Review Article

CULTURE CONDITIONS FOR SCREENING OF NEW ANTIBIOTICS

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Contents

I. Introduction

Screening of antibiotics has been widely performed for about 30 years, and new antibiotics are still being found. However, the possibility of discovering new antibiotics merely by random screening is reduced nowadays, and new approaches are required for finding new antibiotics efficiently[1].

In screening of new antibiotics, three major factors must be considered *i.e.*, detection methods, selection of producing microorganisms, and cultivation methods. These are closely related to each other, and their efficient combination is indispensable for successful screening. Since WAKSMAN intentionally employed *Mycobacterium tuberculosis* 607 as a test organism in seeking anti-tuberculosis drugs and found streptomycin[2], many antibiotics with anti-bacterial, anti-fungal, anti-protozoal, anti-parasitic, anti-viral and anti-cancer activities have been discovered by employing various detection methods such as the use of altered test microbes[3~5]. Minor products have also been detected by highly sensitive detection methods. For example, the discovery of nocardicin A resulted from the utilization of a test microbe highly sensitive to β-lactam antibiotics[6~9]. Though the detection method in screening is important, new substances can be found by combining the detection method with the improved methods of culture selection and cultivation.

The selection of superior producing microorganisms was earnestly pursued by WEINSTEIN *et al.*[10~13]. They thoroughly screened microorganisms of the genus *Micromonospora* which had rarely been studied and found gentamicin and several other antibiotics. With this work as a turning point, studies shifted to methods for effectively isolating actinomycetes other than *Streptomyces* which are less frequent in soil[14~16]. Rare actinomycetes were found to produce many new antibiotics[17~19]. However, since

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rare actinomycetes do not usually produce antibiotics abundantly and grow slowly, research on and development of them are difficult. Moreover, the basic structures of the antibiotics produced by these strains can also be obtained from *Streptomyces*, and thus it is questionable whether it is worth while to emphasize this factor greatly.

The third factor, the cultivation method, has as its purpose to vary fermentation products quantitatively or qualitatively; however, it is not usually emphasized strongly in screening of antibiotics. It is possible, however, to increase the production of a trace antibiotic component by changing the constituents of the medium or the cultivation conditions, resulting in the discovery of a new antibiotic.

In this paper, we describe various factors important in the production of antibiotics together with results obtained in our laboratory, and discuss the significance of the cultivation method in the screening for new antibiotics.

II. Trophophase and Idiophase

Though the production of antibiotics is sometimes evident during growth of the microorganisms, usually the production is actively carried out after growth reaches the stationary phase[50]. DOSKOČIL *et al.*[20] studied growth, synthesis of DNA and RNA, respiration, morphology, utilization of carbon and nitrogen sources, accumulation of pyruvate, and production of oxytetracycline by *Streptomyces rimosus*, and divided the processes into five stages. They indicated that the morphological and physiological properties of the strain are greatly changed before and after antibiotic accumulation begins. Particularly stage 2, in which respiration is high and vegetative growth is accelerating by utilizing constituents of the medium, is well contrasted to stage 5, in which growth stops and the production of antibiotic reaches at maximum. The fermentation period including stage 2 is known "trophophase" and that including stage 5 "idiophase". Conditions for antibiotic production are more restricted than the growth conditions, and thus the efficient conversion from the trophophase to the idiophase is important for the production of antibiotics. The termination of the trophophase does not always lead to the idiophase and thus events that occur during trophophase are important for the production of antibiotics. For example, since the production of antibiotics depends on the production of primary metabolites, it is necessary that precursors and other factors required for antibiotic production are prepared during the trophophase when primary metabolism occurs vigorously[21~23].

The production of antibiotics in an ordinary fermentation in which the growth and production occur in a batch is different from a chemical reaction. Nevertheless, when comparing a fermentation process with a chemical reaction, the growth of producing organisms corresponds to the supply of raw materials and catalysts in the chemical reaction, and the production of an antibiotic corresponds to conversion to the chemically synthesized product. Though the growth of the producing strain is indispensable for fermentation, it is occasionally experienced that despite good growth, the substance sought is not produced; the cause of the change cannot be ascertained in many cases. In our view, the phenomenon is a function of cultivation conditions, and thus the production of antibiotics can be increased by selecting adequate cultivation conditions.

III. Factors Affecting Antibiotic Production

Factors affecting antibiotic production can be divided into the constituents of media and the conditions of cultivation (Table 1).

1. Constituents of Media

Table 1. Factors affecting antibiotic production.

Medium composition	Fermentation conditions
Carbon source	pH
Nitrogen source	Temperature
Inorganic phosphate	Oxygen
Inorganic salts	Others
Trace metals	
Precursors	
Inhibitors	
Inducers	
Others	

Table 2. Inhibition of antibiotic production by glucose.

Actinomycin	*Streptomyces antibioticus*[27]
Indolmycin	*Streptomyces griseus*[28]
Kanamycin	*Streptomyces kanamyceticus*[29]
Mitomycin	*Streptomyces verticillatus*[30]
Neomycin	*Streptomyces fradiae*[31]
Puromycin	*Streptomyces alboniger*[32]
Siomycin	*Streptomyces sioyaensis*[33]
Streptomycin	*Streptomyces griseus*[34]
Streptothricin	*Streptomyces lavendulae*[35]
Bacitracin	*Bacillus licheniformis*[36]
Cephalosporin C	*Cephalosporium acremonium*[37]
Penicillin	*Penicillium chrysogenum*[24]

1-1: Carbon Source: As seen in the penicillin (PC) fermentation, PC production is better in the presence of lactose which is slowly utilized than of glucose which is more rapidly utilized[24]. Such carbon catabolite regulation by rapidly metabolized sugars is known in the production of many antibiotics[21,22,25,26]. In the production of anticapsin by *Streptomyces griseoplanus*, the maximum accumulation is obtained with glucose at a concentration as high as 10%[38], but this is an exceptional case. Table 2 shows some antibiotics the production of which is inhibited by glucose. Beside glucose, inhibition of production by glycerol has been reported in aurantin production by *Bacillus aurantinus*[39] and in the cephamycin fermentation[40]. It is usual that the production of antibiotics is promoted after readily utilizable sugars such as glucose and glycerol have almost been entirely consumed[50].

