.

Gentamicin accumulation, using continuous culture method B, showed minimal accumulation of gentamicin after 30 hours of continuous fermentation (Appendix Table 38).

Comparison of the Various Batch and Continuous Culture Processes.

The various types of batch fermentations performed in this investigation are summarized in Table 15. Primary batch fermentations which had no pH control gave the highest yields of gentamicin at 120 hours of fermentation. Primary batch fermentations, employing automatic pH control, showed slightly lower maximum yields of gentamicin. These maximum yields normally occurred at 96 hours of fermentation. Approximately 60% of the initial glucose was utilized during the time taken for maximum gentamicin accumulation in those primary batch fermentations without automatic pH control. Approximately 46% of the initial glucose was utilized by batch fermentations with automatic pH control during the time taken for maximum gentamicin accumulation.

When compared to primary fermentations, batch fermentations using spent liquor showed lower values of net gentamicin accumulation regardless of the fermentation method. However, those batch fermentations using spent liquor with automatic pH control were proven to be best suited for gentamicin accumulation. In addition, this type of fermentation displayed the greatest capacity for the utilization of residual glucose from primary fermentations. Fortified spent liquor fermentations showed the poorest accumulation of gentamicin. These types of fermentations also showed poor utilization of glucose.

Figure 17 illustrates the yields of gentamicin accumulated by

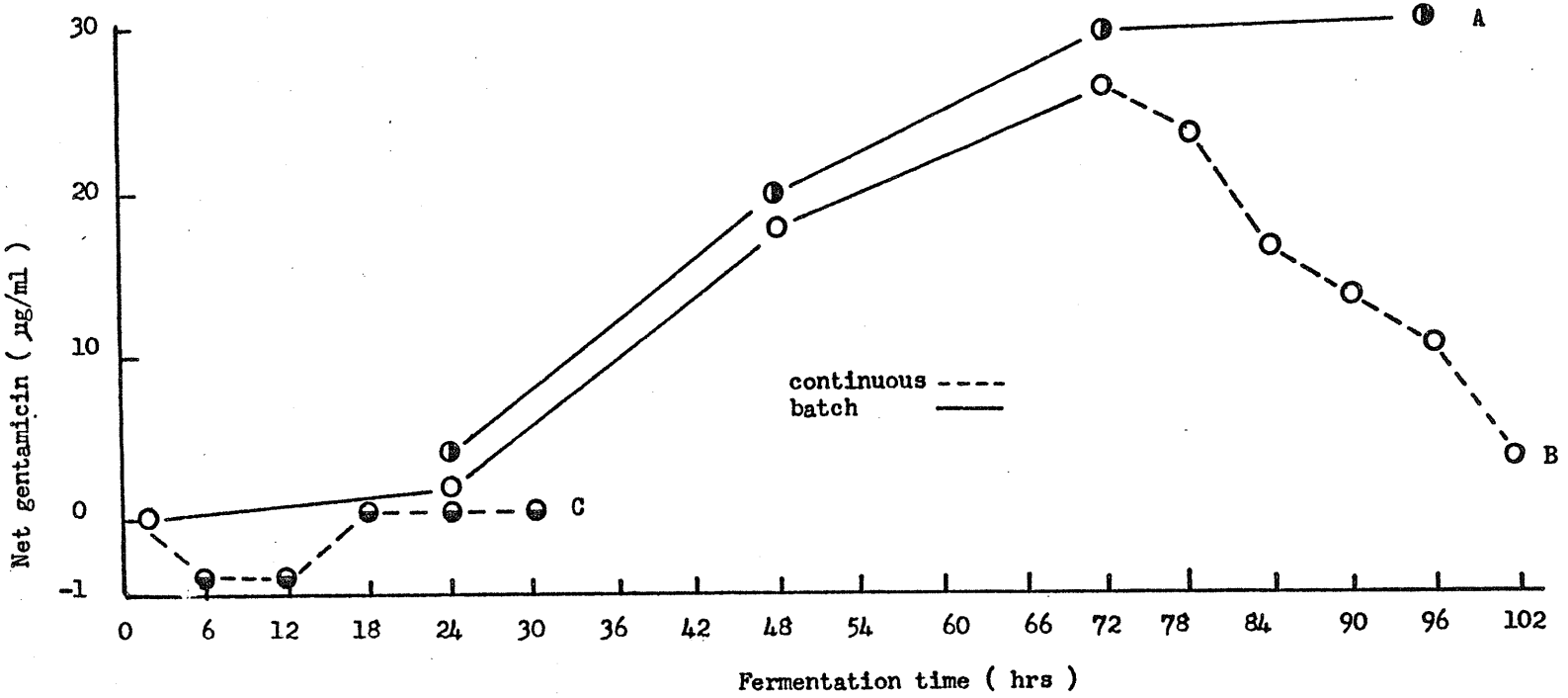
Table 15, Comparison of the various batch type of fermentation processes.

Type of medium	Fermentation volume (liters)	*Fermentation time (hrs)	*pH	*Glucose utilized (mg/ml)	*% Glucose utilized	*Total gentamicin (µg/ml)	*Net gentamicin (µg/ml)	Reference table
Primary batch fermentation.								
medium (B) pH not controlled	7.0	120	7.7	6.21	60	61	60	11
medium (B) pH controlled	7.0	96	6.9	4.62	46	57	54	12
Spent liquor batch fermentation.								
spent liquor pH not controlled	5.0	72	8.3	2.53	51	43	23	13
spent liquor pH controlled	5.0	96	7.1	2.99	58	48	30	appendix 32
** fortified spent liquor pH not controlled	5.0	96	8.9	3.34	36	40	19	14

* Refers to conditions at the time of maximum accumulation of gentamicin .
** Refers to glucose and yeast extract fortification.

Figure 17. Comparison of batch and continuous fermentation processes using spent fermentation liquor for the production of gentamicin.

(cf. Appendix tables 32,37,38)



A. Batch fermentation with automatic pH control.
B. Continuous system A.
C. Continuous system B.

using spent liquor in batch and continuous fermentation processes. Batch fermentation of spent liquor was shown to be far superior - in so far as gentamicin accumulation was concerned - than either method used for continuous fermentation of spent liquor.

A comparison of the various batch type of fermentation combinations is presented in Table 16. The data indicated that two primary fermentations without automatic pH control would give the maximum amounts of gentamicin. Two primary fermentations with automatic pH control would yield slightly lower amounts of gentamicin. Combination fermentations using spent liquor with and without automatic pH control indicated similar total yields of gentamicin. The percent total glucose utilized during fermentation, with the former type of fermentation combination, however, was more efficient.

Table 14. Comparison of the various batch type of fermentation combinations: controlled and uncontrolled pH.

Automatic pH controlled batch fermentations.

Fermentation combinations	Total volume (liters)	Actual volume (liters)	*Total time of fermentation (hrs)	*Total glucose utilized (mg/ml)	*% Total glucose utilized	*Total net gentamicin (µg/ml)
primary fermentation	7.0		96	4.62		54.0
+						
spent liquor fermentation	5.0	7.0	96	2.99	77.4	30.0
primary fermentation	7.0		96	4.62		54.0
+						
primary fermentation	7.0	14.0	96	4.62	46.9	54.0
Uncontrolled pH batch fermentations.						
primary fermentation	7.0		120	6.21		60.0
+						
spent liquor fermentation	5.0	7.0	72	2.53	84.5	23.0
primary fermentation	7.0		120	6.21		60
+						
primary fermentation	7.0	14.0	120	6.21	60.0	60

* Refers to conditions at the time of maximum accumulation of gentamicin.

DISCUSSION

Vegetative growth patterns of M. purpurea in a recommended medium indicated very little accumulation of gentamicin; no gentamicin appeared until 48 hours of growth. This lag in gentamicin production by the vegetative organism may indicate that the accumulation of gentamicin is a property of the mature organism. By contrast, the fermentative growth of the organism showed a relatively high accumulation of gentamicin - 44.0 µg/ml - after 96 hours of fermentation. The gentamicin level noticed at the commencement of fermentative growth may be attributed to the bound gentamicin fraction of the inoculum since no other sources for gentamicin were introduced into the fermentation medium. The inoculum was also washed with sterile saline solution so that gentamicin carry over from the vegetative stage was rendered impossible. The pH of the fermentation, initially adjusted to 6.8, gradually increased as the fermentation progressed, reaching 7.8 - 7.9 at 96 hours. Mycelial weight increased throughout the fermentation without any significant accretion taking place; glucose was utilized slowly with approximately 62% remaining at the end of the fermentation.

Extending the time of fermentation to 144 hours showed only minimal increases in gentamicin accumulation after 96 hours. Abou-Zeid et al. (1974) indicated that maximum gentamicin potencies were reached within 96 hours; thereafter they decreased.

