한국식물보호학회지 : 제19권 제 3 호
Korean J. Pl. Prot. 19(3) : 163~168(1980)

Studies on new antibiotics in Korea IV

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ABSTRACT

The antibiotic bacterium JS7901, one of the eighty three antibiotic microorganisms which have been is olated in the surburbs of CheongJu-city, showed the most effective antimicrobial activities against test organisms, both bacteria and fungi. Among the different culture media Soytone Sugar medium was the most effective for growth and activity of the JS7901 antibiotic bacterium a against both *Escherichia coli* and *Staphyllococcus aureus* by the cylinder plate method. The higher the sugar content, was, the greater the antibiotic amount of substances of JS7901 were produced in the soytone sugar media. The antibiotic bacterium, JS7901 appeared to have a broad activity spectrum showing inhibition in Vitro against gram positive and negative bacteria and plant disease fungi. In general, the active substances were not transferred into organic solvents. Only a small portion of the activity was transferred into ethyl ether and was also adsorbed to active carbon when the cultured broth was also adsorbed to active carbon when the cultured broth was at pH 2.0~4.0.

On adjusting at pH 8.0, the activity disappeared. The crude active substances could be obtained by means of vacuum drying method and still showed strong activity. The dried active cake was solved by solvents and crystallized into various shapes. The active substances were developed on the silica gel plate in the solvent system of n-butanol-acetic acid-water(3 : 1 : 1) and gave 5 pinkish colored spots when sprayed with 0.2% ninhydrine in ethanol. The upper 5th spot, which was the result of using disc plate method with *Escherichia coli* was the strongest of these spots.

INTRODUCTION

For some years we have suggested the utilization antibiotics for controlling plant diseases and hathus worked in the development of the antibic bacterium JS7501 since it was found on a tri dish by chance when culturing *Alternaria lonoes*, a brown spot disease fungus of the tobao plant.

These days only a few known antibiotics including Streptomycin have been used for plant disease control. There appears thus to be a need to try to meet requests for agricultural antibiotics able to control plant diseases which have to the present time been able to be prevented by application of agricultural chemicals. The merits of such antibiotics are their low toxicity their selectivity and accuracy in their effects, the capacity of their residue to easily decompose in nature and these merits would appear to outweigh the negative effect of sometimes including resistance of the pathogens,

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when they are continuously used for many years,

For the purpose of plant disease contol, we have isolated antibiotic bacteria which show strong inhibition against gram negative bacteria, *Escherichia coli*.

This paper refers to further some experiments studying the activity spectrum of JS7501, which from this point we will refer to as JS7901. This new strain(JS7901) was selected during the process of experimentation.

The main purpose of these studies were to find which was the most suitable medium for producing the active substances of the JS7901 and to separate the active substances from the whole broth if possible.

MATERIALS AND METHODS

In general,materials and methods were the same as those in our ealier papers.[8,9,10] Five hundred soil samples were collected from manures, fields, paddy fields and hills n the surburbs of Cheongju-city. Antibiotic microorganisms were isolated from the soil samples by using a reciprocating shaking machine 6cm amplitude and 120 strokes per minute. Sixty m*l* of a medium was placed in a flask of 300m*l* volume and sterilized for 30 minutes at 120°C. One loop full of soil was inoculated into the NBS medium (8gr nutrient broth plus 10gr sugar in 1000 m*l* water) within a period of 72~96 hours the shaking cultured broth was tested by the cylinder plate method on the penicillin assay agar medium with *Escherichia coli*.

The JS7901, one of the strong antibiotic bacteria screened, was cultured by stand culture in the various liquid culture media, to find the most suitable medium for the more active isubstances. The composition of the media is as follows: NBS-0.8% nutrient broth, 0.2% sugar., PS-20% potato, 0.2% sugar., and BS-4% bean, 0.2% sugar., SS-0.4% soytone, 0.2% sugar., CS-0.2% corn steep liquor, 0.2% sugar., and PA(penicillin assay medium)-0.15% beef extract,0.3% yeast extract,0.4% casitone, 0.6% peptone and 0.1% sugar.