The mechanisms of carbon catabolite regulation are known in some cases. They are roughly divided into (1) catabolite repression, in which the production of an enzyme is inhibited as in the production of actinomycin[27] and streptomycin[34], and (2) catabolite inhibition in which the activity of an enzyme is inhibited as in the production of siomycin[33] and indolmycin[28].

Carbon catabolite regulation has been avoided by adding the carbon source periodically[41,42] or gradually[43], but they are both difficult to apply to screening. It is common to avoid the inhibition of production by changing the carbon source in the production medium.

1-2: Nitrogen Source: It is well known that changes in the kind and concentration of nitrogen source influence greatly antibiotic production[44]. For example, as shown in Table 3, antibiotic production is inhibited by a rapidly utilized nitrogen source (NH_4^+, NO_3^-, certain amino acids, *etc.*). Though the phenomenon is not as well studied as that of carbon source regulation, the presence of nitrogen catabolite regulation can be presumed from the following observations. Antibiotic accumulation begins to increase in many cases only after the nitrogen source in the culture broth has been almost entirely consumed. In candihexin production, addition of a nitrogen source in the idiophase, returns the fermentation to the trophophase and production is reduced[42]. Recently, the presence of nitrogen regulation was revealed on the enzymatic level in fermentation of cephamycin[46] and patulin[49].

The inhibition of production by a nitrogen source can be usually avoided by selecting an adequate production medium with the proper kind of nitrogen source. The quantity of nitrogen source is chosen keeping in mind the quantity of carbon source present and this is reflected in the C/N ratio.

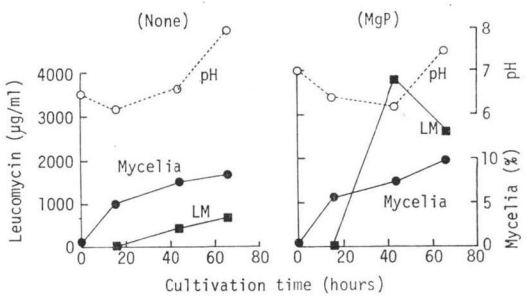
In a study on the biosynthesis of macrolide antibiotics, we found that addition of 1% magnesium

Table 3. Inhibition of antibiotic production by readily utilizable nitrogen sources.

Oleandomycin	*Streptomyces antibioticus*[45]
Erythromycin	*Streptomyces erythreus*[26]
Leucomycin	*Streptomyces kitasatoensis*[51]
Cephamycin	*Streptomyces clavuligerus*[46]
Novobiocin	*Streptomyces niveus*[47]
Candihexin	*Streptomyces viridoflavum*[42]
Fusidin	*Fusidium coccineum*[48]
Patulin	*Penicillium urticae*[49]

Fig. 1. Influence of addition of MgP on leucomycin production in a complex medium[51].

Complex medium: 2% glucose, 0.5% peptone, 0.3% dried yeast cells, 0.5% meat extract, 0.5% NaCl, 0.3% $CaCO_3$, and 0 or 1% $Mg_3(PO_4)_2 \cdot 8H_2O$.



phosphate [$Mg_3(PO_4)_2 \cdot 8H_2O$, MgP], which is an insoluble salt, to a fermentation medium increased the production of macrolide antibiotics[51,52]. Fig. 1 shows the effect of MgP on the production of leucomycin (LM) in a complex medium; the production of leucomycin was increased to 5-fold by adding MgP. By addition of MgP, the amount of mycelia was hardly affected, but the pH curve showed an obvious change.

For studying the action of MgP, an experiment was performed in chemically defined medium. In this medium, the production of LM was increased by about 7-fold by MgP and the pH was markedly lowered. It was confirmed that the increase of antibiotic potency by MgP addition was not due to a change in the leucomycin components. For determining which of the two elements contributed to the effect of MgP, Mg and P were added to the production medium as $MgSO_4$ and K_2HPO_4, and strong inhibition of the LM production were observed in both cases. For determining the effect of pH regulation, $CaCO_3$ and MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer were used as pH regulators and the productivity of LM was studied. Addition of MOPS increased the production a little, but not as much as with MgP. When MgP was added in addition to controlling the pH by MOPS, the production was strongly increased. Thus the effect of MgP was found not to be related directly to the concentration of Mg or P or to pH regulation. Fig. 2 shows the changes in the concentrations of LM and NH_4^+ during the fermentation. In the MgP-adding system, the apparent utilization of NH_4^+ was markedly stimulated and the production of LM was strongly increased following the reduction of the NH_4^+ concentration. Since the amount of NH_4^+ corresponding to the reduced concentration in the figure was detected in the insoluble MgP fraction, it is presumed that MgP would trap NH_4^+ in the culture solution, thus releasing the inhibition of LM production by NH_4^+ and increasing the production.