Shake-flask fermentations, employing various inoculum levels, indicated that a 6% (v/v) inoculum was optimum for the production of gentamicin. Inocula of 8 and 10% showed a loss of growth at 72 and 48 hours, respectively. The observed loss of growth, due to cellular

autolysis, may have been caused by substrate exhaustion, neither residual glucose nor nitrogen was tested, however. Toxic effects exhibited by the accumulation of gentamicin were unlikely since similar levels of gentamicin were shown not to be toxic with the smaller levels of gentamicin were shown not to be toxic with the smaller inoculum levels. Since the pH of the medium was not monitored, it is not known whether this was also a causal factor for the cellular autolysis. Senescence in the inocula was not observed to be a factor for this autolysis since a loss of growth was not noticed with the smaller inoculum levels. Although all the flasks were placed on a rotary shaker, oxygen diffusion into those flasks containing the larger inocula could have been insufficient in providing adequate respiration for all the mycelia. Again, respiration rates were not monitored; thus it is not known exactly if an inadequate respiration rate was also a factor causing autolysis.

Shake-flask fermentations, using spent fermentations liquor, showed a maximum accumulation of gentamicin after 72 hours of fermentation. The net yield of gentamicin - 26.0 μg/ml - represented an additional 67.5% of the amount of gentamicin produced by using spent fermentation liquor. In addition, 67.7% of the residual glucose from the primary fermentation was utilized for growth and gentamicin production. The combined primary and spent liquor fermentations produced a total net gentamicin yield of 64.5 μg/ml with an appropriate total glucose utilization of 80%. Spent liquor fermentations were observed to attain a much higher degree of alkalinity than primary fermentations. It appeared that a pH of 8.8 to 9.0 was critical in spent liquor fermentations; gentamicin yields began to decrease past that point, with a concomitant decrease in the mycelial weight.

Glucose exhaustion did not appear to be a factor in these declines.

The distribution of free and bound gentamicin in fermentations using spent fermentation liquor indicated a relatively high amount of free gentamicin in the broth at the commencement of the fermentation. This high level of free gentamicin was undoubtedly caused by the free gentamicin produced during the primary fermentation. The low level of bound gentamicin, observed at the start of fermentation, was caused by that fraction of gentamicin contained within the mycelia of the inoculum. Approximately 29% of the gentamicin produced after 72 hours of fermentation existed in the broth or free state. At 96 hours, the concentration of free gentamicin rose to approximately 65%, because of mycelial autolysis which occurred at that time. Reiblein et al. (1973) reported that approximately 40% of the total gentamicin produced by M. purpurea could be found in the broth or in the free state; the remainder of the gentamicin was contained within the growing mycelia. The results obtained in spent liquor fermentations were comparable with the distribution of gentamicin in primary fermentations. Any discrepancy of bound and free gentamicin in the spent liquor fermentations can be partially explained by the initial high level of free gentamicin obtained from the primary fermentation liquor. To a lesser extent, mycelial autolysis would also increase the concentration of free gentamicin in the fermentation broth.

Supplementing spent liquor fermentations with cobalt, in the form of cobalt chloride, was shown to be deleterious for gentamicin accumulation. The highest accumulation of gentamicin occurred when spent liquor fermentations had no cobalt supplementation. Charney (1964) indicated that cobalt was not a requisite for the growth of M. purpurea

but rather was a requisite for the increased production of gentamicin. It has been suggested by Charney (1964) that cobalt stimulates the activity of an enzyme which is involved in the biosynthetic pathway of gentamicin. Several enzymes are known to require cobalt as a co-factor; in addition, many microorganisms also require cobalt as a trace element. The optimum concentration of cobalt required by these organisms, however, was dependent upon the initial concentration of cobalt present in the growth medium. Synthetic media normally required less cobalt than did chemically defined media, for the same microorganism.

Supplementing spent liquor fermentations with 0.5 grams per liter calcium carbonate increased gentamicin production. Levels higher than 0.5 grams per liter subsequently decreased gentamicin accumulation. The method used to recover spent fermentation liquor from the primary fermentation involved centrifugation and Millipore filtration; therefore, it would appear that a certain amount of calcium carbonate was removed by virtue of its low solubility properties. Supplementing fermentations using spent liquor would then partially off-set some of that portion of calcium carbonate lost in the preparation of spent liquor. The addition of calcium carbonate did not significantly alter the pH of the fermentation.

Readjusting the pH of spent liquor fermentations to their respective initial pH values every 24 hours indicated that a pH of 6.5 to 7.0 was the optimum for gentamicin accumulation. Spent liquor fermentations initially adjusted to a pH of 7.0, with no pH readjustments, showed slightly lower accumulations of maximum gentamicin levels. The maximum accumulation of gentamicin in spent liquor fermentations which

had no pH readjustments occurred at 72 hours, while those spent liquor fermentations having pH readjustments, showed the maximum accumulation of gentamicin at 96 hours. This manual adjustment of pH every 24 hours was not totally satisfactory and the effect of automatic pH control was further investigated in the latter fermentor studies.

The exact effect of pH on the production of gentamicin is not known. Studies carried out in this investigation, as well as those by Abou-Zeid (1974), indicate that an initial pH of 7.0 to 7.5 was favorable for gentamicin production using fresh medium B. Primary fermentations in this investigation indicated no decreases in the production of gentamicin because of extreme alkaline pH. Those fermentations using spent liquor, however, invariably showed higher alkaline pH values. As indicated earlier, a pH of 8.8 to 9.0 caused both the gentamicin accumulation and the mycelial weight to decrease. It would appear that the high alkaline pH exhibited in spent liquor fermentations provided for sub-optimal growth conditions. Whether the alkaline pH also affected the accumulated gentamicin *per se*, is not known, although Luedemann *et al*. (1963) indicated that gentamicin was stable throughout a pH range from 2 to 12. Other antibiotics have been shown to be affected by pH fluctuations in the growth media. Hoeksema and Smith (1961) have shown that at a pH of 7.5 novobiocin, produced by *Streptomycetes spheroides*, enters the growing cells and produces a toxic effect, while at pH values of 8.5 this phenomenon was not noticed. Hockenhull (1963) has indicated that in the fermentation production of penicillin, the development of pH values over 7.5, especially in the presence of the ammonium ion, quickly lead to the destruction of the antibiotic.

The alkaline pH exhibited in spent liquor fermentations may partially

result from autolytic liberation of ammonia. Ammonium nitrogen was not analyzed in this study. However, alkaline pH caused by ammonium nitrogen has been reported by several researchers including Dulaney and Perlman (1947) in their studies with Streptomyces griseus for the fermentative production of streptomycin.

Whether the accumulation of gentamicin affected the pH is not known. In primary fermentations, the accumulation of gentamicin showed no signs of increasing the pH to the extent that was observed in spent liquor fermentations. Gentamicin, however, is considered a relatively strong basic compound. Spent liquor fermentations were shown to contain a higher concentration of free gentamicin, therefore it may be likely that in these spent liquor fermentations, gentamicin did affect the pH. In addition, the relative simplicity of the growth medium would not afford much buffering capacity.

Varying concentrations of gentamicin sulfate, added to fermentations containing medium (B), indicated no effect on the growth of M. purpurea. The addition of 20.0 µg/ml gentamicin sulfate to fermentations employing medium (B) did, however, cause the net production of gentamicin to decrease. In addition, the amount of gentamicin determined at 24 hours of fermentation, was lower than the amount of gentamicin determined at the start of fermentation. After 24 hours the gentamicin production increased. The fact that 20.0 µg/ml gentamicin sulfate had no effect upon the growth of the organism, but rather on the production of gentamicin, may possibly suggest some type of feedback control mechanism.

Flask fermentations employing various concentrations of spent liquor indicated a progressive decrease in the maximum growth of

M. purpurea as the concentration of spent liquor was increased. A concomitant increase in the pH of the medium also resulted as the concentration of spent liquor was increased. Gentamicin production was observed to be inversely proportional to the initial concentration of spent fermentation liquor. Since the addition of gentamicin sulfate to medium (B), in previous studies, did not significantly alter the growth of M. purpurea, it would seem likely that the free gentamicin portion contained within the spent liquor would also not affect the growth of M. purpurea. Increasing the concentration of spent fermentation liquor did, however, cause greater increases in alkaline pH. The initial concentration of free gentamicin in the spent liquor may have exhibited a collaborative effect with the alkaline pH, i.e., the free gentamicin in the fermentation liquor may have caused gentamicin accumulations to decrease, as was shown with fresh broth, while the alkaline pH may have caused cellular growth to decrease, as was observed in previous studies.

