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CELL WALL COMPOSITION AS AN AID IN THE IDENTIFICATION OF MICRO-ORGANISMS – A STUDY OF BIO-ACTIVE SOIL ACTINOMYCETE D-85

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ABSTRACT

The actinomycetes are well known as a group of filamentous, Gram-positive bacteria that produce many useful secondary metabolites, including antibiotics and enzymes. A classification of any microbial order is a temporary and man-made arrangement in which similar individuals sharing certain common features are grouped together as taxonomic units at different levels in a taxonomic hierarchy. The success of a classification can be measured by the consistency found when different classes of information are used. Cell wall composition has also become widely accepted as an aid in the identification of genera. In many instances however, the analysis of whole-cell hydrolyzates is sufficient for identification. In the present investigation an attempt was made to determine the cell wall composition for selected best isolate(D-85). The selected bioactive actinomycete, D-85 was grown in yeast extract- malt extract broth at 28^{o} C for 48 hrs. The cells were collected by centrifugation at 4000 rpm for 20 min and washed 3 times with sterile water. Then they were analyzed for amino acids and sugars by chromatographic methods. The results indicate that the cell wall of the strain D-85 contained LL-diaminopimelic acid & glycine with xylose and arabinose as diagnostic sugar components. The above data suggested that the strain D-85 belongs to cell wall type I with sugar pattern D. Further studies are in progress, published and presented elsewhere.

KEYWORDS: Actinomycetes, Gram-positive bacteria, Centrifugation, Chromatography, LL-Diaminopimelic acid, Glycine, Xylose, Arabinose.

INTRODUCTION

A cell wall is a rigid, semi-permeable protective layer in some cell types. This outer covering is positioned next to the cell membrane (plasma membrane) in most plant cells, fungi, bacteria, algae, and some archaea. Animal cells however, do not have a cell wall. The cell wall has many important functions in a cell including protection, structure, and support.

Cell wall composition varies depending on the organism. In plants, the cell wall is composed mainly of strong fibers of the carbohydrate polymer cellulose. Cellulose is the major component of cotton fiber and wood, and it is used in paper production. Bacterial cell walls are composed of a sugar and amino acid polymer called peptidoglycan. The main components of fungal cell walls are chitin, glucans, and proteins.

Unlike in plant cells, the cell wall in prokaryotic bacteria is composed of peptidoglycan. This molecule is unique to bacterial cell wall composition. Peptidoglycan is a polymer composed of double-sugars and amino acids (protein subunits). This molecule gives the cell wall rigidity and helps to give bacteria shape. Peptidoglycan molecules form sheets which enclose and protect the bacterial plasma membrane.

The actinomycetes are well known as a group of filamentous, Gram-positive bacteria that produce many useful secondary metabolites, including antibiotics and enzymes. A classification of any microbial order is a temporary and man-made arrangement in which similar individuals sharing certain common features are grouped together as taxonomic units at different levels in a taxonomic hierarchy. The success of a classification can be measured by the consistency found when different classes of information are used. Cell wall composition has also become widely accepted as an aid in the identification of genera. In many instances however, the analysis of whole-cell hydrolyzates is sufficient for identification.[1] Chemical criteria, such as the isomer of diaminopimelic acid (DAP) present in the cell wall and the diagnostic sugar(s) present in the whole-cell hydrolysate, have been used to separate the actinomycete genera into broad chemotaxonomic groups. whole-cell sugar analysis has become a widely used technique to classify and identify actinomycetes.

The whole-cell sugar analysis method can be used to recognize four types of whole cell sugar pattern in aerobic actinomycetes. In these patterns, arabinose, xylose, galactose, and madurose (3-0-methyl-D-galactose) are diagnostic sugars. All the sugars can be identified with conventional chromatographic techniques such as paper chromatography (PC) and thin-layer chromatography (TLC).[2,3]

During our continuous search for antibiotic producing actinomycetes, a potent antibiotic producer was isolated from natural substrates. The isolate D-85 with good antimicrobial activity and occurred as biverticillate sporophores under preliminary morphological studies (Fig.1), it was found to be interesting and it was selected for detailed taxonomic study. In the present investigation an attempt was made to determine the cell wall composition (chemical characteristics) for selected best isolate(D-85) as a part of taxonomic investigations by employing paper chromatographic technique.