1–3: Inorganic Phosphate: When adding a large amount of inorganic phosphate, consumption of carbon and nitrogen sources and respiration are accelerated resulting in good growth, but the production of antibiotics is usually reduced[53]. Doskočil *et al.*[54] reported that in the chlortetracycline fermentation by *Streptomyces aureofaciens* a slight increase of potassium biphosphate (from 0.2 mM to 0.4 mM) greatly accelerated respiration and consumption of sucrose and ammonia nitrogen. Aharonowitz and Demain[55] studied the relation between the concentration of inorganic phosphate in a medium and the production of antibiotics in the fermentation of cephalosporin by *Streptomyces clavuligerus*; the production of cephalosporin was increased with increases in phosphate until the concentration reached 25 mM. Further addition of phosphate progressively decreased the production. As seen

Fig. 2. Influence of addition of MgP ammonia concentration and leucomycin production in a chemically defined medium[51].

Chemically defined medium: 3% glycerol, 0.5% glucose, 1% ammonium lactate, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% K_2HPO_4, 0.5% $CaCO_3$, 1 ml/liter a trace metal solution (each at g/liter, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 2H_2O$), and 0 or 1% $Mg_3(PO_4)_2 \cdot 8H_2O$.

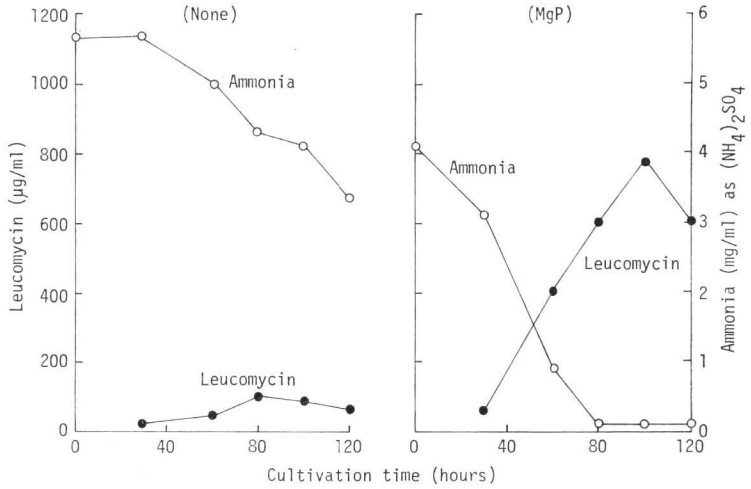
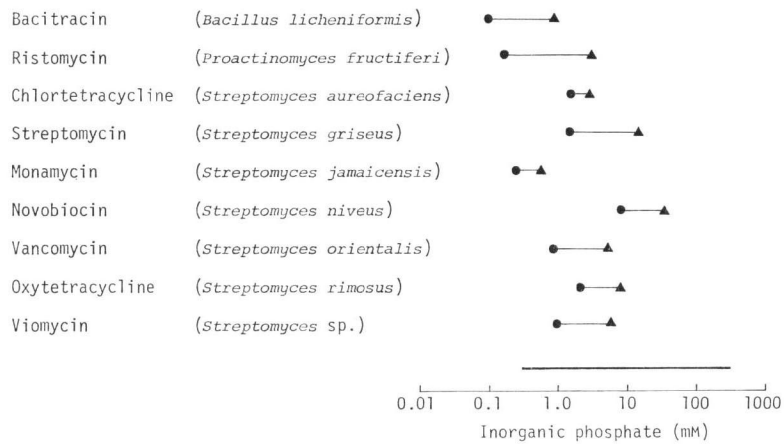


Fig. 3. Relation between antibiotic production and the amount of inorganic phosphate.
●: The maximal production of antibiotic is observed at this concentration or lower.
▲: Production of antibiotic does not occur at this concentration or higher.
——: The range of optimal growth concentrations of both procaryotes and eucaryotes.
(modified from E. D. WEINBERG, Dev. Ind. Microbiol. 15: 70, 1973[53])



in Fig. 3, microorganisms have their optimal phosphate concentration for growth in a range of 0.3 to 300 mM, but the amount of inorganic phosphate adequate for the production of antibiotics is usually much lower than the amount required for growth (<10 mM), similar to the carbon or nitrogen source.

As described above, the amount of inorganic phosphate strongly influences the production of antibiotics, but the precise mechanism has not been clarified. However, it is presumed to be based on the intracellular ATP concentration from the fact that the production of antibiotics usually begins after inor-

ganic phosphate in the culture broth is almost entirely consumed and from the study on the production of candicidin by MARTIN *et al.*[56,57]. The intracellular concentration of ATP is increased and the primary metabolism is accelerated when the concentration of inorganic phosphate in the culture is high. When the amount of inorganic phosphate is lowered, the ATP concentration decreases; it is hypothesized that this decrease derepresses metabolic conversions which are required for the production of antibiotics. The production of streptomycin (SM) is reduced by addition of excessive inorganic phosphate; MILLER and WALKER[58] propose that in the presence of a large amount of inorganic phosphate SM is accumulated in the form of SM-phosphate which has no antibacterial activity. According to this concept, they suggest the possibility that alkaline phosphatase which easily converts SM-phosphate to SM should be utilized in screening of antibiotics.

IMANAKA and coworkers[59~61] carefully considered the concentration of inorganic phosphate in their screening of antibiotics. They performed screening using a medium containing a high (>180 mM) concentration of organic phosphate in which antibiotics are usually not produced and discovered pyrrolnitrin[59], bicyclomycin[60], and thiopeptin[61]. HALL and HASSALL[62] reported that two antibiotics were selectively produced by *Streptomyces jamaicensis* depending on the concentration of inorganic phosphate: with 0.1 mM inorganic phosphate, monamycin, a depsipeptide and with 0.4 mM an antibiotic obviously different from monamycin were obtained. As seen in the above two examples, varying the amount of inorganic phosphate in a production medium is important in screening of antibiotics.