The media used in tests of antimicrobial activity spectrum were different in different test organisms. in human disease and general bacteria were assayed in a period between 16~24 hours at 37°C but test organisms in plant disease fungi and bacteria were investigated after 72 hours at 28°C and in a period of 16~24 hours at 30°C respectively.

In order to allow crystallization of the susbtances, the whole borth of JS7901 was concentrated in vacuum and dried up into cake form, containing active substances. The cake was repeatedly solved by different organic solvents in turn.

Thin layer chromatography was used for separation and identification of the JS7901. The aqueous broth and the crude cake were spotted on the silica gel G (marked by Merk Co.) plates. The plates were developed in the various solvent systems. Spots were colored by spraying 20% H_2SO_4 or 0.2% ninhydrune in ethanol and heat for 10 minutes at 80°C in a dry oven. Thereafter the colored spots were carefully dug out and were poured into a phosphate buffer solution at pH7.0. Six mm paper discs were wet with 10 micro m*l* of the solution and were dried for assaying.

RESULTS

The Isolation of the Antibiotic Microorganism:The test was carried out to find the antibiotic bacteria which most effective inhibited gram negative bacteria, *Escherichia coli*. As shown in table 1, eighty three antibiotc microorganisms were selected from the 500 soil samples collected from 10 places in the surburbs of Chenogju-city. Most of the antibiotic microorganisms were classified into Eubacteria and some others were fungi and Actinomycetes. Of these the JS7801 has shown the most effective antibiotic activity against plant disease pathogens in Vitro.

The Characteristics of Culture: The cultural conditions of JS7901 were contrasted for activity over a range of 6 different media: PS, SS, BS, NBS,PA and CS. As shown in table 2, PS and SS medium appeared to have the strongest activities showing the inhibition zones of 18.1mm in diameter against *Escherichia coli* respectively. NBS and SS medium showed 16.8mm and 15.7mm in diameter of the inhibition zones. The SS medium also showed good

Table 1. Antibiotic microorganism isolated from soil samples collected in around Cheongju-city.

places	antibiotic samples							total
gaduk-myun	70							1
mooneu-myun	114.	117.	118.					3
oaksan-myun	216.	228.	254.	256.	257.	261.	262.	14
	268.	269.	271.	276.	277.	283.	290.	
buckil-myun	292.	296.	302.	305.	310.	315.	336.	8
	339.							
ohchang-myun	346.	352.	353.	354.	355.	356.	357.	18
	359.	361.	390.	391.	393.	394.	395.	
	396.	397.	398.	399.				
jeungpyoung-eub	401.	403.	405.	409.	410.	411.	412.	15
	413.	417.	418.	421.	422.	423.	424.	
	426.							
cheoungju-city	426.	427.	428.	429.	430.	431.	432.	34
	433.	434.	435.	436.	437.	438.	439.	
	440.	441.	442.	443.	444.	455.	466.	
	447.	448.	449.					(83)

The numbers are collected numbers of soil samples.

growth. Therefore the SS medium was considered most suitable for culturing JS7901.

Table 2. Inhibition zones comparing the activities of JS7901 cultured in various liquid media.

culture media	PS	SS	BS	NBS	PA	CS	disc of 10 mcg
pH after cultivation	5.4	5.4	5.4	5.6	5.6	5.6	streptomycin
test organisms							
Escherichis coli	18.1	18.1	16.8	11.4	15.1	13.8	10.6
Staphyllococcus aureus	15.1	15.7	15.1	16.8	13.8	15.4	15.4

To find the factor of increasing activity between oytone and sugar in the above SS media, the S7901 was cultured under shaking culture conditons, and then tested for activity against *Escherichia coli*. As shown in table 3, 20% sugar in the media increased the activities more than 10% sugar in the media, despite varying differences of the oytone content of the media.

