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

BY

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ABSTRACT

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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