1–4: Inorganic Salts: It has been well known for rather long time that addition of inorganic salts such as NaCl to antibiotic production media increases the production. In 1946 RAKE and DONOVICK[63] reported that a marked increase in the production of SM was observed by adding 0.5% NaCl, but addition of a larger amount of NaCl usually inhibits the production. Natural media are generally used for production of antibiotics, but when using such media, the influence of inorganic salts must be considered. For example, meat extract contains a large amount of NaCl as an antiseptic agent, and corn steep liquor, which is acidic because of residual hydrochloric acid used in its preparation, contains a high concentration of salts when neutralized.

Usually the amount of NaCl added is 0.5% or less, but OKAMI *et al.*[64] found that aplasmomycin is produced best in the presence of NaCl as high as 1.0~3.0%. OGATA *et al.*[65] found that the production of antibiotic a-60, a quinone, was accelerated by addition of a high concentration of $MgSO_4$. The optimal concentration of $MgSO_4$ depends on the amount of glycerol as the carbon source; 0.1% $MgSO_4$ is required at 2.0% glycerol and 2.5% at 0.5% glycerol.

As seen in the above examples, it must be considered screening of antibiotics that some antibiotics are produced under conditions as different from the usually known ones.

1–5: Trace Metals: It is obvious that fermentation processes are based on the reactions of enzymes. Not only enzymes and substrates but co-factors such as metals are needed for the reactions to proceed smoothly. Therefore, one can presume that some specific metals will be related to the production of individual antibiotics, and in fact various metals affect the production of antibiotics (Table 4).

In the production of antibiotics from *Micromonospora,* Co^{++} is added in many cases. The components of gentamicin from *Micromonospora purpurea* varied depending on the concentration of the Co^{++} ion. At a high concentration of Co^{++}, *C*-methylation was accelerated to increase the production of the components C_1 and C_2, while the production of C_{1a} and C_{2b} (sagamicin) was inhibited. Addition of an moderate amount of Co^{++} is needed in the production of the components C_{1a} and C_{2b}[66]. A simi-

Table 4. Influence of metals on antibiotic production at a concentration higher than the optimal growth concentration.

Antibiotic	Producing organism	Metal concentration ($\times 10^{-5}$ M)	
		Positive effect	Negative effect
Bacitracin	*Bacillus licheniformis*	Mn (0.07)	Mn (4)
Bacillin	*Bacillus subtilis*	Mn (10*)	
Subtilin	*Bacillus subtilis*	Mn (0.5)	
Actinomycin	*Streptomyces antibioticus*	Fe (10*), Zn (10*)	
Monensin	*Streptomyces cinnamonensis*	Fe (100)	
Neomycin	*Streptomyces fradiae*	Fe (1.0), Zn (0.1)	Fe(15), Zn(1.0), Mn(10)
Candicidin	*Streptomyces griseus*	Fe (4.0*), Zn (4.0*)	
Grisein	*Streptomyces griseus*	Fe (4.0)	
Streptomycin	*Streptomyces griseus*	Fe (1.0), Zn (0.3)	Zn (20)
Chloramphenicol	*Streptomyces venezuelae*	Fe (2.0*), Zn (2.0*)	
Mitomycin	*Streptomyces verticillatus*	Fe (40*)	
Penicillin	*Penicillium chrysogenum*	Zn (0.1), Fe (2.0*)	Zn (3.0), Cu (1.0*)
Griseofulvin	*Penicillium griseofulvum*		Zn (20*)
Patulin	*Penicillium urticae*	Zn (0.1*), Fe (1.5)	

without* mark: 100% effective with concentration
with* mark: 50% effective with the concentration
(modified from E. D. WEINBERG, Adv. Microbiol. Physiol. 4: 1, 1970[63])

lar example involves the addition of Co^{++} in the coumermycin fermentation by *Streptomyces rishiriensis*. Component A_2, a demethylated substance of component A_1, decreased, while A_1 increased greatly[67]. *Streptomyces viridoflavus* produced two kinds of polyene macrolide antibiotics. When adding Zn, candihexin (a hexaene) increased, but not candidin (a heptaene). Interestingly both are supposed to be formed by a similar process of biosynthesis[42].

According to WEINBERG[68,69], among 9 trace metals (V, Cr, Mn, Fe, Co, Ni, Cu, Zn and Mo) said to be indispensable for living creatures, Mn, Fe and Zn are the ones usually important for the production of secondary metabolites. Generally, important metals are said to be Mn for *Bacillus*, Fe for bacteria other than *Bacillus*, Fe and Zn for actinomycetes and Zn for fungi. The amount required for production is larger than that required for growth: the concentrations added for growth are usually about 10^{-7} M Mn, 10^{-7} M Zn, and 2×10^{-7} M Fe, but for production, the concentrations required are usually 10 to 100-fold greater. Addition of further amounts inhibits production. However, considering that the production of primary metabolites is not inhibited by 10^{-3} M concentration of such metals, the range of effective concentration is clearly narrower in the case of secondary metabolites. The production of secondary metabolites such as antibiotics are not indispensable for life, yet it depends on the primary metabolism needed for the growth of a living organism. It is obvious that antibiotics are produced under narrower conditions than those in the production of primary metabolites. This hold true not only for metal concentration but also for most other factors.

1-6: Precursors: It is well known that in the PC fermentation by *Penicillium chrysogenum* a high amount of PC-G is selectively produced by adding phenylacetate as a precursor[70]. Although the addition of precursors has been tried with many antibiotics, it is rather rare for the antibiotic production to increase merely by addition of precursors. The action of a regulatory system is presumed in the secondary metabolism, similar to the primary one. However, among biosynthetic enzymes for the secondary

Table 5. Production of new antibiotics by addition of precursor analogs using wild strains.