Table 3. The comparisions of activities of JS7901 against *E. coli* in media of varying soytone & sugar content.

oytone \ sugar	10%	20%
5%	25	34(mm)
10%	26	34

Antimicrobial Activities: The in Vitro antimicroal spectrum of JS7901 was determined with bactia and fungi by the cylinder plate method. Aqueous both was used for the test after being cultured in SS media for 4 days. The results are shown in table 4. Test organisms are also listed in the table and the inhibition zones were approximately 30cm in diameter each. It became evident that

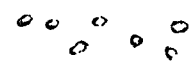
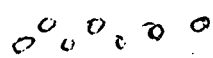
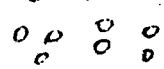
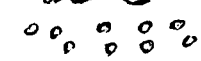
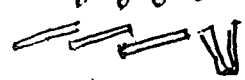
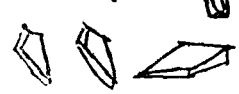
Table 4. The antimicrobiol effects of JS7901 tested by the cylinder plate method.

Test organisms	groups of microorganisms
Bacillus subtillus *Staphyococcus aureus* *Salmonella typhi* *Shigella dysenteriae*	gram positive bucteria
Esherichia coli *Erwinia arodae* *Pseudomonas glycinea* *Xanthomonas oryzae*	gram negative bacteria
Alterraria longipus *Glomerella cingulata* *Helminthosporium oryzae* *Pyricularia oryzae*	plant disease fungi

JS7901 was active against gram positive and negative bacteria as well as plant disease fungi.

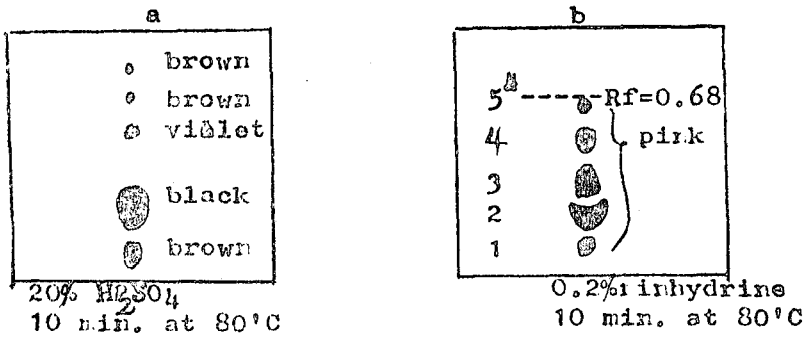
Physical, Chemical and Biological Properties of JS7901: The active substance was not transferred into n-butanol, iso-butanol, n-propanol, iso-propanol, ethylacetate, chloroform, petroleum ether and benzene or acid, neutral and alkali. A small portion of the activity against *Escherichia coli* was transferred into ethyl ether but substantially was left in the aqueous layer. The active substance was adsorbed by active carbon when the broth of pH 2.0~4.0 was applied and this part was eluded by washing with 50% acetone. A some portion of the activity was left without adsorption. The activity of aqueous broth disappeared above pH 8.0 in alkaline. The active substance seemed to precipitate at higher alkaline levels. The whole broth of JS7901 was evaporated in vacuo and the concentrated brownish cake was obtained. The cake was soluted and crystallized in turn by various organic solvents. Only number 10 was left in liquid in butanol. The results are shown

Table5. Crystallization of crude substances dried under vacunum.

isolate numbers	color & shapes	Shapes under microscope	soluble	Insoluble
1	crystalline		water methanol	ether, chloroform ethanol, aceton, petroleum ether, propanol, buthano l ethylacetate.
2	white crystal.		water methanol	〃
3	milky crystal.		water	(methanol) 〃
4	milky crystal.		water	(methanol) 〃
5	yellow crystal.		water methanol	〃
6	yellow crystal.		water methanol	〃
7	brownish powder		water methanol	〃
8	white powder		water methanol	(methanol) 〃
9	amorphous		water methanol	〃
10	brown liquid		water methanol	〃
11	white crystal.		water methanol	〃
12	white crystal.		water methylacetate	〃