Spent liquor fermentations fortified with glucose and yeast extract were shown to favor growth and gentamicin production when compared to similar fermentations having no nutrient fortification. The data indicated that a net maximum production of 29.0 µg/ml gentamicin resulted with nutrient fortification, as opposed to a net maximum production of 23.0 µg/ml gentamicin produced without nutrient fortification. Approximately 59% of the residual, primary fermentation glucose was utilized in those fermentations without nutrient fortification. Approximately 45% glucose was utilized in those fermentations which had glucose fortification. Fortifying spent liquor fermentations, at least with glucose and yeast extract, indicated little nutritional

benefits since both glucose and Kjeldahl nitrogen were not limiting factors in the fermentations which had no nutrient fortification. The fact that glucose was more efficiently utilized by spent liquor fermentations which had no glucose fortification indicated that the fortification offered only little advantage in increasing the yields of gentamicin. It was observed, however, that those spent liquor fermentations which had nutrient fortification showed smaller increases in pH, especially after 48 hours of fermentation. This resulted in the increase in gentamicin accumulation. It is not known exactly how the addition of glucose or yeast extract affected the pH differences between fortified and non-fortified spent liquor fermentations. The acid-base properties of yeast extract, i.e. amino acid and peptides, could conceivably offer some type of buffering capacity to the medium. The main species of amino acids found in yeast extract i.e., aspartic, glutamic, leucine, lysine and alanine, however, do not show significant buffering capacity at pH 8.0 (Bridson and Brecker, 1970).

Fermentations using a 14-liter fermentor without pH control gave a maximum accumulation of 60.0 µg/ml gentamicin at 120 hours of fermentation when fresh medium (B) was used. The greatest accretion of gentamicin was noted at 48 hours of fermentation; thereafter it increased at a steady rate until the termination of the fermentation. Glucose and Kjeldahl nitrogen decreased progressively; Kjeldahl nitrogen increased slightly at 120 hours. This was presumably because of the cellular autolysis which was indicated by the loss in mycelial weight. The pH of the fermentation, initially adjusted to 7.0, showed a slight decrease at 24 hours; thereafter it continually increased.

The decrease in pH at 24 hours was not typical in any shake-flask fermentation performed in this study and may have been caused by the temporary accumulation of organic acids brought about by proliferous growth. Approximately 60% of the initial glucose was utilized during the fermentation. Studies conducted throughout this investigation have shown that mycelial growth and gentamicin activity were unrelated, although the increasing mycelial weight in any one fermentation study usually correlated with increasing levels of accumulated gentamicin. The decline in mycelial weight, however, did not always bring about a concomitant decrease in gentamicin levels. In many instances, a decline in mycelial weight still exhibited increasing values for gentamicin. Since the total amount of gentamicin was monitored, increases in gentamicin production could not be attributed to gentamicin released through autolysis. Gentamicin production perhaps could continue through senescence. Legator and Gottlieb (1953), in their studies with chloramphenicol, found that peak antibiotic production occurred 48 hours after maximum growth was attained. Dulaney and Perlman (1947) also reported that streptomycin production continued to increase despite a decline in the mycelial weight. Similar findings have been reported for other antibiotics including penicillin (Brown and Peterson, 1950).

Fermentor studies using automatic pH control indicated that the maximum yield of gentamicin accumulated was slightly lower than in those fermentor studies having no pH control. Gentamicin accumulation in the former case occurred at 96 hours of fermentation when the pH was regulated to 7.0. The maximum mycelial weight, however, was higher than similar fermentor studies without pH control. The results of these

fermentor studies using fresh media indicate that an initial pH of 7.0 is optimum for mycelial growth but that a slightly higher pH is indicated for optimum gentamicin accumulation. Since the normal pH range for uncontrolled pH fermentations is from 7.0 to approximately 7.7, it would appear that a pH of 7.4 to 7.5 would be the optimum for gentamicin accumulation. The higher pH value required for the optimum gentamicin production could slow down mycelial growth and allow more suitable allocation of nutrients toward gentamicin biosynthesis rather than mycelial biosynthesis.

Batch fermentor fermentations using spent liquor without pH control produced a net yield of 23.0 µg/ml gentamicin at 72 hours of fermentation; this was comparable to the net gentamicin yields produced in shake-flask fermentations using spent liquor. A similar pH increase was observed. The initial pH of the fermentation was adjusted to 7.0, and it was shown to increase steadily as the fermentation progressed, reaching a final pH of 9.0 at 96 hours. The increase in pH, within any 24 hour period, was shown to be much higher than those pH increases observed in similar fermentations using fresh medium. Once the pH of the spent liquor increased past 9.0, decreases in mycelial weight and gentamicin accumulation were observed.

Fermentor fermentations using spent liquor with automatic pH control adjusted to 7.0, showed maximum accumulation of gentamicin - 30.0 µg/ml - after 72 to 96 hours. No decreases in mycelial weight were observed in this fermentation. Approximately 58% of the residual glucose from the primary fermentation was used. Similar fermentor fermentations without automatic pH control, used approximately 51%

of the residual glucose from the primary fermentation. Maintaining a constant pH in spent liquor fermentations was found to be advantageous for increased production of gentamicin; the efficiency of glucose utilized was also improved. The absence of a high alkaline pH, as observed in those spent liquor fermentations with automatic pH control, definitely stimulated gentamicin production and mycelial growth. The presence of a high alkaline pH, as observed in spent liquor fermentations without automatic pH control, is therefore definitely deleterious for maximum gentamicin production and deferred mycelial autolysis.

Fortifying spent liquor fermentations, with glucose and yeast extract without automatic pH control gave slightly lower values for accumulated gentamicin than did similar fermentations without nutrient fortification. Approximately 36% glucose was utilized in these fermentations as compared to 51% utilized by similar fermentations without fortification. Both types of fermentations showed similar pH ranges during the fermentation period; no buffering of the pH was observed by fortification as was the case in the shake-flask fermentations. The lack of buffering capacity in these fermentations can perhaps be attributed to the more vigorous growth of the organism than that observed in shake-flask fermentations. Since nutrient fortification of spent liquor only slightly increased the levels of accumulated gentamicin, with a less efficient utilization of glucose, it was decided that any further studies involving nutrient fortification should be omitted.

Final studies in this investigation centered on continuous culture processes using both medium (B) and spent liquor. The aim of this

particular study was not to run a continuous fermentation *per se*, but to compare the yields of gentamicin accumulated and the amounts of glucose utilized with batch fermentations employing the same media.

Initial studies, performed with medium (B), indicated that the continuous fermentation produced far less amounts of gentamicin than did batch fermentations. The yields of gentamicin decreased throughout most of the continuous fermentation, showing only a slight increase after 126 hours. The initial level of gentamicin - 63.0 μg/ml - obtained at 96 hours of primary batch fermentation was never maintained during the continuous fermentation. The growth of *M. purpurea*, however, through most of the continuous fermentation, did maintain its initial level. Glucose accumulated gradually as the continuous fermentation progressed. The uneconomic utilization of glucose and the loss of gentamicin in the harvested broth indicated that a continuous run fermentation was not as practical as a batch fermentation, at least within the parameters of this investigation. Since mycelial growth and gentamicin production are unrelated, other parameters under investigation should include methods of increasing the gentamicin level *per se*. Only one dilution rate, $D = 0.0416\ hr^{-1}$, was used, and this corresponds to complete medium exchange within 24 hours. A faster exchange rate was considered wasteful with respect to glucose utilization, while a smaller exchange rate was not possible with the equipment available. Although the Kjeldahl nitrogen was not monitored in the fermentation, a rapid supply of nitrogenous substances, offered by a high medium exchange, could conceivably favor protein synthesis and new cell formation; thus the supply of carbohydrate

would be directed to the formation and subsistence of mature mycelia.

Continuous spent liquor fermentations were also shown to be disadvantageous for the accumulation of gentamicin, at least within the parameters of this study. Method A exhibited a steady decline in gentamicin accumulation when the continuous process was initiated. Approximately 88% of the gentamicin found at the start of the continuous process was lost during the remainder of the continuous fermentation. In method B the yields of gentamicin accumulated were minimal and far inferior to method A.

Examining the data obtained from the various batch-type of fermentations, it was observed that primary fermentations using fresh medium (B) accumulated the highest amounts of gentamicin after 120 hours of fermentation without automatic pH control. Spent liquor fermentations, on the other hand, required automatic pH control in order to achieve maximum gentamicin yields; these occurred after 96 hours of fermentation. The use of spent fermentation liquor obtained from primary gentamicin fermentations resulted in an additional 30.0 µg/ml gentamicin. Whether the extra amount of gentamicin produced through the use of spent liquor is warranted will depend upon the relative cost and time required to achieve this additional gentamicin when compared to the relative cost required to run another primary fermentation. If a comparison were made among the total volumes of fermentation broth used, the efficiency of glucose utilized and the total gentamicin produced, it would appear that two primary fermentations run simultaneously, or consecutively, would be superior to a primary fermentation followed by a spent liquor fermentation, or both run simultaneously. The two primary fermentations would be

superior insofar as to the total amount of gentamicin they produced; however, the advantages of reduced fermentation volumes and increased glucose utilization in relation to the total cost of the fermentation operation, can be evaluated only after precise cost figures are determined.