New antibiotic	Original antibiotic	Precursor analogue	Producing organism
Desalicetin 2′-(4-aminosalicylate)	Celesticetin	4-Aminosalicylic acid	*Streptomyces caelestis*[71]
Quinazomycin	Echinomycin	Quinazol-4-one-3-acetic acid	*Streptomyces* sp. X-53[72]
5-Fluoropolyoxins L & M	Polyoxins L & M	5-Fluorouracil	*Streptomyces cacaoi*[73]
New pyrrolnitrin derivatives	Pyrrolnitrin	5-Fluorotryptophan *etc.*	*Pseudomonas aureofaciens*[74]
Neoviridogrisein II	Viridogrisein	D-Proline	*Streptomyces* sp. P-8648[75]
3-*O*-Oleandorosyl-5-*O*-desosaminyl erythronolide A oxime	Oleandomycin	Erythronolide A oxime	*Streptomyces antibioticus*[76]
Dihydronovobiocin	Novobiocin	Dihydronovobiocic acid	*Streptomyces* sp.[77]

metabolites such as antibiotics, some have a low substrate specificity. Thus when adding analogs as precursors, they are sometimes incorporated to give new products. Some examples are shown in Table 5. ARAI *et al.*[78] reported that by adding NaCN to the production medium, the production of saframycin, which has a CN group in the molecule, was greatly increased. Many antibiotics contain halogens, S or P in the molecules. It may be possible to vary the amount of such elements and direct the production of antibiotics, but such a report has not yet been published.

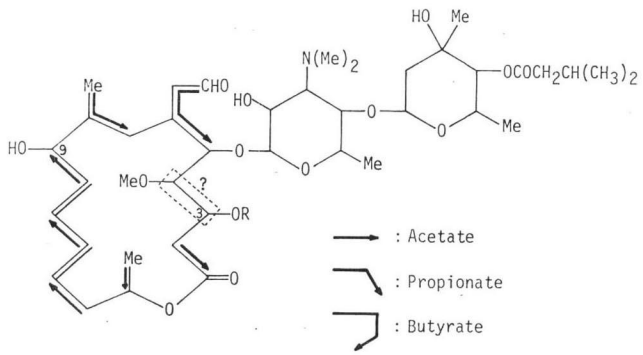
Mutational biosynthesis[80] (or mutasynthesis[81]), which was employed in the discovery of hybrimycins by SHIER *et al.*[79], utilizes idiotrophs (antibiotic-blocked mutants) and the precursor method. The technique is based on the theory of BIRCH[82], and many new substances have been produced by it: the kinds of antibiotics produced are aminoglycosides, aminocyclitols, macrolides, novobiocin, β-lactams and other antibiotics[81,83,84]. Mutational biosynthesis requires the isolation of idiotrophs. New product can also be produced with wild strains by using selective inhibitors for biosynthesis of antibiotics such as cerulenin. ŌMURA *et al.* proposed to call such biosynthesis of new products using idiotrophs or biosynthetic inhibitors as "hybrid biosynthesis"[85].

1-7: Inhibitors: Upon adding ethionine, an analog of methionine, and sulfa drugs affecting one-carbon transfer reactions to antibiotic fermentations, demethylated derivatives of the original antibiotics are produced. These include 7-chloro-6-demethyltetracycline in the tetracycline fermentation[86,87], and *N*-demethyllincomycin in the lincomycin fermentation[88,89].

It is known that chloramphenicol (CP) inhibits protein biosynthesis, but does not inhibit biosynthesis of peptide antibiotics. The production of actinomycin[90] and polymyxin[91], which are peptide antibiotics, is reported to increase by CP addition. This action is thought to be due to interruption of protein synthesis and promotion of the flow to peptide antibiotic. The increase in production of aminoglycoside antibiotics such as streptomycin and kanamycin by addition of cell wall synthesis inhibitors such as bacitracin would be similar to the above phenomenon: *i.e.*, there are precursors which are common to the biosynthesis of aminoglycoside antibiotics and cell walls and when inhibiting the route to the cell wall biosynthesis, metabolism will progress towards the production of antibiotics[92,93].

ŌMURA *et al.*[94,95] studied biosynthesis of LM with ^{13}C-precursors and ^{13}C NMR, and obtained the results shown in Fig. 4. The origin of the carbon skeleton at positions 3 and 4 is still unknown. To study the origin of these carbon skeletons, various organic acids were added to the production medium. It was found that by addition of 0.1% butyrate, LM production increased about 2-fold[96]. The

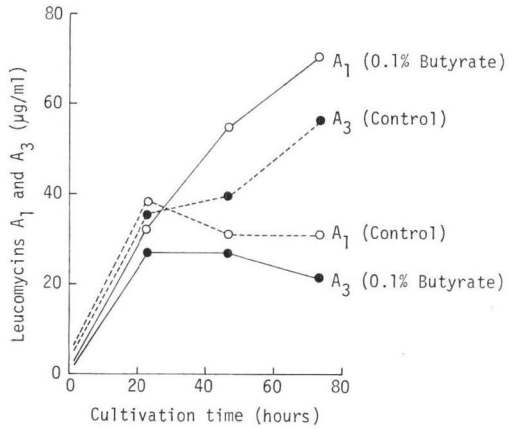
Fig. 4. Structure of leucomycins and biosynthesis of the lactone ring of leucomycins elucidated by using ^{13}C precursors and ^{13}C NMR[94,95].



Leucomycin	R
A_1	H
A_3	$COCH_3$

increase was due to a shift in production of LM components, as shown in Fig. 5. In the system containing no butyrate, LM-A_3 was predominant, while with butyrate the predominant component was LM-A_1 which has a stronger antibacterial activity than LM-A_3. The influence of butyrate on the acylation of LM-A_1 to LM-A_3 was studied and butyrate was found not to inhibit the enzymatic reaction itself but to repress the production of enzymes[97,98]. Butyrate also inhibited acetylation of spiramycin I to II[99].