MATERIALS AND METHODS

The isolate D-85 was selected on the basis of preliminary morphological and antimicrobial activity studies out of 359 isolates isolated from different natural substrates on selective media. The whole -cell hydrolysates were prepared by the following procedure;

The isolate D-85 was allowed to grow in sterile conical flasks contained sterile yeast extract-malt extract broth[4] at 28^{o} C for 48 hours on a rotary shaker at 200rpm. All chemicals used in the present investigation were analytical and laboratory grade.

The cells were collected by centrifugation at 4000 rpm for 20 minutes and washed three times with sterile water. Then they were analyzed for amino acids and sugars by descending paper chromatographic methods.

i) Detection of Amino acids

About 10 mg of cells were hydrolyzed for 2 hours with 1 ml of 6 N HCl in a closed screw capped tube held at 100^{o}C in a water bath. The contents were filtered after cooling through Whatman no.1 filter paper. The solid material on the filter paper was washed with 3 drops distilled water. The liquid hydrolyzate was transferred into watch glass and dried three consecutive times on a steam bath and removed HCl. The residue was taken up in 0.3 ml of distilled water and used for chromatographic study along with the following standards: meso & LL – DAP and glycine.

The solvent system used for amino acids was n-butanol-pyridine-water-glacial acetic acid (60:40:30:3). The descending chromatographic technique was used for 6 to 8 hours. The developed chromatogram was air dried and it was sprayed with 0.4% ninhydrin reagent heated at 100^{0}C for 5 to 10 minutes. The purple /reddish brown/yellow colour spots appeared were marked and R_f values were calculated and compared with known standards. The results are given in Table.1.

Preparation of Ninhydrine reagent: 0.4g of Ninhydrine was dissolved in 100ml of water saturated with n-butanol.

ii) Detection of Sugars

About 10 mg of cells were hydrolyzed in 1 ml of 2 N HCl in closed screw capped tube held at 100^{o}C in a water bath for 2 hours and the rest of the procedure is same as mentioned above. The solvent system used for sugars was ethylacetate-pyridine-water (3.6:1:1.15) and descending paper chromatographic technique was used. The developed chromatogram was air dried and sprayed with aniline acid phthalate reagent and heated at 100^{0} C for 5 to 10 minutes. The following standards were used: arabinose, galactose, xylose and madurose.

Aniline acid phthalate reagent: 3.3 g of phthalic acid was dissolved in 2 ml of aniline and it was made up to 100 ml with water saturated n- butanol.

For detection of carbohydrates and reducing sugars, the dried chromatogram was sprayed with a solution of aniline acid phthalate reagent and heated for 5 minutes. The developed reddish-brown spots were marked and calculated the R_f values as shown in table.2.
(Hexoses- yellowish brown; pentoses- reddish-brown, Madurose-yellowish brown)

iii) Paper Chromatography

Chromatography is a technique that is used to separate and to identify components of a mixture. This analytical technique has a wide range of applications in the real world since many substances are mixtures of chemical compounds. In the present investigation whatman no.1 chromatographic paper 10x25 cm was used and followed the procedure as reported in the literature.

In paper chromatography, the sample mixture was applied(5-10µl) onto a baseline with capillary tube along with standards . Components of the mixture were carried along with the solvent down the paper to varying degrees, depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent. The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually consisting of a mixture of water and an organic liquid. The paper is called the stationary phase while the solvent is referred to as the mobile phase.[5]

Performing a chromatographic experiment is basically a three-step process:
1) Application of the sample,
2) Saturation of chromatographic tank with solvent mixture previously, "developing" the chromatogram by allowing the mobile phase to move down the paper, and
3) Calculating Rf values and making conclusions.

In order to obtain a measure of the extent of movement of a component in a paper chromatography experiment, we calculated an "R_f value" for each separated component on the developed chromatograms in case of aminoacids and sugars separately.

An R_f value is a number that is defined as: distance travelled by component from application point /distance travelled by solvent from application point.

The distance travelled by the spot was measured to the middle of the spot. The results are shown in table 1 & 2 for aminoacids and sugars respectively.

RESULTS AND DISCUSSION

As reported in the literature cell wall composition has also become widely accepted as an aid in the identification of genera. Four cell wall types are widely accepted (I-IV), other cell wall patterns have been proposed.[6-9] As shown in table 3 & 4.

Table 1: R_f Values of Aminoacids present in the whole cell hydrolysate(Test)of isolate D-85.