SUMMARY

Preliminary studies using Micromonospora purpurea in a spent fermentation liquor medium showed that sufficient amounts of gentamicin were accumulated to warrant additional studies. Shake-flask fermentations established that calcium carbonate, but not cobalt, was essential in obtaining maximum yields of gentamicin in spent liquor. In addition, it was established that the control of pH from 7.0 to 7.2, by means of automatic pH regulators, enhanced the accumulation of gentamicin.

The accumulation of gentamicin and the growth of M. purpurea was shown to be inversely proportional to the initial concentration of the spent liquor used in the fermentation. An increase in pH toward the alkaline side apppeared to be one factor causing decreased amounts of accumulated gentamicin. This phenomenon also affected the growth of the organism. pH values of 8.8 or higher, in spent liquor fermentations, decreased the accumulation of gentamicin. Supplementing glucose and yeast extract into spent liquor fermentations afforded no great advantage in increasing the levels of gentamicin.

Studies using a continuous fermentation process showed this method to be ineffective in achieving a constant production of gentamicin regardless of the type of medium used. Batch fermentations were found to be the superior mode of fermentation. Primary fermentations without pH control followed by spent liquor fermentations with pH control gave the best cumulative amounts of gentamicin.

CONTRIBUTIONS TO KNOWLEDGE

The major contributions to knowledge made by this study are:

1. Gentamicin, a wide-spectrum antibiotic, was produced in a submerged fermentation process utilizing spent liquor obtained from primary gentamicin fermentations.
2. Conditions of spent liquor fermentation established that calcium carbonate, but not cobalt, was required as a necessary supplementary agent. Automatic pH control from 7.0 to 7.2 was necessary in order to realize maximum yields of gentamicin in spent liquor fermentation. It was also shown that spent liquor, depending upon its concentration, exhibited inhibitory effects toward the growth of M. purpurea and toward its ability to produce gentamicin.
3. Primary batch fermenations exhibited the highest accumulation of gentamicin; continuous fermentation processes using both fresh and spent broth were unsatisfactory.
4. Primary batch fermentations without pH control, followed by batch spent liquor fermentations with pH control gave the highest cumulative yields of gentamicin.

RECOMMENDATIONS FOR FUTURE INVESTIGATIONS

Possible Research Areas:

1. To study the growth requirements necessary to achieve maximum production of gentamicin in spent liquor fermentations.
2. To study the inhibition parameters governing the growth of M. purpurea and its ability to produce gentamicin both in primary and spent liquor fermentations.
3. To investigate the use of physico-chemical methods as a means of eliminating the build-up of toxic compounds.
4. To investigate a cyclical fermentation process using spent liquor.

BIBLIOGRAPHY

ABOU-ZEID, A., EISSA, M. and HASSAN, M. 1976. Influence of some compounds on gentamicin formation by Micromonospora purpurea. J. Appl. Chem. Biotech. 26:318.

ABOU-ZEID, A., EISSA, A.I. and SALEM, H.M. 1974. The fermentative production of gentamicin by Micromonospora purpurea. J. Appl. Chem. Biotech. 24:655.

A.O.A.C. 1965. Official Methods of Analysis (10th ed.). Association of Official Agricultural Chemists, Washington, D.C.

BARBER, M. and GARROD, L. 1963. Neomycin, kanamycin and paromycin. In Antibiotic and Chemotherapy. E. and S. Livingstone Ltd., London.

BERDY, J. 1961. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv. Appl. Microbiol. 18:309.

BRIDSON, E.Y. and BRECKER, A. 1970. Design and formulation of microbiological media. In Methods in Microbiology. Vol. iii. (J.R. Norris and D.W. Ribbons, eds.). Academic Press, New York.

BROWN, W.E. and PETERSON, W.H. 1950. Factors affecting production of penicillin in semi-pilot plant equipment. Ind. Eng. Chem. 42:1769.

CHARNEY, W. 1964. Manufacture of gentamicin. U.S. Pat. No. 3,136,704.

COOPER, D., MARIGLIANO. H.M., YUDIS, M.D. and TRAUBEL, T. 1969. Recent developments in the chemistry of gentamicin. J. Infec. Dis. 119:342.

CROWE, C.C. and SANDERS, E. 1974. Rosamicin: evaluation in vitro and comparison with erthromycin and lincomycin. Antimicrob. Agents Chemother. 5:272.

CROWE, C.C. and SANDERS, E. 1973. Sisomicin: evaluation in vitro and comparison with gentamicin and tobramycin. Antimicrob. Agents Chemother. 3:24.

DAVIES, J.D. 1971. Second international symposium on gentamicin: Chemistry and Microbiology. J. Infec. Dis. 124:S-7.

DAVIES, J.D., BENVENISTE, R., KVITEK, K., OZANNE, B. and YAMADA, T. 1969. Aminoglycosides: biological effects of molecular manipulation J. Infec. Dis. 119:351.

DAVIES, J.D. 1967. Structure-activity relationships among the aminoglycoside antibiotics. Antimicro. Agents Chemother.-1967, 297.

DULANEY, E.L. and PERLMAN, D. 1947. Antibiotic mechanism. Bull. Torrey Bot. Club. 74:504.

FRANKLIN, T.J. and SNOW, G.A. 1971. Suppression of gene function 2. In Biochemistry of Antimicrobial Action. Academic Press, New York.

FRIED, H.M. and STOCKTON, D.L. 1973. Pollution change. In National Conference on Complete Waste Reuse. American Institute of Chemical Engineers.

GALE, E.F., CUNDLIFFE, E., REYNOLDS, P.E., RICHMOND, M.H. and WARING, M.J. 1972. Bacterial resistance to antibiotics. In The Molecular Basis of Antibiotic Action. John Wiley and Sons Inc., New York.

GALMARINI, O.L., and DEVOFEU, V. 1961. Curamycin, isolation and characterization of some hydrolysis products. Tetrahedron. 16:76.

GARROD, P.L., LAMBERT, H.P. and O'GRADY, F. 1973. Other aminoglycosides. In Antibiotic and Chemotherapy. E. and S. Livingstone Ltd, London.

GOTTLIEB, D. and SHAW, P. 1967. Antibiotics Vol. 1. Antibiotics, Mechanism of Action. Springer-Verlag, New York.

GROVE, D.C. and RANDALL, W.A. 1955. Assay methods of antibiotics. Antibiotic Monograph no. 2. Medical Encyclopedia, New York.

HAHN, F.E., JAMES, E. and SARNE, G.S. 1969. Mechanism of action of gentamicin. J. Infec, Dis. 119:364.

HAYES, W. 1965. Sex factors and episomes. In The Genetics of Bacteria and Their Viruses (3rd ed.). John Wiley and Sons Ltd., New York.

HOCKENHULL, D.J.D. 1963. Antibiotics. In Biochemistry of Industrial Microorganisms (C. Rainbow and A.H. Rose, eds.). Academic press, New York.

HOEKSEMA, H. and SMITH, C.G. 1961. Novobiocin biosynthesis. Prog. Ind. Micro. 3:91.

JARET, R., MALLAMS, A. and REIMANN, H. 1973. The Megalomycins. 1V. The structures of megalomycins A, B, C_1 and C_2. J. Chem. Soc. Perkin Trans. 13:1374.

KANTOR, N. and SELZER, G. 1968. Chromatographic separation and bioassay of the gentamicin complex. J. Pharm. Sci. 57:2170.

LEE, B.K., TESTA, R.T., WAGMAN, G.H., HU, C.M., McDANIEL, L. and SCHAFFNER, C. 1973. Incorporation of L-methionine-methyl-^{14}C into gentamicins. J. Antibiotics. 26:728.

LEGATOR, M. and GOTTLIEB, D. 1953. Dynamics of chloramphenicol synthesis. Antibiotics and Chemotherapy. 3:809.

LUEDEMANN, G.M. 1969. Micromonospora taxonomy. Adv. Appl. Micro. 11:101.

LUEDEMANN, G.M. and BRODSKY, B.C. 1965. Micromonospora carbonacea sp. n.,

an everninocicin-producing organism. Antimicrob. Agents Chemother.-1965, 47.

LUEDEMANN, G.M. and BRODSKY, B.C. 1964. Taxonomy of gentamicin-producing Micromonospora. Antimicrob. Agents Chemother.-1974, 116.

LUEDEMANN, G.M. and WEINSTEIN, M.J. 1963. Gentamicin and method of production. U.S. Pat. No. 3,091,572.