Fig. 5. Influence of butyrate on production of leucomycin A_1 and leucomycin A_3[96,98].



The relationship between antibiotics and producing microorganisms presents many interesting problems[100~102]. When discussing the productivity of antibiotics, there must be a separate consideration of autotoxic antibiotics which inhibit the producing microorganisms themselves and xenotoxic antibiotics such as antimycin, PC, polyene macrolides and polyoxins which have no target site in the producing microorganisms. In the former group, a more self-resistant mutant strain often shows an increase in production. Although there is a feedback inhibition in certain fermentations *e.g.*, the aurodox fermentation[103], there exists production acceleration in the erythromycin fermentation by erythromycin itself (*i.e.*, autostimulation)[104]. However, there is known no report dealing with the exploitation of feedback inhibition or the effect of autostimulation in antibiotic screening. There are only cases in which production of a known antibiotic has been improved by such manipulations. For example, a mutant strain resistant to feedback inhibition by an analogue of the primary metabolite precursor increased production of the precursor and showed an increase in production of pyrrolnitrin[105] or maridomycin[106]. KOMINEK[142] reported that the use of continuous dialysis-extraction in the cycloheximide fermentation by *Streptomyces griseus* resulted in relief from end-product inhibition and a twofold titer increase.

1-8: Inducers: Similar to their roles in primary metabolism, inducers and inhibitors influence the biosynthesis of antibiotics, and work together to control the production of substances. However, to

reveal the presence of inducers is more difficult experimentally than inhibitors, and this is the reason that our understanding of inducers is meagre compared with inhibitors.

There are interesting reports on inducers which are produced by antibiotic-producing microorganisms. For example, a small amount of A Factor induces SM production[107] and a lactone with the formula $C_{12}H_{22}O_4$ induces staphylomycin production[108]. However, they have not been utilized as additives to production media in routine screening.

Table 6. Influence of butyrate and glucose on the bioconversion of leucomycin A_1 to leucomycin A_3[98].

Addition to growth medium	Conversion of LM-A_1 into LM-A_3 (%)
None	0
Butyrate (0.1%)	0
Glucose (2%)	39.8
Glucose (2%)+Butyrate (0.1%)	10.9

Growth medium; 2% soluble starch, 0.5% peptone, 0.5% meat extract, 0.3% yeast extract, 0.5% NaCl, 0.3% $CaCO_3$. Incubation medium; 2% glucose, 0.5% NaCl, 20 μg/ml cerulenin.

In the previous section, the production of A_1 and A_3 components of LM was shown to vary depending on the addition of butyrate to the medium. Such variation of components has also been shown to depend on medium ingredients especially glucose[97]. Table 6 shows the influence of glucose and butyrate on the bioconversion of LM-A_1 to LM-A_3. LM-producing cells were cultivated in four kinds of media and cerulenin was added to the intact cell-reaction system to inhibit the *de novo* synthesis of the lactone ring of LM. The substrate LM-A_1 was added to the reaction mixture to determine the conversion rate from LM-A_1 to LM-A_3 (Table 6). Inactive cells were produced in a production medium containing soluble starch as a carbon source, but activity was induced by adding glucose; the induction was inhibited by butyrate. Although catabolite inhibition of antibiotic production by glucose is known[27~37], but this is the only report that suggests induction of an antibiotic-biosynthesizing enzyme by glucose.

1-9: Other Factors: Elasnin produced by *Streptomyces noboritoensis* KM-2753 is a selective inhibitor of human granulocyte elastase[109,110], and is formed from 12 acetates as seen in Fig. 6[111]. From the regularity of incorporation of these acetates, the elasnin skeleton is presumed to be constructed from four linear C_6 acids. Various linear fatty acids from C_6 to C_{20} were added at 10 μg/ml expecting a pre-

Fig. 6. Structure of elasnin and incorporation of [1,2-^{13}C]acetate into the elasnin carbon skeleton[111].

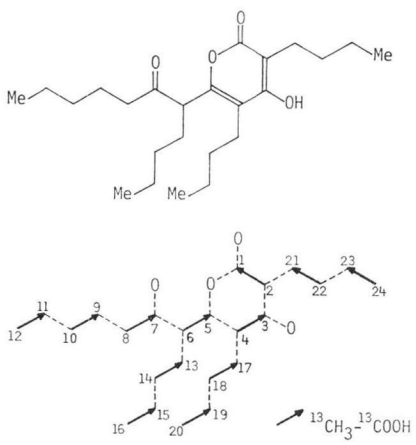


Table 7. Influence of addition of various long-chain fatty acids on elasnin production by *Streptomyces noboritoensis* KM-2753[112].

Long-chain fatty acid	Trivial name	Elasnin production (mg/ml)
None		0.31
10:0	Capric acid	0.06
11:0	Undecanoic acid	0.35
12:0	Lauric acid	5.12
13:0	Tridecanoic acid	0.34
14:0	Myristic acid	3.81
14:1 (*cis*-9)	Myristoleic acid	0.68
15:0	Pentadecanoic acid	0.98
16:0	Palmitic acid	2.29
16:1 (*cis*-9)	Palmitoleic acid	0.44
18:0	Stearic acid	1.61
18:1 (*cis*-11)	Vaccenic acid	0.31
20:1	Eicosanoic acid	1.27

Table 8. Influence of pH before sterilization on kinamycin production by *Streptomyces murayamaensis*[116].