S.No	Amino acids (Standard)	Distance travelled by the component(Cm)	Distance travelled by the solvent front (Cm)	R_f value
1	meso -DAP	3.2	16	0.2
2	LL-DAP	3.8	16	**0.237**
3	Glycine	4.2	16	**0.26**
4	Test	3.7 4.3	16	**0.23** **0.268**

As indicated in the table.1, the whole cell hydrolysate of D-85 contained LL-DAP and glycine and they were present in the cell wall as major aminoacids.

Table 2: R_f Values of Sugars present in the cell hydrolysate of isolate D-85.

S.No	Sugars (Standard)	Distance travelled by the component(Cm)	Distance travelled by the solvent front (Cm)	R_f value
1	L-Arabinose	7.3	15	**0.486**
2	D-Xylose	8.8	15	**0.586**
3	D-Galactose	5.5	15	0.366
4	Madurose	8.4	15	0.56
	Test	7.2 8.7	15	**0.48** **0.58**

The table 2. explores that the cell wall of D-85 contained L-arabinose, and D-xylose as major sugars.

Table 3: Major Components of cell wall types of actinomycetes accepted.[6-9]

Cell wall type	DAB	LYSINE	ORNITHINE	Aspartic acid	Glycine	meso DAP	LL-DAP	Arabinose	Galactose	Gram reaction
I					+		+			+
II					+	+**				+
III						+				+
IV						+		+	+	+
V		+	+		*					+
VI		+		+	*					+
VII	+	+		+	*					+
VIII				+	*					+
D + +										

*Glycine is variably presentin these groups, +many aminoacids present
** hydroxy DAP may also be present, DAB=2,4-diaminobutyric acid, DAP=2,6 diaminopimelic acid
All preparations contained major amounts of alanine, glutamicacid, glucosamine and muramic acid

Table 4: Whole cell sugar patterns of aerobic actinomycetes accepted.[6]

Pattern	Arabinose	Galactose	Xylose	Madurose
A	+	+	-	-
B	-	-	-	+
C	-	-	-	-
D	+	-	+	-

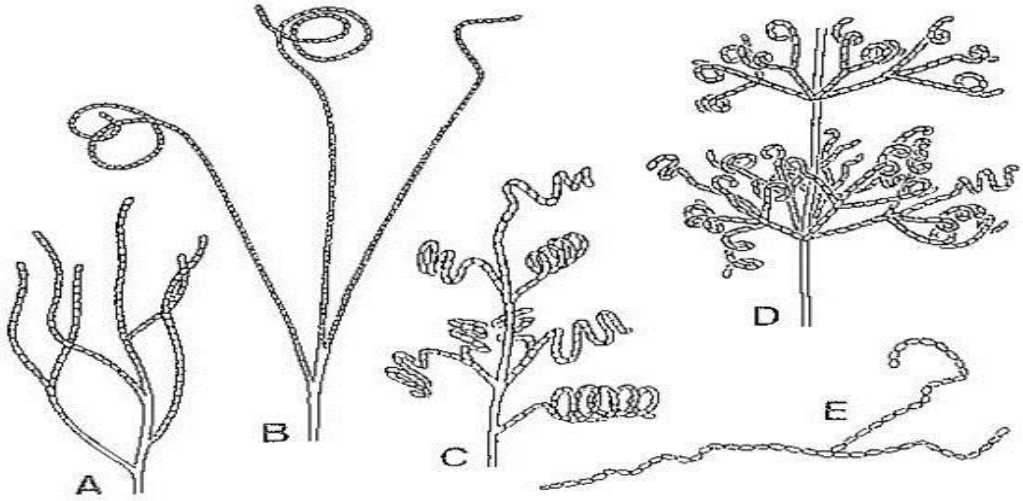

Figure 1: Spore production in long chains - Streptomyces: A) Rectiflexbiles type, B)Retinaculiaperti type, C) Spira type, D) Verticillati type, E) Fragmenting branched (Nocardiopsis)

CONCLUSIONS

- Based on the above results and literature review, it was suggested that the isolate D-85 belongs to cell wall type I with sugar pattern D. Further studies are in progress published and presented elsewhere.
- The method can be used as a routine laboratory procedure requiring a minimal amount of equipment and effort.

LIMITATIONS

- Although cell wall analyses provide information of great taxonomic value they are time consuming and cannot be recommended when large number of isolates have to be examined.

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