MALLAMS, A. 1973. The megalomicins. 111. Rhodosamine, a new dimethylamino sugar. J. Chem. Soc. Perkin Trans. 1:1369.

MARQUEZ, J.A., WAGMAN, G.H. and COPPER, D.J. 1968. Separation of the components of the gentamicin complex. U.S. Pat. No. 3,651,042.

MILANESI, G. and CLIFERRI, O. 1966. Studies on the mechanism of action of gentamicin. Effect on protein synthesis in cell-free extracts of Escherichia coli. Biochem. 5:3926.

NEWTON, B.A. 1965. Mechanism of antibiotic action. Ann. Rev. Microbiol. 19:209.

ODEN, E.M., WAGMAN, G.H. and WEINSTEIN, M.J. 1968. Gentamicin. In Analytical Microbiology (F. Kavanagh, ed.) Academic Press, New York.

ODEN, E.M., STANDER, H. and WEINSTEIN, M.J. 1964. Microbiological assay of gentamicin. Antimicrob. Agents Chemother.-1964,8.

PARKER, C.R. 1972. Analysis By Atomic Absorption Spectroscopy. Varian Techtron Pty. Ltd., Springvale, Australia.

PERLMAN, D. and O'BRIEN. 1956. Utilization of carbohydrate by strains of Streptomycetes griseus. J. Bact. 72:214.

PERLMAN, D. 1953. Physiological studies on the Actinomycetales. Bot. Rev. 19:46.

PETERSON, H. and PETERSON, M. 1954. The broad-spectrum, polypeptide and other antibiotics. In Industrial Microbiology. Chemical Publishing Co. Inc., New York.

PORTER, N.J. 1974. Cultural conditions for antibiotic producing microorganisms. In Methods in Enzymology (G. Perlman and L. Lorand, eds). 21:43. Academic Press, New York.

PRAMER, D. 1955. Antibiotics against plant diseases. Scientific American. 192:82.

PRESCOTT, S.C. and DUNN, C.G. 1959. Antibiotics. In Industrial Microbiology. (3rd ed). McGraw-Hill, New York.

PRICE, K.E., GODFREY, J.C. and KAWAGUCHI, H. 1974. Effect of structural modification on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine. Adv. Appl. Microbiol. 18:191.

RAKE, G. and DONOVICK, R. 1946. Studies on the nutritional requirements of Streptococcus griseus for the formation of streptomycin. J. Bact. 52:223.

REIBLEIN, W.J., WATKINS, P.D. and WAGMAN. G.H. 1973. Binding of gentamicin and other aminoglycosidic antibiotics to mycelium of various Actinomycetes. Antimicrob. Agents Chemother. 4:602.

REIMANN, H. and JARET, R. 1972. Structure of rosamicin, a new macrolide from M. rosaria. J. Chem. Soc. Chem. Comm. 23:1270.

RINEHART, K.L. 1964. Structure and function. In The Neomycins and Related Antibiotics. John Wiley and Sons. Inc., New York.

ROSSELET, J.P., MESECK, E. and MARQUEZ, J.A. 1963. Isolation, purification and characterization of gentamicin. Antimicrob. Agents Chemother. 1963,14.

SCHAFFNER, C.P., DONOVAN, J.E. and MAEHR, H. 1964. The separation and differentiation of the gentamicin antibiotic complex. In Proceedings of the International Congress on Antibiotics. Prague, Czechoslovakia.

SCHONFELD, H., GRENZAC, H. and DEWECK, B. 1971. Purification of the newer antibiotic complexes. Antibiotics and Chemotherapy. 17:256.

SMITH, D. 1969. R-factors for aminoglycoside antibiotics. J. Infec. Dis. 378:119.

SYLVESTER, J. and COGHILL, R.D. 1954. Antibiotics. In Industrial Fermentation. Chemical Publishing Co. New York.

UMEZAWA, H. 1964. Kanamycin. In Recent Advances in Chemistry and Biochemistry of Antibiotics. Microbiological Chemistry Research Foundation, Tokyo, Japan.

WAGMAN, G.H., BAILEY, J.V. and WEINSTEIN, M.J. 1975. Binding of aminoglycoside antibiotics to filtration materials. Antimiro. Agents Chemother. 7:316.

WAGMAN, G.H., MARQUEZ, J.A., WATKINS, P.D., BAILEY, J.V., GENTILE, F. and WEINSTEIN, M.J. 1973. Neomycin production by Micromonospora species 69-683. J. Antibiotics. 26:732.

WAGMAN, G.H., MARQUEZ, J.A., BAILEY, J.V., COOPER, D., WEINSTEIN, M.J., Tkach. R. and DANIELS. P. 1972. Chromatographic separation of some minor components of the gentamicin complex. J. chromatogr. 70:171.

WAGMAN, G.H. TESTA, R.T. and MARQUEZ, J.A. 1970. Antibiotic 6640: fermentation, isolation and properties. J. Antibiotics. 23:555.

WAGMAN, G.H. and BAILEY, J.V. 1969. Use of silicic acid - glass sheets for bioautography of antimicrobial substances. J. Chromatogr. 41:263.

WAGMAN, G.H., BAILEY, J.V. and MILLER, M.M. 1968. Differential ninhydrin

chromatographic assay of the gentamicin complex. J. Pharm. Sci. 57:1319.

WAGMAN, G.H., MARQUEZ, J.A. and WEINSTEIN, M.J. 1968. Chromatographic separation of the components of the gentamicin complex. J. Chromatogr. 34:210.

WAGMAN, G.H. and WEINSTEIN, M.J. 1966. A chemically defined fermentation medium for the growth of Micromonospora purpurea. Biotech. Bioengineer. 3:259.

WAGMAN, G.H., LUEDEMANN, G.M. and WEINSTEIN, M.J. 1964. Fermentation and isolation of everninomicin. Antimicro. Agents Chemother. - 1964, 33.

WAITZ, J.A., MOSS, E.L., ODEN, E.M., WAGMAN, G.H. and WEINSTEIN, J.J. 1975. Antibiotic 6640: biological studies with antibiotic 6640, a new broad-spectrum aminoglycoside antibiotic. Antimirco. Agents Chemother. 2:265.

WAITZ, J.A., MOSS, E.L., ODEN, E.M., WAGMAN, G.H. and WEINSTEIN, M.J. 1972. Biological activity of Sch. 14342, an aminoglycoside antibiotic coproduced in the gentamicin fermentation. Antimicro. Agents Chemother. 2:464.

WAITZ, J.A., MOSS, E.L., ODEN, E.M. and WEINSTEIN, M.J. 1970. Biological studies with antibiotic 6640, a new broad-spectrum aminoglycoside antibiotic. J. Antibiotics. 23:559.

WAKSMAN, S.A. 1961. Classification, identification and description of genera and species. In The Actinomycetes, Vol. 11. The Wilkins Co. Baltimore, Md.

WAKSMAN, S.A. 1958. Neomycin. The Williams and Wilkins Co. Baltimore, Md.

WAKSMAN, S.A., GEIGER, W. and BUGIE, E. 1947. Micromonosporin, an antibiotic substance from a little-known group of microorganisms. J. Bact. 53:355.

WEINSTEIN, M.J., WAGMAN, G.H., MARQUEZ, J.A., TESTA, R.T. and WAITZ, J.A. 1975. Verdamicin, a new broad-spectrum aminoglycoside antibiotic. Antimicro. Agents Chemother. 7:246.

WEINSTEIN, M.J., MARQUEZ, J.A., TESTA, R.T., WAGMAN, G.H., ODEN, E.M. and WAITZ, J.A. 1970. Antibiotic 6640, a new Micromonospora-produced aminoglycoside antibiotic. J. Antibiotics 23:551

WEINSTEIN, M.J., WAGMAN, G.H., MARQUEZ, J.A., ODEN, E.M., TESTA, R.T. and WAITZ, J.A. 1968. Preliminary studies on megalomicin, a new Micromonospora-produced macrolide antibiotic. Antimicro. Agents Chemother. - 1968, 260.

WEINSTEIN, M.J. WAGMAN, G.M., ODEN, E.M. and WAGMAN, G.H. 1967. Biological activity of the antibiotic components of the gentamicin complex. J. Bact.

94:789.

WEINSTEIN, M.J., LUEDEMANN, G.M., ODEN, E.M. and WAGMAN, G.M. 1965a. Biological properties of gentamicin A. Antimicro. Agents Chemother. - 1965, 816.

WEINSTEIN, M.J., WAGMAN, G.H., ODEN, E.M., LUEDEMANN, G.M., SLOANE, P., MURAWSKI, A. and MARQUEZ, J.A. 1965b. Purification and biological studies of everninomicin B. Antimicro. Agents Chemother. - 1965, 821.

WEINSTEIN, M.J., LUEDEMANN, G.M., ODEN, E.M. and WAGMAN, G.H. 1963. Gentamicin, a new antibiotic complex from Micromonospora. J. Med. Chem. 6:463.