Temperature (°C)	pH*	pH**	Age (hours)	pH	Mycelium (ml/10 ml)	Residual sugar (%)	Kinamycin formed (mcg/ml)
24	6.0	6.1	30	5.8	2.0	1.37	1.7
	7.0	6.3	72	5.9	2.8	trace	34.4
	8.0	6.5	72	5.9	2.5	0.38	41.2
27	6.0	6.1	72	5.8	2.9	trace	4.3
	7.0	6.3	72	5.9	2.9	trace	22.0
	8.0	6.5	72	6.0	2.3	0.38	44.0

* pH of medium before sterilization.
** pH of medium after sterilization.
Medium: 2% glucose, 2% soybean meal, 0.3% NaCl.
Fermentation conditions: fermentor, 5 liters; aeration, 5 liters/minute; agitation, 600 r.p.m.; inoculum size, 10% (v/v).

cursor effect on the productivity of elasnin (Table 7). Addition of *n*-C_6, *n*-C_8 and *n*-C_{10} acids caused cell-lysis of the producing microorganism, and addition of the odd number acids *n*-C_{11}, *n*-C_{13} and *n*-C_{15} did not increase the yield. However, addition of *n*-C_{12}, *n*-C_{14} and *n*-C_{16} fatty acids, particularly *n*-C_{12}, lauric acid, increased greatly the production of elasnin[112]. If lauric acid is a precursor of elasnin, it might be converted into *n*-C_6 acid by β-oxidation and incorporated into elasnin. However the 16-fold increase of production by addition of such a small amount of lauric acid as 10 μg/ml must be caused by some action other than that of a precursor, for example, change of the membrane permeability or an inducing effect.

Although pH of a medium is often noted after sterilizing, but since the medium has been subjected to severe conditions of high pressure sterilization before use, the pH before sterilization is also of importance. Aminocarbonyl reactions of sugars and amino acids under heating conditions especially in the alkaline pH has been particularly noted by the food industry. The formation of a substance[113] accelerating the growth of lactic acid bacteria from L-lysine and glucose and formation of psicose[114] from glucose by high pressure sterilization have also been reported. In this regard, it is of interest that the fermentation of kinamycin, a quinone antibiotic having a N-CN group[115], which was discovered by the authors' laboratory was not reproducible initially. However, as shown in Table 8, when the pH before sterilization was adjusted to the alkaline side, product formation could be stabilized[116]. Since the pH after sterilization in this medium was about 6 in either case, the problem was not the initial pH, but presumably some substances, produced at pH 8 under high pressure sterilization, which is related to the production.

2. Culture Conditions

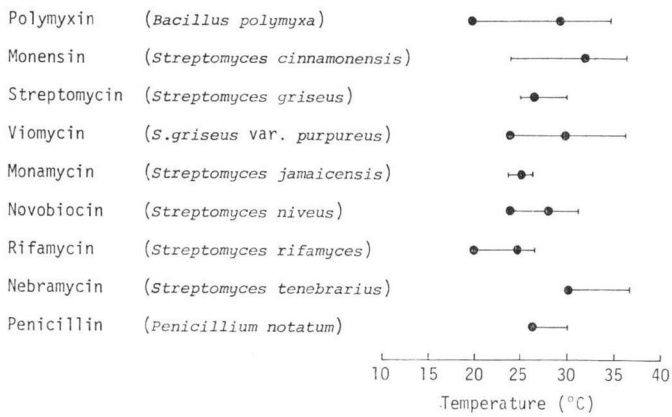
2-1: pH: The pH of cultivation affects not only the growth but the production as well as do the medium constituents and the temperature. The inhibition of antibiotic production by glucose or K_2HPO_4 is not only to the above-described regulatory controls but also to the effect on pH during cultivation. When keeping the pH of the culture broth at about 6.0 by addition of $CaCO_3$, K_2HPO_4 or $NaHCO_3$ in the fermentation of helvolic acid and cerulenin by *Cephalosporium caerulens*, production of the former increased, but that of the latter was little affected[117] (Table 9). HORIKOSHI[118~120] studied the production of various enzymes by alkalophilic bacteria isolated from a Na_2CO_3-containing medium (pH 10), but there is no report on the production of antibiotics of such bacteria. Screening of antibiotic produc-

Table 9. Influence of addition of $CaCO_3$ and K_2HPO_4 on production of cerulenin and helvolic acid by *Cephalosporium caerulens*[117].

$CaCO_3$ or K_2HPO_4 (mg/ml)		Addition time (hours)	pH	Antibiotics (μg/ml)	
				Cerulenin	Helvolic acid
$CaCO_3$	2.0	0	6.4	130	165
	6.0	0	6.4	110	150
	10.0	0	6.6	130	230
	10.0	24	6.2	140	230
K_2HPO_4	0.5	0	4.8	140	48
	2.0	0	6.2	110	320
	5.0	0	6.4	69	230
	1.0	24	5.8	132	280
	2.5	24	6.2	191	370
Control (no addition)			4.4	200	21

Medium: 1% glucose, 3% glycerol, 0.5% peptone and 0.2% NaCl.

Fig. 7. Relation between the antibiotic production and the production temperature.
●: Optimal production temperature.
|——|: The temperature range in which production is seen.
(modified from E. D. WEINBERG, Dev. Ind. Microbiol. 15: 70, 1973[53])



tion by alkalophilic bacteria, actinomycetes and fungi would be interesting. An alkalophilic fungus was found to produce an antibiotic which is active against Gram-positive bacteria and inactivated by penicillinase[121].

2-2: Temperature: It is known that thermophilic actinomycetes such as *Thermoactinomyces* produces new antibiotics at temperatures higher than 40°C, but *Streptomyces* usually produces antibiotics at temperatures near 27°C (Fig. 7). Generally the range of a good growth temperatures is supporting good growth as wide as 25 degrees, but the temperature range adequate for good production of secondary metabolites is narrow *i.e.*, 5~10 degrees.