Thin Layer Chromatography was applied for finding the active spots on the silica gel plates. A solvent system, n-butanol-acetic acid-water(3 : 1 : 1) was most suitable for separation of various spots. The spots of the substances including active substances were developed on the silica gel plates in the TLC chamber during 3~4 hour period and the spots were colored by spraying 20% H_2SO_4(Fig.1a) and 0.2% ninhydrine (Fig. 1b) respectively and putting them in a dry oven for 10 minutes at 80°C, as shown in Fig. 1. The spots colored by spraying 20% H_2SO_4 did not show any inhibition

Fig. 1. TLC respones of JS7901 on silica gal G plate.



solvent system: n-butanol: acetic acid: water=3 : 1 : 1

Table 6. Inhibition zones of TLC fractions colored by 0.2% ninhydrine against *E. coli* in Vitro.

fraction number / repeat	1	2	3	4	5th spot
I	—	10.85	11.40	9.30	15.35
II	—	10.30	11.90	9.90	15.60
average	—	10.58	11.65	9.60	15.48

when the spots were assayed. However the pinkish spots colored by spraying 0.2% ninhydrine showed inhibition zones against *Escherichia coli* (using the paper disc method) followed by the 3rd, 2nd and 4th spot respectively.

Attempts of separate the active substances with four types of ion exchanger failed due to technical errors

DISCUSSION

This work, together with our previous work (8.10) demonstrated the feasibility of separation and refining the active substances of the antibiotic bacterium JS7901, which had previously been named as antibiotic bacterium JS7501 when the bacterium was first found as a wild strain in 1975. Recently we have isolated 83 antibiotic microorganisms with the aim of finding bacteria which form antibiotic substances inhibiting gram negative bacteria. The isolates were significantly effective against gram negative bacteria *Escherichia coli* but further future studies of plant disease fungi will also be required for furture effective plant disease control,

Tests made to discover the best media for producing activity of JS7901 against test organisms showed that the most effective media against *Escherichia coli* were the SS media whilst the NBS media were best against *Staphyllococcus aureus*. These results would seem to show different antibiotic substances in the whole broth of JS7901. It also coincided with the response of TLC tests showing 4 different active spots colored by ninhydrine on the silica gel plate.

The solvent system, n-butanol-acetic acid-water (3 : 1 : 1) were spiramycin 0.08, oleandomycin 0.29, amaromycin 0.38, erythromycin 0.39, picromycin 0.41, carbomycin 0.55, leucomycin 0.58, tylosin 0.59, tertiomycin B 0.63, tertiomycin A 0.86; nystatin 0.18, pimaricin 0.34, unamycin A 0.39, amphorthericin A 0.33, pentamycin 0.67, trichomycin 0.17, actinomycin C 0.68, actinomycin J 0.73; etamycin 0.66, pyridomycin 0.38, telomycin 0.44, amophormycin 0.53; thiolutin 0.65, aureothricin 0.58; acidomycin 0.74 and enteromycin 0.73. (4) The Rf value of the upper 4th spot was 0.68 as shown in Fig. 1b as well as actinomycin 0.68. However it is anticipated that comparisons with known antibiotics would be made after the active substances of JS7901 were able to be completely purified in further study.

摘 要

本實驗은 韓國產抗生物質 또는 抗生菌의 開發利用을

目的으로 한 基礎硏究로서 主로 前年度의 繼續硏究와 아울러 土壤試料로부터 새로히 抗生細菌을 分離하였다 淸州部近의 土壤試料 500點으로부터 83點의 抗生菌을 分離하였으며 이들은 主로 gram음성균인 *Escherichia coli*에 有効한 것을 選別하였다. 이들에 대한 硏究는 앞으로 繼續할 것이며 수년간 계속 연구중인 우수항생균 JS7501은 그동안 數次에 걸쳐 再選別하였기 때문에 JS7901로 改稱하여 本試驗에 供試하였으며 항균작용범위도 재확인하였으며 주로 추출을 목적으로 용매추출, 흡착, TLC, 이온교환 수지 등을 총동원하였으나 TLC에 의하여 활성이 확인된 4 spot를 분리할 수 있었다. JS7901의 배양배지로서는 Soytone Sugar배지가 가장 높은 항균력을 보였으며 Soytone보다는 Sugar가 활성물질 증가와 관계가 있었다.

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