WEINSTEIN, M.J., LUEDEMANN, G.M., ODEN, E.M. and WAGMAN, G.H. 1963. Gentamicin, a new broad-spectrum antibiotic complex. Antimicro. Agents Chemother. - 1963, 1.

APPENDICES

Appendix table 1. The vegetative growth of M. purpurea in a starch based medium (B) at 35°C.

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual carbohydrate as glucose (mg/ml)	Residual carbohydrate as starch (mg/ml)	Gentamicin (µg/ml)
Trial 1					
0	6.8	*	27.84	25.06	0.0
24	6.9	0.78	27.00	24.30	0.0
48	7.1	1.59	26.22	23.59	1.0
72	7.2	2.58	24.56	22.10	2.0
96	7.5	4.02	21.67	19.50	4.0
Trial 11					
0	6.8	*	27.84	25.06	0.0
24	6.9	0.69	26.97	24.27	0.0
48	7.2	1.66	25.79	23.21	1.0
72	7.4	2.98	24.01	21.60	2.0
96	7.6	4.62	21.79	19.61	5.0

* Mycelial growth too small to calculate.

Appendix table 2. The fermentative growth of M. purpurea in a glucose based medium (B) at 30°C .

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Gentamicin (µg/ml)
Trial I				
0	6.8	1.56	10.67	3.0
24	7.1	2.89	9.43	11.0
48	7.3	3.68	8.45	18.0
72	7.5	4.43	7.02	29.0
96	7.9	4.74	6.46	42.0
Trial II				
0	6.8	1.78	10.67	3.0
24	7.0	2.59	9.40	9.0
48	7.3	3.96	8.32	19.0
72	7.5	4.40	7.12	30.0
96	7.8	4.69	6.78	45.0

Appendix table 3. The effect of increasing the time of fermentation on the production of gentamicin.

Trial 1		Trial 11	
Fermentation time (hrs)	Gentamicin (µg/ml)	Fermentation time (hrs)	Gentamicin (µg/ml)
0	4.0	0	4.0
24	8.0	24	9.0
48	22.0	48	27.0
72	30.0	72	37.0
96	36.0	96	42.0
120	41.0	120	44.0
144	40.0	144	44.0

Appendix table 4. Size of inoculum and production of gentamicin.

Trial 1

Initial inoculum (% v/v)		Fermentation time (hrs) 0	24	48	72	96
2	Mycelial weight (mg/ml)	0.88	1.67	2.49	3.06	3.09
	Gentamicin (µg/ml)	*	8.0	15.0	24.0	31.0
4	Mycelial weight (mg/ml)	1.46	2.01	3.05	3.95	4.65
	Gentamicin (µg/ml)	*	7.0	16.0	27.0	38.0
6	Mycelial weight (mg/ml)	2.50	3.20	4.50	5.21	6.61
	Gentamicin (µg/ml)	*	9.0	26.0	46.0	50.0
8	Mycelial weight (mg/ml)	3.20	4.80	5.90	6.42	6.10
	Gentamicin (µg/ml)	*	18.0	32.0	46.0	55.0
10	Mycelial weight (mg/ml)	4.18	5.25	6.04	5.50	5.00
	Gentamicin (µg/ml)	*	25.0	29.0	32.0	43.0

* Not determined.

Appendix table 5. Size of inoculum and production of gentamicin.

Trial 11

Initial inoculum (% v/v)		Fermentation time (hrs) 0	24	48	72	96
2	Mycelial weight (mg/ml)	0.76	1.56	2.42	3.12	4.42
	Gentamicin (µg/ml)	*	8.0	14.0	27.0	36.0
4	Mycelial weight (mg/ml)	1.52	2.21	3.19	4.25	5.10
	Gentamicin (µg/ml)	*	10.0	21.0	33.0	42.0
6	Mycelial weight (mg/ml)	2.46	3.09	4.10	5.32	6.09
	Gentamicin (µg/ml)	*	11.0	29.0	52.0	61.0
8	Mycelial weight (mg/ml)	3.33	4.65	5.56	6.00	5.42
	Gentamicin (µg/ml)	*	16.0	29.0	48.0	51.0
10	Mycelial weight (mg/ml)	4.00	5.73	6.54	5.67	5.00
	Gentamicin (µg/ml)	*	22.0	35.0	42.0	42.0

* Not determined.

Appendix table 6. The effects of using spent liquor for the production of gentamicin.

Fermentation time (hrs)	pH	Residual glucose (mg/ml)	Mycelial weight (mg/ml)	Gentamicin (µg/ml)
Primary fermentation.				
0	7.0	10.22	2.12	2.0
24	7.3	9.56	3.24	6.0
48	7.4	8.00	4.10	17.0
72	7.6	7.12	5.06	30.0
96	7.5	6.00	4.64	46.0
Spent liquor fermentation.				
0	6.9	6.25	1.80	15.0
24	7.5	5.00	2.58	27.0
48	8.0	3.78	3.20	37.0
72	8.6	3.00	2.54	40.0
96	9.1	1.89	2.00	26.0

Appendix table 7. The effects of using spent liquor for the production of gentamicin.

Fermentation time (hrs)	pH	Residual glucose (mg/ml)	Mycelial weight (mg/ml)	Gentamicin (µg/ml)
Primary fermentation.				
0	6.9	10.09	2.45	4.0
24	7.2	9.40	3.44	10.0
48	7.3	8.10	4.40	21.0
72	7.5	7.00	5.00	35.0
96	7.8	5.85	4.35	37.0
Spent liquor fermentation.				
0	6.8	6.12	2.00	18.0
24	7.4	5.14	3.10	25.0
48	8.0	3.42	4.06	36.0
72	8.6	2.98	3.40	45.0
96	9.2	2.10	2.60	34.0

Appendix table 8. The distribution of bound and free gentamicin in primary fermentations.

Fermentation time (hrs)	Total gentamicin (µg/ml)	Free gentamicin (µg/ml)	Bound gentamicin (µg/ml)
Trial 1			
0	6	0	6
24	13	3	10
48	30	11	19
72	41	13	28
96	48	17	31
Trial 11			
0	5	0	5
24	11	2	9
48	26	10	16
72	37	14	23
96	45	16	29

Appendix table 9. The distribution of bound and free gentamicin in fermentations employing spent fermentation liquor.

Fermentation time (hrs)	Total gentamicin (µg/ml)	Free gentamicin (µg/ml)	Bound gentamicin (µg/ml)
Trial 1			
0	18	15	3
24	28	19	9
48	39	22	17
72	47	26	21
96	36	24	12
Trial 11			
0	18	16	2
24	30	17	13
48	43	23	20
72	50	25	25
96	34	24	10

Appendix table 10. Influence of cobalt on the production of gentamicin in primary and spent liquor fermentations.

Trial 1

Fermentation time (hrs)	Gentamicin (µg/ml)			
	A	B	C	D
0	4	18	16	16
24	14	24	26	22
48	26	36	33	30
72	39	47	42	38
96	50	39	41	40

A. Primary fermentation containing recommended level of cobalt.
B. Spent liquor fermentation with no Co supplementation.
C. Spent liquor fermentation with 0.16 µg/ml cobalt supplementation.
D. Spent liquor fermentation with 0.32 µg/ml cobalt supplementation.

Appendix table 11. Influence of cobalt on the production of gentamicin in primary and spent liquor fermentations.

Trial 11

Fermentation time (hrs)	Gentamicin (µg/ml)			
	A	B	C	D
0	6	20	16	16
24	12	26	22	23
48	28	40	31	32
72	37	50	44	41
96	48	41	40	40

A. Primary fermentation containing recommended level of cobalt.
B. Spent liquor fermentation with no cobalt supplementation.
C. Spent liquor fermentation with 0.16 µg/ml cobalt supplementation.
D. Spent liquor fermentation with 0.32 µg/ml cobalt supplementation.

Appendix table 12. Influence of calcium carbonate on the production of gentamicin in primary and spent liquor fermentations.

		Fermentation time (hrs)				
	Trial 1	0	24	48	72	96
A.	pH	6.9	7.1	7.3	7.5	7.8
	Gentamicin (µg/ml)	4	12	33	48	59
B.	pH	6.9	7.0	7.5	8.1	8.8
	Gentamicin (µg/ml)	21	28	37	44	41
C.	pH	6.9	7.0	7.4	8.1	8.7
	Gentamicin (µg/ml)	19	25	43	47	46
D.	pH	6.9	7.0	7.4	8.2	9.1
	Gentamicin (µg/ml)	19	24	44	46	43

A. Primary fermentation with recommended level of $CaCO_3$.
B. Spent liquor fermentation with no $CaCO_3$ supplementation.
C. Spent liquor fermentation with 0.5 gram/liter $CaCO_3$ supplementation.
D. Spent liquor fermentation with 1.0 gram/liter $CaCO_3$ supplementation.