Usually, cultivation for antibiotic production is performed under one constant temperature from the beginning to the end, but the temperature adequate for growth is not always the same as that for production. When a PC-producing strain was grown at 30°C and then shifted to 20°C for production a highly effective process was obtained[122]. *Streptomyces* sp. No. 81 strain, which produces antibiotic M-81 at

27°C, forms cryomycin at the low cultivating temperature of 12°C.[123~125] Thus temperature must be considered separately for growth and for production. It would be of interest in antibiotic screening to use temperature shifts.

2-3: Oxygen: Many antibiotic-producing microorganisms require oxygen for growth. The water solubility of oxygen is very low and scale-up of antibiotic fermentation is based on dissolved oxygen in a cultivation medium[126~128]. For determining the conditions for large-scale cultivation of individual antibiotics, aeration and agitation conditions are selected depending on the optimal concentration of dissolved oxygen, but there are only few reports of precise research on the relation between production of antibiotics and the oxygen concentration.

In the fermentation of *Cephalosporium acremonium*, PC-N production decreases and cephalosporin C increases as dissolved oxygen is increased; it is caused by the oxidative conversion of PC-N to cephalosporin C. The reaction is accelerated by increasing the oxygen partial pressure in a cell free system[129]. *Streptomyces lavendulae* No. 314 produces streptothricin as the main product using 250 rpm agitation in a jar-fermentor. Satellite antibiotics, mimosamycin and chlorocarcins, are produced only under high concentration of dissolved oxygen (*i.e.*, agitation, 550 rpm)[130]. PLICHON *et al.*[131] found that in the oxytetracyline fermentation by *Streptomyces varsoviensis*, the production of tetracycline increased on increasing the oxygen concentration.

The optimal level of dissolved oxygen may be different between growth and production. Therefore, it will be neccessary to cultivate not under one aeration or agitation condition, but perhaps with shifts in this parameter. In the daunorubicin fermentation, after cultivating under vigorous aeration and agitation condition for 4 to 6 days, agitation is stopped and the culture is allowed to stand overnight. The glycosidic bond of daunorubicin is then cleaved to form 7-deoxy-daunorubicinol aglycone[132].

NASH[133] reported that in the erythromycin fermentation, introduction of CO_2 did not affect growth but inhibited the production, and presumed that CO_2 was contributing to lactone formation.

2-4: Others: Besides the factors described above, there are others that affect antibiotic production, such as pressure, oxidation-reduction potential and light.

It is frequently experienced in the study of antibiotics that they are not produced in a shaking culture but produced on an agar plate. In the production of fumaramidmycin, the difference is due to the breakdown of the antibiotic under the two conditions. The antibiotic producing activity is almost the same on both agar plates and in shaking culture, but the decomposition of the antibiotic is less on agar. Also fumaramidmycin easily diffuses into the agar plate so that it may avoid contact with decomposing enzymes[134,135].

Antibiotic SF-1993 (*N*-carbamoyl-D-glucosamine) is produced by filamentous hyphae of *Streptomyces halstedii*, but not produced by hyphae which are well fragmented. On agar plates fragmented hyphae are not observed; they are seen in shaking culture. This antibiotic can be produced in a shaking culture by using a diluted medium or mutants having non-fragmented hyphae[136,137].

OKANISHI *et al.*[138] found in their study on the relation between plasmids and the production of antibiotics that producing organisms in which plasmids were cured did not produce antibiotics in a shaking culture but only on agar plates containing fatty acids such as oleate or palmitate.

Although agitation is usually considered only from the viewpoint of oxygen supply, it may have other effects.[139] Cellular damage by agitation affects the production of maridomycin by *Streptomyces hygroscopicus*[140]. In the bicyclomycin fermentation by *Streptomyces sappronensis*, the producing

microorganism suffers a reduction in antibiotic production by increased agitation, concomitant with an absence of aerial mycelium. The phenomenon is thought to be connected with a loss of plasmids by agitation[141].

IV. Final Remarks

Various factors affecting the production of antibiotics have been discussed in this review. Different methods of cultivation can be developed by combinations of these individual factors. If the desired effects can be obtained by merely adding substances to the production medium, the development will require only simple operations and be advantageously utilized in screening. However, even when selecting media for improving the productivity of antibiotic, empirical experiments to choose the kinds and the concentrations of carbon, nitrogen, and phosphate sources are usually employed (although sometimes data treatment by computor is employed). Unfortunately, additives to media which can be broadly applied to production of a wide range of antibiotics are not known. We have found that addition of MgP greatly increased the production of LM by 5 to 10-fold. The increase is presumed to be caused by decrease of NH_4^+ in the medium due to addition of MgP. Of importance is that the production of many antibiotics belonging to the macrolide, β-lactam, and aminoglycoside types was also increased. Thus the discovery of MgP as an ammonia reducing agent suggests a novel fermentation technique which is to be called "nitrogen limiting fermentation" and applied generally to fermentations.

In antibiotic screening, it is particularly necessary to consider the components of a production medium. Besides ordinary methods for changing the composition based on the kind of carbon and nitrogen sources and their quantitative balance represented by the C/N ratio, one must pursue the development of production media from the theoretical viewpoint. The study of PC has played a pioneer role in antibiotic science and has had a great influence on the media and cultivation methods used for antibiotic production. However, it is unfortunate that there have been no studies which improve upon the information obtained from the PC fermentation. Genetic engineering is merely beginning to be directly applied to antibiotic production, and much progress is expected in the future. Antibiotics are important in medicine and they have been earnestly studied as therapeutic substances, but, the fundamental study on their fermentation is unsatisfactory. If fundamental studies on the regulatory mechanisms and biosynthetic pathways of antibiotic production are developed and these data are applied intelligently, improved methods for not only producing antibiotics but for new antibiotic screening will be established.

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