Appendix table 13. Influence of calcium carbonate on the production of gentamicin in primary and spent liquor fermentations.

		Fermentation time (hrs)				
	Trial 11	0	24	48	72	96
A.	pH	6.9	7.1	7.3	7.5	7.8
	Gentamicin (µg/ml)	4	12	33	48	59
B.	pH	6.9	7.0	7.5	8.1	8.7
	Gentamicin (µg/ml)	21	28	37	47	46
C.	pH	6.9	7.0	7.4	8.1	8.7
	Gentamicin (µg/ml)	19	25	43	47	46
D.	pH	6.9	7.0	7.4	8.2	9.1
	Gentamicin (µg/ml)	19	24	44	46	43

A. Primary fermentation with recommended level of $CaCO_3$.
B. Spent liquor fermentation with no $CaCO_3$ supplementation.
C. Spent liquor fermentation with 0.5 gram/liter $CaCO_3$ supplementation.
D. Spent liquor fermentation with 1.0 gram/liter $CaCO_3$ supplementation.

Appendix table 14. The effect of pH adjustment on the production of gentamicin using spent fermentation liquor.

	Fermentation time (hrs)				
Trial 1	0 *	24	48	72	96
pH	5.5	5.8	5.7	6.2	6.9
Gentamicin (µg/ml)	14	12	16	22	30
pH	6.0	6.3	6.5	7.0	7.2
Gentamicin (µg/ml)	14	14	18	26	33
pH	6.5	6.9	7.0	7.3	7.5
Gentamicin (µg/ml)	14	15	22	31	44
pH	7.0	7.5	7.7	7.6	8.2
Gentamicin (µg/ml)	14	17	26	34	47

* These values represent initial pH values of the media . pH values other than at the commencement of fermentation represent pH values of the fermentation prior to adjustment.

Appendix table 15. The effect of pH adjustment on the production of gentamicin using spent fermentation liquor.

	Fermentation time (hrs)				
Trial 11	0 *	24	48	72	96
pH	5.5	5.7	5.9	6.0	6.6
Gentamicin (µg/ml)	14	14	17	20	25
pH	6.0	6.6	6.7	6.8	7.0
Gentamicin (µg/ml)	14	15	20	27	36
pH	6.5	7.0	7.2	7.3	7.6
Gentamicin (µg/ml)	14	18	25	36	48
pH	7.0	7.5	7.8	8.0	8.4
Gentamicin (µg/ml)	14	20	30	37	48

* These values represent initial pH values of the media. pH values other than at the commencement of fermentation represent pH values of the fermentations prior to adjustment.

Appendix table 16. Gentamicin production at pH 7.0 using spent fermentation liquor with/without pH adjustment.

Fermentation time (hrs)	pH		Gentamicin (µg/ml)	
	A	B	A	B
Trial 1				
0	7.0	7.0	15	15
24	7.4	7.4	25	21
48	8.0	8.0	39	30
72	8.8	8.1	44	41
96	9.1	8.1	30	44
Trial 11				
0	7.0	7.0	15	15
24	7.4	7.5	23	20
48	8.0	7.8	37	28
72	8.7	7.9	41	37
96	9.2	8.2	32	45

A. No pH adjustment
B. pH adjustment every 24 hours. The values shown represent the pH of the fermentations before they were adjusted back to pH 7.0.

Appendix table 17. The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

Trial 1

Gentamicin sulfate added to medium (µg/ml)		Fermentation time (hrs) 0	24	48	72	96
0.0	Mycelial weight (mg/ml)	1.85	2.60	3.75	4.75	4.70
	Residual glucose (mg/ml)	9.56	8.21	7.11	5.92	5.12
1.0	Mycelial weight (mg/ml)	1.85	2.74	3.66	4.80	4.65
	Residual glucose (mg/ml)	9.56	8.12	7.23	6.00	5.55
5.0	Mycelial weight (mg/ml)	1.85	2.70	4.01	5.11	4.85
	Residual glucose (mg/ml)	9.56	8.30	7.00	5.89	5.55
10.0	Mycelial weight (mg/ml)	1.85	2.77	3.99	5.00	4.95
	Residual glucose (mg/ml)	9.56	8.34	7.18	5.77	5.22

Appendix table 17(continued). The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

Gentamicin sulfate added to medium (µg/ml)		Fermentation time (hrs) 0	24	48	72	96
20.0	Mycelial weight (mg/ml)	1.85	2.68	3.78	4.79	5.00
	Residual glucose (mg/ml)	9.56	8.22	7.65	5.21	4.93

Appendix table 18. The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

Trial 11

Gentamicin sulfate added to medium (µg/ml)		Fermentation time (hrs) 0	24	48	72	96
0.0	Mycelial weight (mg/ml)	1.85	2.76	3.96	4.89	4.40
	Residual glucose (mg/ml)	9.56	8.01	7.00	5.79	5.08
1.0	Mycelial weight (mg/ml)	1.85	2.65	3.72	4.72	4.70
	Residual glucose (mg/ml)	9.56	8.22	7.12	6.34	5.77
5.0	Mycelial weight (mg/ml)	1.85	2.70	3.82	4.92	5.00
	Residual glucose (mg/ml)	9.56	8.30	7.22	6.00	5.12
10.0	Mycelial weight (mg/ml)	1.85	2.77	3.99	5.09	4.79
	Residual glucose (mg/ml)	9.56	8.12	7.04	5.85	5.00

Appendix table 18(continued). The effect of gentamicin sulfate on the growth of M. purpurea in medium (B).

Gentamicin sulfate added to medium (µg/ml)		Fermentation time (hrs) 0	24	48	72	96
20.0	Mycelial weight (mg/ml)	1.85	2.70	3.92	5.00	5.09
	Residual glucose (mg/ml)	9.56	8.43	7.40	5.78	5.12

Appendix table 19. The effect of gentamicin sulfate on the production of gentamicin by M. purpurea in medium (B).

Fermentation time (hrs)	Gentamicin * (µg/ml) A	B
Trial I		
0	21	2
24	17	16
48	24	27
72	35	36
96	49	45
Trial II		
0	21	2
24	19	13
48	21	25
72	32	39
96	44	47

A. Gentamicin sulfate standard (20.0 µg/ml) added at the start of fermentation.

B. No addition of gentamicin sulfate at the start of fermentation.

* Refers to the total amount of gentamicin present at time of assay.

Appendix table 20. The growth pattern of M. purpurea in varying concentrations of spent fermentation liquor.

Trial 1	Fermentation time (hrs)				
	0	24	48	72	96
A. pH	6.9	7.3	7.6	7.9	8.5
Mycelial weight (mg/ml)	1.98	3.10	4.21	4.90	4.65
B. pH	6.9	7.5	7.9	8.7	9.1
Mycelial weight (mg/ml)	1.98	3.00	3.70	3.65	2.70
C. pH	6.9	7.5	8.0	8.6	9.1
Mycelial weight (mg/ml)	1.98	2.70	3.00	2.70	2.50
D. pH	6.9	7.6	8.1	8.8	9.2
Mycelial weight (mg/ml)	1.98	2.40	3.10	2.80	2.30
E. pH	6.9	7.8	8.6	9.2	9.2
Mycelial weight (mg/ml)	1.98	2.10	2.10	1.66	1.40

A. Fermentation using 75% fresh medium (B) and 25% spent fermentation liquor.
B. " 50% " 50% "
C. " 25% " 75% "
D. " 0% " 100% "
E. Fermentation using concentrated (lyophilized) spent fermentation liquor.

Appendix table 21. The growth pattern of M. purpurea in varying concentrations of spent fermentation liquor.

Trial 11	Fermentation time (hrs)				
	0	24	48	72	96
A. pH	6.9	7.4	7.6	8.0	8.4
Mycelial weight (mg/ml)	1.98	3.42	4.75	5.20	5.00
B. pH	6.9	7.7	8.0	8.6	9.1
Mycelial weight (mg/ml)	1.98	2.89	3.50	3.55	2.85
C. pH	6.9	7.7	8.1	8.6	9.2
Mycelial weight (mg/ml)	1.98	2.58	3.45	2.99	2.60
D. pH	6.9	7.6	8.0	8.7	9.1
Mycelial weight (mg/ml)	1.98	2.99	3.20	2.90	2.35
E. pH	6.9	7.7	8.9	9.3	9.3
Mycelial weight (mg/ml)	1.98	2.20	1.87	1.47	1.40

A. Fermentation using 75% fresh medium (B) and 25% spent fermentation liquor.
B. " 50% " 50% "
C. " 25% " 75% "
D. " 0% " 100% "
E. Fermentation using concentrated (lyophilized) spent fermentation liquor.

Appendix table 22. The production of gentamicin using varying concentrations of spent fermentation liquor.

Fermentation time (hrs)	Gentamicin (µg/ml)				
	A	B	C	D	E
Trial 1					
0	7	12	16	18	26
24	15	15	19	24	30
48	28	22	25	32	36
72	46	38	38	39	36
96	30	24	25	30	27
Trial 11					
0	7	12	16	18	26
24	12	13	17	26	33
48	26	25	27	35	37
72	44	38	40	39	*
96	32	31	29	29	22

* Sample was not avaible for analysis.

A. Fermentation using 75% fresh medium (B) and 25% spent fermentation liquor.
B. Fermentation using 50% fresh medium (B) and 50% spent fermentation liquor.
C. Fermentation using 25% fresh medium (B) and 75% spent fermentation liquor.
D. Fermentation using 0% fresh medium (B) and 100% spent fermentation liquor.
E. Fermentation using concentrated (lyophilized) spent fermentation liquor.

Appendix table 23. Fermentation pattern of *M. purpurea* using spent fermentation liquor with no glucose and yeast extract fortification.

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)
Trial 1				
0	6.9	1.90	4.92	0.90
24	7.5	2.60	3.40	0.81
48	8.0	3.70	2.15	0.76
72	8.4	3.33	1.90	0.79
96	9.2	2.60	1.78	0.80
Trial 11				
0	7.0	1.78	4.88	0.85
24	7.5	2.64	3.62	0.80
48	8.1	3.56	2.34	0.74
72	8.7	3.00	2.00	0.76
96	9.3	2.52	1.89	0.77

Appendix table 24. Fermentation patterns of M. purpurea using spent fermentation liquor with glucose and yeast extract fortification.

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)
Trial 1				
0	6.9	1.95	10.61	1.46
24	7.6	2.80	8.76	1.35
48	7.9	3.36	*	*
72	8.1	4.20	6.80	1.29
96	8.6	4.00	5.10	1.31
Trial 11				
0	6.9	1.78	10.44	1.42
24	7.7	2.65	8.92	1.38
48	8.1	3.40	7.77	1.33
72	8.3	3.92	7.10	1.30
96	8.7	3.80	6.42	1.30

* Samples were unavailable for analysis.

Appendix table 25. The production of gentamicin using spent fermentation liquor with and without glucose and yeast extract fortification.

Fermentation time (hrs)	Gentamicin (µg/ml) A.	B.
Trial 1		
0	17	16
24	22	25
48	34	40
72	41	46
96	37	41
Trial 11		
0	19	17
24	24	26
48	30	37
72	38	44
96	41	44

A. Fermentations using spent liquor with no glucose and yeast extract fortification.

B. Fermentation using spent liquor with glucose and yeast extract fortification.

Appendix table 26. Batch fermentation pattern of *M. purpurea* in medium (B).

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	7.1	1.90	9.78	1.45	1.0
24	6.8	3.20	7.11	1.27	16.0
48	7.1	3.56	6.79	1.11	41.0
72	7.3	4.98	5.05	0.96	52.0
96	7.6	5.23	4.35	0.69	58.0
120	7.8	4.90	4.12	0.66	61.0

Appendix table 27. Batch fermentation pattern of *M. purpurea* in medium (B).

Trial 11

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.11	10.98	1.32	1.0
24	6.8	3.87	8.21	1.12	12.0
48	7.3	4.70	7.05	0.78	38.0
72	7.4	4.90	6.12	0.62	49.0
96	7.6	5.16	5.00	0.54	56.0
120	7.6	5.00	4.14	*	61.0

* Sample not available for analysis.

Appendix table 28. Batch fermentation pattern of *M. purpurea* in medium (B) with automatic pH control.

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.33	9.83	4
24	7.0	3.27	8.32	12
48	7.2	4.78	7.00	35
72	7.0	5.90	5.23	54
96	6.9	6.15	4.98	66
120	7.1	6.00	4.66	56

Appendix table 29. Batch fermentation pattern of M. purpurea in medium (B) with automatic pH control.

Trial 11

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Gentamicin (µg/ml)
0	7.0	1.88	10.34	2
24	7.0	2.34	9.46	9
48	6.8	4.51	7.32	34
72	6.8	5.10	6.68	40
96	6.9	5.27	6.60	47
120	7.7 *	5.10	6.27	54

* pH was not maintained at this time period due to pH controller failure.

Appendix table 30. Batch fermentation pattern of M. purpurea employing spent fermentation liquor.

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	7.0	2.28	4.78	0.91	21
24	7.4	3.16	3.94	0.87	25
48	7.7	4.42	3.12	0.72	37
72	8.1	4.40	2.68	0.60	46
96	8.9	3.87	2.44	0.64	41

Appendix table 31. Batch fermentation pattern of M. purpurea employing spent fermentation liquor.

Trial 11

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.8	2.08	5.16	0.89	18
24	7.3	3.45	4.34	0.96	21
48	7.9	4.47	3.33	0.78	32
72	8.5	3.78	2.79	0.52	39
96	9.1	2.35	*	0.59	*

* These samples were unavailable for analysis.

Appendix table 32. Batch fermentation pattern of M. purpurea employing spent fermentation liquor with automatic pH control.

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Gentamicin (µg/ml)	Residual glucose (mg/ml)
0	6.9	1.78	18	5.17
24	7.2	2.00	22	*
48	7.1	3.67	37	*
72	7.3	4.45	47	*
96	7.1	4.90	48	2.18

* These samples were not analyzed.

Appendix table 33. Batch fermentation pattern of M. purpurea employing spent fermentation liquor with glucose and yeast extract fortification.

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.15	9.55	1.34	20
24	7.4	3.22	8.16	1.21	21
48	7.8	4.67	7.04	1.06	28
72	8.3	4.90	6.43	0.89	37
96	8.8	4.92	6.08	0.81	39

Appendix table 34. Batch fermentation pattern of M. purpurea employing spent fermentation liquor with glucose and yeast extract fortification.

Trial 11

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
0	6.9	2.53	9.01	1.14	22
24	7.0	2.70	8.66	*	20
48	7.6	3.76	7.20	*	28
72	8.2	4.20	6.13	*	37
96	8.9	4.40	5.79	0.87	41

* Tests were not performed.

Appendix table 35. Fermentation pattern of M. purpurea using medium (B) in a continuous culture process.

Trial 1

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjeldahl nitrogen (mg/ml)	Gentamicin (µg/ml)
Primary batch fermentation.					
0	6.9	2.43	10.67	1.33	4.0
96	7.9	5.07	4.44	0.89	63.0
Continuous culture process.					
102	7.8	4.14	5.34	*	56.0
108	7.8	4.00	5.65	*	56.0
114	7.6	4.32	5.55	*	54.0
120	7.5	5.18	5.79	1.07	47.0
126	7.5	5.89	5.68	*	40.0
132	7.5	5.67	6.16	*	39.0
138	7.6	5.34	6.74	*	41.0
142	7.7	5.42	6.77	1.02	44.0

* Tests not performed.

Appendix table 36. Fermentation pattern of M. purpurea using medium (B) in a continuous culture process.

Trial 11

Fermentation time (hrs)	pH	Mycelial weight (mg/ml)	Residual glucose (mg/ml)	Kjelhahl nitrogen (mg/ml)	Gentamicin (µg/ml)
Primary batch fermentation.					
0	6.9	2.02	10.17	1.27	3.0
96	7.7	4.92	4.54	0.72	57.0
Continuous culture process.					
102	7.4	4.23	5.07	*	51.0
108	7.5	4.35	5.27	*	50.0
114	7.5	5.18	5.77	*	50.0
120	7.3	5.55	6.40	0.99	41.0
126	7.2	5.35	6.56	*	40.0
132	7.4	5.12	6.23	*	42.0
138	7.4	4.45	6.78	*	44.0
142	7.5	4.22	6.44	0.91	47.0

* Tests not performed.

Appendix table 37. Continuous fermentation process A using spent fermentation liquor with automatic pH control.

Fermentation time (hrs)	Total gentamicin (µg/ml)	Net gentamicin (µg/ml)
Spent liquor batch fermentation .		
0	14.0	0.0
24	16.0	2.0
48	31.0	17.0
72	40.0	26.0
Continuous process A.		
78	37.0	23.0
84	30.0	16.0
90	27.0	13.0
96	24.0	10.0
102	17.0	3.0

Appendix table 38. Continuous fermentation process B using spent fermentation liquor with automatic pH control.

Continuous process B.

Fermentation time(hrs)	Total gentamicin (µg/ml)	Net gentamicin (µg/ml)
0	15.0	0.0
6	14.0	- 1.0
12	14.0	- 1.0
18	16.0	1.0
24	16.0	1.0
30	16.0	1.0